European Cell Death Organization (ECDO)

''Jožef Stefan'' Institute

15th ECDO Euroconference on Apoptosis

October 26-31, 2007, Portoroz, Slovenia

Programme and Book of Abstracts

This publication is available at the conference website
http://www.ecdo.eu/portoroz/index.html

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MARIE CURIE ACTIONS

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Welcome

It is my great pleasure and honour to welcome all of you at the 15th Euroconference on Apoptosis in Portorož.

This is the first time that the Euroconference is organized in Slovenia. Although a cell death meeting was never organized in Slovenia, cell death was a regular topic for more then a decade on International Symposia on Proteinase Inhibitors and Biological Control and on International Conferences on Cysteine Proteinases, organized in Slovenia every second year since 1983.

The program of this Meeting linking basic science with clinical applications stresses the importance and trends in cell death research. At this year's meeting there are 400 participants from 6 different continents. I would like to thank all of the invited speakers, poster presenters and participants who contribute to the quality of the Meeting, as well as express my thanks to EC for the financial support within the framework of the Marie Curie Conferences and Training Courses, which have made the meeting possible. At this point I would express my sincere gratitude to ECDO Scientific Board, who made a very attractive program with a lot of space for young researchers, and especially to ECDO Secretary Veronique Vandevoorde for her invaluable work in preparing a very exciting conference. I believe that scientific meetings like this provide a great opportunity to strengthen our knowledge in this area and, by our discussions together, stimulate future research.

I wish you a very pleasant stay in Slovenia.

October 11, 2007

Boris Turk
Chairman of the Conference

Evaluation form

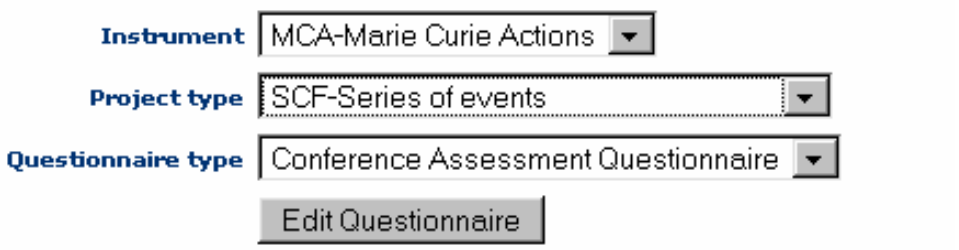
Also this year's Euroconference is organised within the framework of the EC Marie Curie Programme 'Europtosis'. Thanks to the financial support of the EC, a substantial number of participants received a grant to attend this meeting.

In view of this European programme, ECDO would appreciate it very much if you could fill in the **evaluation questionnaire online** upon your return. This will only take about 5 minutes of your time and the total procedure is outlined on the next page.

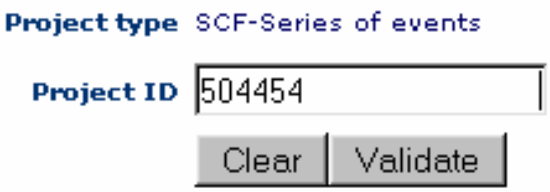
Thank you for your cooperation!

The ECDO board

1 Choose the URL: http://webgate.cec.eu.int/sesam/index.do
2. In the left banner, select "**MCA Questionnaires**"
3. The " **Select questionnaire type**" page is presented below. The "Instrument", "Project type" and "Questionnaire type" options you need to select are filled in on the example



4. "Edit questionnaire"
5. The "**Project identification**" page is presented. The "Project ID' number you have to fill in is **"504454".**



6. After pressing the "**Validate**" button, you will reach the "**MCA Conferences and Training Courses Assessment Questionnaire**".
7. Please read carefully the instructions, complete the questionnaire, and submit your evaluation by pressing the "submit" button.

CONFERENCE VENUE:

St. Bernardin Resort & Convention Centre
Obala 2
6320 Portoroz
Slovenia

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REGISTRATION

To collect the conference materials you are kindly requested to register at the Conference Secretariat in the Grand Hotel Bernardin.

Opening hours of the registration desk

October 26 (Friday)	14:00 – 18.00
October 27 (Saturday)	08.00 – 18.00

Participants are kindly requested to wear their name badge during all events of the meeting. It allows entry to all sessions, welcome reception, meals,..

CONFERENCE SECRETARIAT

If you need any help during the conference you can find the local organiser and the ECDO secretary at the registration desk. They will help you in all practical aspects of conference participation.
In case of emergency please call one of these mobile phone numbers:
+32-475-783851 (Veronique Vandevoorde) or +386-51348925 (Boris Turk)

LECTURE ROOMS

All lectures will be given in the **Europe Convention Hall** on the top floor of the Grand Hotel Bernardin.
Poster presentation will take place next to the meeting room. Please visit the information boards at the registration desk for further information on the poster sessions.

INTERNET CONNECTION

Wireless Internet access is available at the St. Bernardin Conference Center and is paying (a card can be bought in the hotel). Additionally, a few PCs with internet access will be available to the conference delegates. Histrion Hotel has an internet room where you can connect to the Web through one of the available computers.

MEALS AND REFRESHMENT

Breakfast will be served at the hotel of lodging.

Lunches and dinners during the meeting will be served at the **Grand Hotel Bernardin** for the all participants/hotel guests of the St. Bernardin Hotels. Lunch and dinner time is indicated in the conference programme.

Note:

- For participants arriving on **October 25 or 26**, dinner will be served in the hotel of lodging. The dinner time at the Grand Hotel Bernardin is from 19.00 – 21.00 and at the Histrion Hotel from 18.30 – 21.00. Hotel guests of the Vile Park Hotel can have the dinner at the Histrion Hotel.
- On **October 27**, dinner has to be taken before the official opening of the meeting. It will be served from 18.30 on.
- Gala dinner on Tuesday evening **October 30** will start at 19.30.

Refreshments are included in the registration fee for all registered participants.

INCOMING MESSAGES AND MESSAGE BOARD

Messages received by the desk will be posted on the message board located at the registration desk. Participants may also use this board to leave messages to other delegates.

INSTRUCTIONS FOR SPEAKERS

We support OFFICE 2003 (Powerpoint 2003 or XP). If you want to use your own laptop, or you have a Macintosh, be sure that there is a VGA connector for an external monitor.
We prefer Powerpoint presentations. Your Powerpoint presentation should be on USB stick or CD. Slide presentations are not supported.
Short orals take 15 minutes (discussion included). Hence, try to prepare a talk of 10-12 min.
You will be able to upload your presentation in the lecture room before your presentation. A technician will be of your assistance for the file upload in the conference hall.

INSTRUCTIONS FOR POSTER PRESENTERS

To allow maximal viewing of the posters, we kindly ask the presenting authors to exhibit their poster **during the entire meeting**.

Poster should be made in portrait and the measurements of the posterboards are **200 cm (height) x 100 cm (width)**. Please prepare your poster accordingly.
Posters should be readable by viewers one meter away. The poster should contain the title of the submitted abstract, the author(s)' name(s) and affiliation at the top. The organisers will provide mounting materials to fix posters.

Posters should be mounted at the beginning of the meeting and removed by 19:00 on October 30 (Tuesday). We cannot take responsibility for the posters not removed by evening time on Tuesday October 30.

Two fixed poster sessions are scheduled during the meeting (see final programme). Presenting authors are requested to be near their poster during one of the two sessions.

Poster session 1 : Sunday October 28: Uneven poster numbers (P-1,-3,…)
Poster session 2: Monday October 29: Even poster numbers (P-2, -4,…)

Nature Reviews Molecular Cell Biology donates a poster prize consisting of a 1-year print and online subscription to the journal.

SOCIAL PROGRAMME

The scientific programme of the conference will be completed with social events, allowing some time for informal discussions for the participants.

Welcome Reception
The Welcome reception will be held at the St. Bernardin Hotel at 21:00 on Saturday, October 27.

Gala Dinner
The gala dinner will be held on Tuesday evening, October 30 in the Grand Hotel Bernardin.

Excursions
On Monday afternoon, three separate excursions are proposed to the conference delegates: A guided visit to the **caves of Postojna**, to the **Lipica Stud Farm**, or to **the salt lakes**. These excursions will take place according to interest, with a minimal requirement of 20 participants per excursion. More information is available at the conference website (section 'Practical info'>'Excursions').
These excursions are **NOT** included in the conference registration fee and have to be booked on site on a personal basis by Saturday October 27 at the latest. An excursion booking list will be available at the registration desk.

SPONSORS

Poster Prize is sponsored by **Nature Reviews Molecular Cell Biology**, and consists of a 1-year print and online subscription to the journal.

ACCOMPANYING PERSONS' PROGRAMME

Accompanying persons programmes can be organized with the staff of the Hotel.

SMOKING

Smoking is NOT allowed in the St. Bernardin Resort & Convention Centre.

TRANSPORT TO/FROM THE AIRPORT

Transport from the Ljubljana or Trieste airport to the conference venue (and return) on scheduled hours during the days of arrival (October 26-27) and departure (October 31) is included in the registration fee for those participants reporting their detailed flight schedule via the online form. The exact time schedule of the shuttle busses is available at the conference website.

There is a taxi service available at the St. Bernardin hotel. Contact: suntours @siol.net, +386-(0)40-710 560

TELEPHONE SERVICE

Public telephones are available in the St. Bernardin Resort & Convention Centre.

INSURANCE

Participants are strongly advised to make their own insurance arrangements. The organisers cannot accept any liability for personal injuries sustained, or for loss or damage to property belonging to participants and accompanying persons, either during or as a result of the conference.

FOREIGN EXCHANGE, BANKING AND PAYING FACILITIES

The Slovenian currency is the Euro. Currency exchange booths are available at the airport terminals and in a large number of hotels, including the hotels of the St. Bernardin Resort. Usual opening hours of Slovene banks are: Monday-Friday: 8:00-12:00 and 14:00-18:00, Saturday: 8:00-12:00.

Participants are expected to pay for the hotel room upon check-out, in cash or by credit card. Following credit cards are accepted by the hotel: VISA, American Express, MasterCard, Diners Club Card.

4th Training Course on

Concepts and Methods in Programmed Cell Death

Portoroz, Slovenia, October 27, 2007

Programme

Chair: Richard Lockshin (New York, USA)

9:00-10:00	**Seamus Martin** (Dublin, Ireland) Caspase-dependent and independent cell death pathways
10:00-11:00	**Hans-Uwe Simon** (Bern, Switzerland) Non-caspase proteases in cell death
11:00-11:30	Coffee
11:30-12:30	**Laszlo Fesus** (Debrecen, Hungary) Apoptosis and chronic inflammation disease
12:30-13:30	**Gabriel Nunez** (Ann Arbor, USA) Role of inflammasome in fighting against infection
13:30-15:00	Lunch
15:00-16:00	**Roberta Gottlieb** (La Jolla, USA) Cell death and ischemia
16:00-17:00	**Klaus-Michael Debatin** (Ulm, Germany) Cell death and treatment of cancer

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MARIE CURIE ACTIONS

Conference Programme

15th EUROCONFERENCE ON APOPTOSIS

October 27- October 30, Portoroz, Slovenia

Saturday evening October 27

Chairs: **Klaus-Michael Debatin (Ulm, Germany)**
Boris Zhivotovsky (Stockholm, Sweden)

20:00 **Official Opening**

20:00-21:00: **ECDO honorary lecture**

Marie-Lise Gougeon (Paris, France)
Apoptosis : an HIV strategy of immune evasion

21:00 **Welcome reception**

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October 28: Sunday morning

Session 1: Cell death in small organisms

Chair: Boris Zhivotovsky (Stockholm, Sweden)

9:00-9:30 **Hermann Steller (New York, USA)**
Coordinate Regulation of Cell Death and Tissue Regeneration

9:30-10:00 **Pascal Meier (London, UK)**
Inhibitor of Apoptosis (IAPs) Proteins in Ubiquitin-mediated Signalling

10:00-10:15 **Benoît Meslin (Lyon, France)**
Place of metacaspases like proteins in Plasmodium falciparum apoptosis

10:15-10:30 **Florian Baumgartner (Innsbruck, Austria)**
Role and function of the apoptosis-regulator PIDD

10:30-11:00 Coffee break

Session 2: Cell death proteases

Chair: Boris Turk (Ljubljana, Slovenia)

11:00-11:30 **Guy S. Salvesen (LaJolla, USA)**
Caspases: Activation and Substrates

11:30-12:00 **Judy Liebermann (Boston, USA)**
Many Ways to Die - Granzyme A Activates a Caspase-independent Mitochondrial Cell Death Pathway

12:00-12:15 **Sebastien Conus (Bern, Switzerland)**
Cathepsin D initiates neutrophil apoptosis and its inhibition blocks the resolution of inflammation

12:15-12:30 **Markus Rehm (Dublin, Ireland)**
Quantitative real time analyses of the signaling dynamics during apoptosis execution

12:30-14:30 Lunch

October 28: Sunday afternoon

Session 3: Cell death signalling

Chair: Wim Declercq (Ghent, Belgium)

14:30-15:00 **Michael Lenardo (Bethesda , USA)**
Germline N-Ras mutation causes a human lymphoproliferative syndrome

15:00-15:30 **Luca Scorrano (Padova, Italy)**
Keeping mitochondria and endoplasmic reticulum in shape and together: a matter of life and death

15:30-15:45 **Martin Ott (Stockholm, Sweden)**
The mitochondrial TOM complex is required for tBid/Bax-induced cytochrome c release

15:45-16:00 **Jerry E. Chipuk (Memphis, USA)**
Pro-apoptotic BAX and BAK require endoplasmic reticulum-derived sphingolipid metabolism to induce mitochondrial outer membrane permeabilization

16:00-16:30 Coffee break

Session 4: Autophagy and necrosis in model organisms

Chair: Peter Vandenabeele (Ghent, Belgium)

16:30-17:00 **Pierre Golstein (Marseille, France)**
Autophagic and necrotic cell death in a model system

17:00-17:30 **Nektarios Tavernarakis (Heraklion, Greece)**
Autophagy and necrotic cell death in *C. elegans*

17:30-17:45 **Tom Vanden Berghe (Ghent, Belgium)**
Necrotic cell death, a live experience

17:45-18:00 **Grégory Bellot (Nice, France)**
Early molecular initiation steps of hypoxia-induced autophagy: involvement of BNIP3 and BNIP3L, two HIF-induced BH3-only proteins

18.00 -19:30 Dinner

20:00 Poster session

October 29: Monday morning

Chair: Boris Turk (Ljubljana, Slovenia)

ECDO Keynote Lecture

9:00-10:00 **Aaron Ciechanover (Haifa, Israel)**
The Ubiquitin Proteolytic System: From Basic Mechanisms and onto Human Diseases and Drug Targeting

10:00-10:30 Coffee break

Session 5: Cell death and disease

Chair: Laszlo Fesus (Debrecen, Hungary)

10:30-11:00 **Martina Müller-Schilling (Heidelberg, Germany)**
One, two, three - p53, p63, p73 and chemosensitivity

11:00-11:30 **David Huang (Parkville, Australia)**
The Bcl-2 family of molecular switches controlling cell survival and death

11:30-11:45 **Valeria Piddubnyak (Paris, France)**
Positive regulation of apoptosis by HCA66, a new Apaf-1 interacting protein, and its putative role in the physiopathology of NF1 microdeletion syndrome patients

11:45-12:00 **Andreas Villunger (Innsbruck, Austria)**
Characterization of the BH3-only protein Bmf in cell death induction and disease

12:00-12:15 **Shazib Pervaiz (Singapore, Singapore)**
Bcl-2 Induces Pro-oxidant State by Engaging Mitochondrial Respiration in Tumor Cells

12:15-12:30 **Laura Ciarlo (Rome, Italy)**
CD95/Fas triggers the “journey” of GD3 to mitochondria via its association with microtubules

12:30-19:00 Lunch and free time

19.00 -20:00 Dinner

20:00 Poster session

October 30: Tuesday morning

Session 6: Autophagy in health and disease

Chair: Hans-Uwe Simon (Bern, Switzerland)

9:00-9:30 **Vojo Deretic (Albuquerque, USA)**
Autophagy in immunity: A cell santiation and survival process

9:30-10:00 **Adi Kimchi (Rehovot, Israel)**
System level analysis of programmed cell death: switching between apoptosis, autophagy and necrosis

10:00-10:15 **Goran Petrovski (Debrecen, Hungary)**
Phagocytosis of cells dying through autophagy evokes a pro-inflammatory response in macrophages

10:15-10:30 **Ellen Wirawan (Ghent, Belgium)**
Caspase dependent cleavage of Beclin1 during apoptosis

10:30-11:00 Coffee break

Chair: Mauro Piacentini (Rome, Italy)

11:00-11:30 **Beth Levine (Dallas, USA)**
Crossroads of Autophagy and Apoptosis: Regulation and Corpse Clearance

11:30-12:00 **Guido Kroemer (Villejuif, France)**
Self-eating and self-killing. Crossroads of autophagic and apoptotic pathways

12:00-12:15 **Muriel Priault (Bordeaux, France)**
Differential regulation of pro-survival autophagy by Bcl-2 and Bcl-xL

12:15-12:30 **Michael Dewaele (Leuven, Belgium)**
Molecular effectors of autophagy and apoptosis in ER-photodamaged cells

12:30-14:30 Lunch

October 30: Tuesday afternoon

Session 7: Cell death and cancer

Chair: Klaus-Michael Debatin (Ulm, Germany)

14:30-15:00 **Patrick Mehlen (Lyon, France)**
Dependence receptors as tumor suppressors: mechanisms, proof of concept and possible hints for therapy.

15:00-15:30 **Henning Walczak (Heidelberg, Germany)**
TRAIL-induced apoptosis: biochemical mechanism and identification of a physiological role in metastasis prevention

15:30-15:45 **Venturina Stagni (Rome, Italy)**
ATM kinase activity modulates Fas-sensitivity through the regulation of FLIP in lymphoid cells

15:45-16:00 **Meike Vogler (Leicester, United Kingdom)**
Targeting BCL2 with ABT-737: a promising strategy to kill leukaemia cells

16:00-16:30 Coffee break

Chair: Peter Krammer (Heidelberg, Germany)

16:30-17:00 **Simone Fulda (Ulm, Germany**
Targeting apoptosis pathways for cancer therapy

17:00-17:15 **Domagoj Vucic (South San Francisco, USA)**
IAP antagonists inhibit tumor growth and induce TNF-dependent apoptosis

17:15-17:30 **Valeria Tomati (Genoa, Italy)**
A novel Bcl-XL peptide inhibitor as a potential anti-leukaemic agent

17:30-18:00 **Eileen White (Piscataway, USA)**
Regulation of Apoptosis and Autophagy in Cancer Progression and Therapy

18.00 -18.15 Concluding remarks

19:30 Gala dinner

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Lecture Abstracts

Invited speakers

Abstracts in this section are in **alphabetical** order by the name of the invited speaker (underlined).

The presentations of the Training Course are not included in this section. They will be available as PDF files on the conference website http://www.ecdo.eu/portoroz/index.html.

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ECDO Keynote Lecture

The Ubiquitin Proteolytic System: From Basic Mechanisms through Human Diseases and onto Drug Targeting

Aaron Ciechanover

Cancer and Vascular Biology Research Center, Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

Between the sixties and eighties, most life scientists focused their attention on studies of nucleic acids and the translation of the coded information. Protein degradation was a neglected area, considered to be a non-specific, dead-end process. While it was known that proteins do turn over, the large extent and high specificity of the process - whereby distinct proteins have half-lives that range from a few minutes to several days - was not appreciated. The discovery of the lysosome by Christian de Duve did not significantly change this view, as it was clear that this organelle is involved mostly in the degradation of extracellular proteins, and their proteases cannot be substrate-specific. The discovery of the complex cascade of the ubiquitin pathway revolutionized the field. It is clear now that degradation of cellular proteins is a highly complex, temporally controlled, and tightly regulated process that plays major roles in a variety of basic pathways during cell life and death, and in health and disease. With the multitude of substrates targeted, and the myriad processes involved, it is not surprising that aberrations in the pathway are implicated in the pathogenesis of many diseases, certain malignancies and neurodegeneration among them. Degradation of a protein via the ubiquitin/proteasome pathway involves two successive steps: (a) conjugation of multiple ubiquitin moieties to the substrate, and (b) degradation of the tagged protein by the downstream 26S proteasome complex. Despite intensive research, the unknown still exceeds what we currently know on intracellular protein degradation, and major key questions remain unsolved. Among these are the modes of specific and timed recognition for the degradation of the many substrates, and the mechanisms that underlie aberrations in the system that lead to pathogenesis of diseases. The recent discovery of modification by ubiquitin-like proteins along with identification of “non-canonical” polyubiquitin chains that serve non-proteolytic functions, have broadened the scope of the system beyond proteolysis and set new challenges in for biologists and proteomic experts. Major challenges in the field are clearly (i) identification of the cellular proteins tagged by ubiquitin and ubiquitin-like proteins, (ii) identification of the downstream elements recognized by these chains, and (iii) deciphering the structure of the different ubiquitin and ubiquitin-like chains that tag the different proteins.

Autophagy and Immunity Related GTPse IRGM in Intracellular Cleansing and Cellular Life and Death

Vojo Deretic

University of New Mexico School of Medicine, Albuquerque, NM87131, USA

Autophagy is a ubiquitous biological process for cleansing of the eucarytotic cell's interior, whereby portions of the cytoplasm, including large objects such as organelles, get sequestered into a double membrane organelle called autophagosome for delivery to lysosomes and degradation of the captured cytoplasmic constituents. The process of autophagy plays many functions, including feeding the cells under starvation conditions and ensuring their survival upon withdrawal of growth factors. Furthermore, damaged or surplus organelles, e.g. leaky mitochondria or overproliferated peroxisomes, are removed by autophagy, thus potentially preventing unscheduled cell death. Autophagy has been implicated in cancer, neurodegeneration including Alzheimer's, Huntington's and Parkinson's diseases, development, aging and longevity. Most recently, autophagy has been shown to play a role in innate and adaptive immunity. In this presentation, I will first give a very brief introduction to autophagy as a biological process, and cover its role in cellular homeostasis with the specific emphasis on the immune system and innate immunity. Next, I will present results indicating that the innate immunity related GTPases (IRG; a.k.a. p47 GTPases), believed for a long time to play a role (by a hitherto unknown mechanism) in defense against intracellular microbes, act through autophagy. After that, I will specifically address the role of human IRGM, implicated in tuberculosis and Crohn's disease along with Atg16, another autophagy factor. Finally, I will present unpublished information regarding IRGM effects on cell viability.

Targeting apoptosis pathways for cancer therapy

Simone Fulda

University Children's Hospital, Eythstr. 24, Ulm 89075, Germany

One of the key advances in cancer research in recent years is the recognition that killing of tumor cells by anticancer therapies commonly used in the treatment of cancer, e.g. chemotherapy or γ-irradiation, is to a large extent mediated by triggering cell death pathways including apoptosis in cancer cells. Thus, failure to undergo apoptosis may result in primary or acquired resistance of cancers to current treatment approaches. Understanding the molecular events that regulate apoptosis in response to anticancer therapy and how cancer cells evade apoptotic cell death, provides novel perspectives for a rational approach to develop molecular therapeutics that target cell death pathways in human cancers.

Autophagic and necrotic cell death in a model system

Pierre Golstein

Centre d'Immunologie de Marseille-Luminy, 13288 Marseille cedex 9, France

A few model organisms have helped greatly in our understanding of cell death but, at the same time, might have constrained it. Investigations in simple biological models, taken for instance outside the animal kingdom, may benefit from less interference from other cell death mechanisms and from better experimental accessibility, while providing phylogenetic information. We previously showed that the protist Dictyostelium discoideum is a genetically tractable model for developmental autophagic vacuolar cell death. Further studies showed that the procedure that induced autophagy, vacuolization and death in wild-type cells led in atg1 autophagy gene mutant cells to impaired autophagy and to no vacuolization, demonstrating that atg1 is required for vacuolization. Unexpectedly, however, cell death still took place, with a non-vacuolar and centrally condensed morphology. We further investigated this developmentally-induced cell death occurring in an autophagy mutant, and found that it included a stereotyped sequence of events characteristic of necrotic cell death. Of additional interest, developmental stimuli and classical mitochondrial uncouplers triggered a similar sequence of events. A genetic analysis through random mutagenesis contributed to an analysis of corresponding signaling pathways. An overview of the autophagic and necrotic cell death mechanisms and their signaling pathways in Dictyostelium will be presented, as well as the main conclusions we can draw from these studies so far.

HIV-induced Cell Death : a Viral Strategy of Immune Evasion

Marie-Lise Gougeon

Antiviral Immunity, Biotherapy and Vaccine Unit, Institut Pasteur, 28 rue du Dr. Roux, 75015 Paris, France

Viruses have evolved numerous mechanisms to evade the host immune system, and one of the strategies developed by HIV is to activate apoptotic programs in order to destroy immune effectors. Not only does the HIV genome encode for proapoptotic proteins, which kill both infected and uninfected lymphocytes, but it also creates a state of chronic immune activation responsible for the exacerbation of physiological mechanisms of clonal deletion.

HIV infects cells of the immune system and HIV-infection is characterized by the gradual loss of $CD4^+$ T cells and a progressive immune deficiency that leads to opportunistic infections, and ultimately death. Although our understanding of CD4 T cell homeostasis is still incomplete and controversial, a growing body of evidence points to HIV-driven lymphocyte apoptosis as an important contributor to the destruction of the immune system Because of persistent expression of viral particles, this infection results in high turnover rates of T cells, leading to increased T cell proliferation that is physiologically controlled by increased apoptosis. In addition, HIV has developed strategies to trigger the apoptotic machinery in both infected and non-infected cells, inducing the destruction of the effectors of the immune system. Death receptors, such as CD95, TNF-RI and –II or the TRAIL system, are activated in patients' T cells and are contributing to the destruction of HIV-specific CD4 T helper cells and cytotoxic T cells (CTL), required for the immune defense against HIV-infection. Syncytia arising from the fusion of cells expressing the HIV envelope protein with cells expressing the receptors CD4/CXCR4 undergo apoptosis through a mitochondrion-dependent pathway, contributing to the destruction of CD4 T cells. HIV-encoded proteins, such as gp120, Tat, Nef, Vpr, trigger apoptosis in both infected and bystander cells through several mechanisms, including the down-regulation of Bcl-2, the release of cytochrome-c, caspase activation, the up-regulation of death receptors and their ligands (CD95/CD95L, TNF-R/TNF) etc…

Exacerbated T cell apoptosis contributes to HIV disease evolution, and a positive correlation is found between the rate of apoptosis in CD4 T cells and their susceptibility to CD95-induced apoptosis. In addition, premature T cell apoptosis is not detected in non-pathogenic models of simian infections (SIV in African green monkeys, or HIV in chimpanzees) while it strongly occurs in the pathogenic models (SIV in macaques). New potent antiviral therapies (HAART) to HIV^+ persons leads rapidly to the suppression of HIV viral load in the blood and lymphoid organs and to a concomitant rise in the number of CD4 T cells. This restoration is attributable to decreased apoptosis due to the down-regulation of proapoptotic HIV proteins, the reduction of virus-driven immune activation, and probably the anti-apoptotic properties of some HIV drugs. Understanding the viral strategies involved in the destruction of HIV-specific effectors is particularly important since no currently available therapies can efficiently restore virus-specific immunity.

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The Bcl-2 family of molecular switches controlling cell survival and death

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A central outstanding issue in the regulation of apoptosis by the Bcl-2 family is whether its BH3-only members initiate apoptosis by directly binding to the essential cell death mediators Bax and Bak, or whether they can act indirectly, by engaging their pro-survival Bcl-2–like relatives. Contrary to the direct activation model, we show that Bax and Bak can mediate apoptosis without discernable association with the putative BH3-only activators (Bim, Bid and Puma), even in cells with no Bim, Bid or Puma. These results indicate that BH3-only proteins induce apoptosis primarily by engaging the multiple pro-survival relatives guarding Bax and Bak.
Thus, the critical role of the pro-survival Bcl-2 proteins is to restrain Bax and/or Bak. This conclusion is reinforced by our recent structural study of anti-apoptotic proteins expressed by certain viruses. Many viruses express anti-apoptotic proteins to counter host defense mechanisms that would otherwise trigger the rapid clearance of infected cells. For example, adenoviruses and some γ-herpesviruses express homologs of pro-survival Bcl-2 to subvert the host's apoptotic machinery. Myxoma virus, a double-stranded DNA virus of the pox family, harbors anti-apoptotic M11L, its virulence factor. Analysis of its 3-dimensional structure reveals that despite lacking any primary sequence similarity to Bcl-2, it adopts a virtually identical protein fold. This allows it to associate with BH3 domains, especially those of Bax and Bak. We found that M11L acts primarily by sequestering Bax and Bak, thereby blocking the killing action of these essential cell death mediators. These findings expand the family of protein sequences that act like Bcl-2 to block apoptosis.
Our more recent work focuses on how Bax/Bak become activated during apoptosis and relevant results from these studies will be discussed.

Funding: Leukemia and Lymphoma Society, Australian NHMRC, Australian Research Council, the U.S. NCI and the Cancer Council of Victoria.

System level analysis of programmed cell death: switching between apoptosis, autophagy and necrosis

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The molecular network of programmed cell death (PCD) comprises a few independent functional modules driven by different sets of genes. This includes the type I caspase-dependent apoptosis, type II autophagic cell death, and type III programmed necrosis. Different strategies undertaken in our laboratory led to the discovery of novel proteins which function within these modules, and revealed a few unexpected 'system level' principles. Among the positive mediators of autophagic cell death, we identified the calcium/calmodulin Ser/Thr kinase, DAPk. Another positive mediator of the autophagic cell death module was identified as a new isoform of p19ARF, named smARF, the product of internal initiation of translation from the p19ARF open reading frame. smARF is a short lived protein which localizes to the mitochondria and dissipates the mitochondrial membrane potential. In the mitochondria smARF binds to p32 protein and this interaction stabilizes the protein thus increasing its cellular effects including the mitochondrial damage and autophagy. To perform function-based systems level analysis of the entire molecular network of PCD (comprising about 150 genes) we developed a high throughput platform that measures the functional weight of the network's individual nodes. This approach is based on quantifying the extent to which combinatorial perturbations of genes, caused by RNA interference and chosen by hypothesis driven decisions, influence the overall network's performance. It was found that a high degree of plasticity exists in the PCD network, as another way to achieve robustness, including unpredicted switches between apoptosis, necrosis and autophagy.

Autophagy regulation by cytoplasmic p53

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Multiple cellular stressors including activation of the tumor suppressor protein p53 can stimulate autophagy. In addition, as shown here, deletion, depletion or inhibition of p53 can induce autophagy, as determined in human cells subjected to the knockout, knockdown or pharmacological inhibition of p53. Enhanced autophagy improved the fitness of p53-deficient cancer cells in conditions of hypoxia and nutrient depletion, allowing them to maintain high ATP levels and clonogenic survival. Inhibition of p53 caused autophagy in enucleated cells, and cytoplasmic, not nuclear, p53 was able to repress the enhanced autophagy of p53-/- cells. Multiple different autophagy inducers, including starvation, rapamycin and toxins affecting the endoplasmic reticulum stimulated the degradation of p53 by a pathway relying on the E3 ubiquitine ligase HDM2 and the proteasome. Avoidance of p53 degradation prevented the activation of autophagy, indicating that p53 depletion is a prerequisite for the induction of autophagy. These results have far reaching implications for the cancer-associated dysregulation of p53 and autophagy.

Germline N-Ras mutation causes a human lymphoproliferative syndrome

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Signaling networks regulating cell proliferation and death play a crucial role in maintaining self-tolerance, lymphocyte homeostasis, and preventing neoplasia. We are using a human genetic approach to understand abnormalities of lymphocyte homeostasis and immunological tolerance as manifested in human clinical disorders. We describe an NRAS mutation in a patient with autoimmune lymphoproliferative syndrome (ALPS) manifested as defective lymphocyte apoptosis, autoantibody formation, lymphoproliferation and B-cell lymphoma. This mutation induced the accumulation of active, GTP-bound NRAS, which suppressed cytokine withdrawalinduced ymphocyte apoptosis in vitro by decreasing the pro-apoptotic protein BIM through augmented RAF/MEK/ERK pathway signaling. The association of NRAS ith ALPS contrasts with the developmental abnormalities associated with human RAS and HRAS mutations and defines a novel genetic basis for ALPS and the risk of lymphoma. Therapeutics that modulate NRAS or its effectors might be beneficial in selected disorders of immune homeostasis and autoimmunity.

Crossroads of Autophagy and Apoptosis: Regulation and Corpse Clearance

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Apoptosis and autophagy are both tightly regulated biological processes that play a central role in tissue homeostasis, development, and disease. Increasing evidence suggests that there is extensive interplay between these two pathways. Previously, we have shown that the cellular anti-apoptotic protein, Bcl-2, inhibits Beclin 1-dependent autophagy and autophagy gene-dependent cell death. We have also shown that the autophagic machinery is required for the generation of engulfment signals and apoptotic corpse clearance during mammalian embryonic development. In this meeting, we will discuss new data identifying a biochemical mechanism by which starvation disrupts the Bcl-2/Beclin 1 complex to activate autophagy and new data describing a role for autophagy genes in apoptotic corpse clearance during *C. elegans* embryonic development.

Many Ways to Die: A Caspase-independent Mitochondrial Pathway Triggered by Granzyme A

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The killer lymphocyte protease granzyme A (GzmA) triggers caspase-independent target cell death with morphological features of apoptosis. We previously showed that GzmA acts directly on mitochondria to generate reactive oxygen species (ROS) and disrupt the transmembrane potential ($\Delta\Psi_m$), but does not permeabilize the mitochondrial outer membrane. Mitochondrial damage is critical to GzmA-induced cell death since cells treated with superoxide scavengers are resistant to GzmA. Here we find that GzmA accesses the mitochondrial matrix to cleave the complex I protein NDUFS3, an iron-sulfur subunit of the NADH:ubiquinone oxidoreductase after Lys^{56} to interfere with NADH oxidation and generate superoxide anions. Target cells expressing a noncleavable mutant of NDUFS3 are resistant to GzmA-mediated cell death, but remain sensitive to GzmB.

The Dependence Receptor notion: When apoptosis regulates tumor progression and metastasis

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Dependence receptors share the ability to induce apoptosis in settings of absence of their respective ligands. As such, they create cellular states of dependence on their respective ligands. We reported that DCC (Deleted in Colorectal Cancer), a candidate tumor suppressor that is a receptor for netrin-1, an axon guidance cue, is such a prototype dependence receptor. A similar behavior was also found for other netrin-1 receptors, UNC5H (UNC5H1-3), hence suggesting that netrin-1 is not only a guidance cue but also survival factor via its receptors DCC and UNC5H. We then have proposed that UNC5H and DCC may be considered as tumor suppressors that would induce apoptosis of tumor cells that would grow out of ligand availability. Along this line, both DCC and UNC5H expression appears drastically inhibited in numerous carcinomas including colorectal tumors. Moreover, ectopic expression of netrin-1 or inactivation of UNC5H3 in mice gut leads to an increased tumorigenesis. Here we will provide an overview of the implication of the netrin-1 dependence receptors in primary cancer and metastasis and will describe how this may be used to propose novel therapeutic approaches.

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Inhibitor of Apoptosis (IAPs) Proteins in Ubiquitin-mediated Signalling

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The covalent attachment of ubiquitin to target proteins influences various cellular processes including protein turnover, cell-surface receptor signaling, DNA damage responses and cell survival. Ubiquitylation culminates in the conjugation of ubiquitin, either as a single moiety (monoubiquitylation), or as Lys48- or Lys63-linked polyubiquitin chains. Although ubiquitylation may regulate protein function through conformational changes, the most common mode of regulation by ubiquitin-conjugation involves specific “ubiquitin-receptors” that recognise ubiquitylated proteins and link them to downstream biochemical processes.

Although, members of the IAP family are best known for their ability to regulate caspases and cell death, it is now clear that IAPs also control a number of caspase-independent processes, which includs NF-kappaB signaling and innate immunity. Here, we will discuss our latest findings regarding the function of IAPs in ubiquitin-mediated signalling events.

One, Two, Three – p53, p63, p73 and chemosensitivity

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The discovery of the p53-related genes p63 and p73 raised the possibility that they may be cancer-associated genes and as a consequence that p53 is not the only determinant in predicting response of human tumors to chemotherapeutic drugs, but instead the status of a network that contains p53, p63 and p73.
The identification and characterization of the p53- p63- p73-network provides evidence of a tight link between developmental processes and tumorigenesis. p63 and p73 are important for normal development and differentiation, but are also implicated in human carcinogenesis. The p53 family genes produce multiple isoforms that vary in composition of the NH_2- and C-termini. The ΔN isoforms can oppose the transactivation capabilities of the full length proteins. The finding that a significant percentage of tumors select for dominant negative p63- and/or p73-isoforms argues for their oncogenic role.
Like p53, TAp63 and TAp73 activate genes exerting roles in different steps of the apoptosis program. Accordingly, chemosensitivity is influenced by the interactions between the different isoforms of the p53 family members.
Distinct post-translational modifications and interactions with cofactors further modulate the transcriptional activity of the p53 family members in response to particular stress signals.
Thus, therapeutic modulation of TAp63/ΔNp63, TAp73/ΔNp73 and mutant p53 levels might be used to target the large percentage of human tumors that harbor p53 mutations and/or overexpress ΔNp63 or ΔNp73. Interfering with the expression or function of ΔNp63 and/or ΔNp73 and/or mutant or inactivated p53 in tumor cells may render such tumors more responsive to therapy and reduce their aggressiveness and metastatic capacity.

Proteolytic pathways in cell death

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The form of cell death known as apoptosis is primarily a dismantling mechanism that results in the removal of unwanted cells in vivo. The pathways that converge on the execution of this cell death program require the participation of members of the caspase family of cysteine proteases. Apoptosis is initiated by ligation of death receptors (extrinsic pathway), or developmental cues, stress and genomic damage (intrinsic pathway). These events result in the activation of apical (or initiator) caspases, and converge on the direct activation of effector (or executioner) caspases. Thus, a minimal two step activation cascade is at the heart of apoptosis. The pathways are regulated by endogenous caspase inhibitors – members of the IAP family of zinc finger proteins, which operate as direct fast binding inhibitors. Determining how apoptosis is controlled affords the opportunity to understand the fundamental processes that regulate proteolytic pathways. This talk will concentrate on biochemical and structural elucidations of caspase activation and inhibition, cast in the context of apoptosis regulation, and conclude with recent advances in the determination of the in vivo substrates of specific limited proteolysis.

Keeping mitochondria and endoplasmic reticulum in shape and together: a matter of life and death

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Mitochondria are complex organelles whose internal structure and cytosolic organization is controlled by a growing number of “mitochondria-shaping” proteins. These include mitochondrial proteins like Mitofusin (Mfn) 1 and 2, Optic Atrophy 1 (Opa1); as well as cytosolic ones like the dynamin-related protein 1 (Drp1) and its receptor on the outer mitochondrial membrane Fis1. These proteins influence not only the shape of mitochondria, but also the function of the organelle and integrated cellular signalling cascades, including apoptosis. We undertook a genetic approach to elucidate function and regulation of these proteins. Our recent data indicate that Opa1 and Parl, an inner membrane rhomboid protease, regulate the cristae remodelling pathway of apoptosis. Similarly, excess fission promoted by Fis1 results in alteration in the shape of the cristae, mitochondrial dysfunction and ultimately death. Surprisingly, apoptosis depends on adequate levels of Ca^{2+} in the endoplasmic reticulum (ER), strengthening the interaction between these two organelles in controlling signalling cascades like apoptosis. The role of mitochondria-shaping proteins in the cross-talk between mitochondria and ER is further substantiated by the finding that Mitofusin 2 regulates the shape of the ER and more importantly, the physical and functional connection between ER and mitochondria.

Coordinate Regulation of Cell Death and Tissue Regeneration

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Inhibitor of Apoptosis Proteins (IAPs) are a conserved family of cell death regulators that can bind to and inhibit caspases. Many IAPs function as ubiquitin ligases to stimulate degradation of important apoptotic proteins, including caspases. However, IAPS must be inactivated in cells that are doomed to die. Work in *Drosophila* first demonstrated that this is accomplished by Reaper-family proteins. Reaper induces apoptosis by both disrupting caspase-IAP interactions, and also by specifically stimulating the degradation of *Drosophila* IAP1 (Diap1). Diap1 is also a key regulator for compensatory proliferation. In many metazoans, stress-induced apoptotic cell death can be compensated for by extra-proliferation of neighboring cells. Inactivation of Diap1 stimulates activation of the JNK pathway, which in turn induces transcriptional activation of the mitogens Wingless and Dpp in doomed cells. These findings show that apoptotic cells can activate signaling cascades to stimulate tissue regeneration.

We have also generated mice lacking the mammalian IAP-antagonist ARTS. Deletion of the *Sept4* gene, which encodes ARTS causes elevated XIAP protein levels in certain tissues and defects in the caspase-mediated elimination of bulk cytoplasm during spermiogenesis. *Sept4*-Null mice also have cell death defects and show increased spontaneous tumor formation. These observations provide the first direct *in vivo* evidence for a critical physiological role of IAP regulation in the control of cell death and tumor suppression in mammals.

Autophagy and necrotic cell death in *Caenorhabditis elegans*

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While the crosstalk between autophagy and apoptotic cell death, as well as the contribution of autophagy in clearing expanded polyglutamine-repeat proteins have been studied extensively, the role of autophagy in necrotic cell death remains elusive. Non-apoptotic mechanisms contribute critically to cell death accompanying neuronal injury (as occurs during stroke and ischemia, for example), and cell death consequent to neurodegenerative disease. An assortment of genetic lesions and treatments are available in *C. elegans*, which trigger degenerative, necrotic cell death that occurs independently of apoptosis-regulatory or executor genes, and is similar to mammalian excitotoxic cell death. We have used this exceptionally well-characterized model of necrosis in *C. elegans* to dissect the role of autophagy in necrotic cell death. We find that necrosis induced by diverse insults is suppressed in genetic backgrounds, deficient for a key regulator of autophagy induction. In addition, specific pharmacological inhibition of autophagy results in similar protection from necrotic cell death. Therefore, induction of autophagy is required for necrotic cell death in *C. elegans*. Furthermore, impairment of autophagosome nucleation, expansion and completion, suppresses cell death. Similarly, inhibition of Atg protein complex disassembly and retrieval from mature autophagosomes protects from necrosis. Our observations indicate that autophagosome formation and maturation is required for necrosis in *C. elegans*. We utilized a reporter fusion of GFP with LGG-1/Atg8, a protein associated with the autophagosome membrane to visualize autophagosomes in dying neurons during necrosis. We find that excessive autophagosome formation is induced early during necrotic cell death in nematode neurons. Finally, we show that autophagy synergizes with lysosomal proteolytic mechanisms to facilitate necrotic cell death in *C. elegans*. Suppression of necrosis by lysosomal aspartyl protease or V-ATPase deficiency is enhanced by conditions that impede the autophagic process. We propose that runaway autophagy is an important, conserved mechanism of cellular destruction during necrotic cell death.

TRAIL-induced apoptosis: biochemical mechanism and identification of a physiological role in metastasis prevention

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TRAIL/Apo2L (TNF-related apoptosis-inducing ligand) is a promising anticancer agent due to its ability to selectively induce apoptosis in tumour cells but not in most non-transformed cells. To appreciate its full clinical potential it is important to understand the biochemical principles which govern sensitivity and resistance to apoptosis induction by TRAIL. In this talk the mechanisms of TRAIL sensitivity versus resistance will be addressed in cancer cell lines as well as in primary human hepatocytes and in primary human cancer cells. Then, data on the identification of a specific function of TRAIL-R in the suppression of metastasis from autochthonous murine tumours will be presented. Finally, a short overview on the current clinical developments with TRAIL receptor agonists, especially their potential in novel combinatorial treatments, will be discussed.

Role of Autophagy in Cancer

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Autophagy is a bulk degradation process that promotes survival under metabolic stress, but can also be a means of cell death if executed to completion. Monoallelic loss of the essential autophagy gene *beclin1* causes susceptibility to metabolic stress, but also promotes tumorigenesis. This raises the paradox that the loss of a survival pathway enhances tumor growth where the exact mechanism is not known. We have found that autophagy is a survival pathway utilized by tumor cells to survive metabolic stress. Tumor cells with defect in apoptosis survive long-term metabolic deprivation through autophagy that when inhibited by allelic loss of the *beclin1*, *atg5* deficiency or PI-3 kinase pathway activation, promotes necrotic cell death due to metabolic catastrophe. In vivo tumor necrosis brought about by defects in apoptosis and autophagy under conditions of metabolic stress is associated with inflammation and accelerated tumor growth. Thus autophagy can function as a tumor suppression mechanism by limiting necrosis and inflammation. Failure to sustain metabolism through autophagy is also associated with increased DNA damage, gene amplification and aneuploidy, and this genomic instability may promote tumorigenesis. Thus autophagy maintains metabolism and survival during metabolic stress that serves to protect the genome and prevent chronic inflammation, explaining the paradox that the loss of a survival pathway leads to tumor progression. Identification of this novel role of autophagy may be important for rational chemotherapy and therapeutic exploitation of autophagy inducers as potential chemopreventive agents.

Lecture Abstracts

Short Oral Communications

Abstracts in this section are in **alphabetical** order by the name of the presenting speaker (underlined).

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Role and function of the apoptosis-regulator PIDD

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PIDD (p53 induced protein with a death domain) is a protein implicated in the cellular responses to genotoxic stress. Autoproteolytic cleavage of PIDD in response to DNA-damage leads to the generation of two different fragments. The shorter fragment, PIDD-C, forms a complex with RAIDD and procaspase-2 in the cytoplasm – the so-called "PIDDosome", which leads to the activation of caspase-2 that contributes to apoptotic cell death. The longer fragment, PIDD-CC, was reported to associate with RIP1 and NEMO in the nucleus. The formation of this complex leads to the activation of NF-κB and is considered to promote cell survival enabling DNA repair.
Thus PIDD can act as a switch between the pro- and the antiapoptotic signalling pathways in response to genotoxic stress, but the mechanisms that tilt the balance towards life or death remain unknown.
To study the function of PIDD, constitutive knockout mice were generated and analyzed.
Pidd$^{-/-}$, *caspase-2*$^{-/-}$, *p53*$^{-/-}$ and wildtype MEFs were exposed to genotoxic stress to examine, whether loss of one of these proteins allows clonal survival after DNA-damage or thermal stress. Colony forming assays using cells exposed to UV-irradiation or heat-shock revealed that neither the lack of PIDD nor caspase-2 provided a survival advantage when compared with wildtype MEFs. However, p53-deficient cells were still able to form colonies.
In order to determine whether loss of PIDD or caspase-2 is able to provide a short-term survival advantage we investigated apoptosis induction in MEFs by AnnexinV/PI staining and FACS analysis. However, no differences in the viability of the *pidd*$^{-/-}$ and *pidd*$^{+/+}$ cells were observed. Surprisingly, *caspase-2*$^{-/-}$ MEFs displayed even higher sensitivity to heat shock or UV irradiation.
In conclusion, our data suggests that activation of caspase-2 by the PIDDosome is redundant in cell death signalling and that the true physiological function of caspase-2 remains to be identified.

Keywords: apoptosis, PIDD, caspase-2

Early molecular initiation steps of hypoxia-induced autophagy: involvement of BNIP3 and BNIP3L, two HIF-induced BH3-only proteins

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Hypoxic stress is often associated with tumour's microenvironment. Cell response to oxygen decrease is under the control of the "hypoxia-inducible factor" which drives the induction or repression of genes controlling multiple cell functions such as angiogenesis, metabolism, survival/cell death… This hypoxia-signalling pathway leads to the adaptation of cancer cells to such a hostile environment in which they can survive. Interestingly amongst HIF-1a target genes we can found BNIP3 and BNIP3L, two pro-apoptotic BH3-only protein members of the Bcl2 family. Although they have been described as promoters of a necrosis-like cell death rather than apoptosis their precise role remains highly confused. In addition it appears that BNIP3/BNIP3L-mediated cell death following hypoxia requires also secondary stresses such as acidosis to occur.

Recently we have observed that hypoxia alone is not sufficient to triggered cell death. While normal cells (CCL39) and tumour cell lines (MCF7, PC3, LS174) exposed for 48h to hypoxia (from 1 to 0.1% O2) fully express the pro-apoptotic BNIP3 and BNIP3L markers, they display minimal cell death (less than 5%). However, hypoxic stress clearly induced autophagy, a cell rescue process, in all cells tested. Thus we hypothesized that the induction of BNIP3/BNIP3L in a hypoxic microenvironment contributes to survival rather than cell death by inducing autophagy. We have observed that silencing of either BNIP3 or BNIP3L had little effect while silencing of both proteins suppressed hypoxia-induced autophagy.

Here we have investigated the role of BNIP3 and BNIP3L in the molecular events leading to autophagy. First we observed that as described in starvation-induced autophagy, the dissociation of the Bcl2/Beclin 1 complex is a characteristic of hypoxia-induced autophagy required for the autophagic initiation pathway. We found that both BNIP3 and BNIP3L target preferentially the evolutionary conserved Atg6/Beclin 1, key of autophagy initiation. Our results indicate that the couple BNIP3/BNIP3L participate to the dynamics of Beclin 1-containing complexes therefore unlocking the Bcl2 repression of Beclin 1.

In conclusion these results show that BNIP3 and BNIP3L act as pro-autophagic factors in hypoxia (48h). Hence this scheme represents a mean for tumour cells to adapt to hypoxic microenvironment.

Keywords: Hypoxia, Autophagy, BNIP3/BNIP3L, Beclin 1

Pro-apoptotic BAX and BAK require endoplasmic reticulum-derived sphingolipid metabolism to induce mitochondrial outer membrane permeabilization

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Mitochondria are physically associated with the endoplasmic reticulum (ER), yet little is known on how the mitochondrial-ER network impacts on mitochondrial outer membrane permeabilization (MOMP), the ultimate decision in the initiation of apoptosis. Dissociation of the ER from intact mitochondria inhibited BID- and BIM-induced cytochrome c release. Biochemical purification of a neutral sphingomyelinase activity that correlated with MOMP suggested that sphingolipid metabolism may coordinate BAX/BAK activation and subsequent cytochrome c release. Using minimal ER-derived lipids and enzymes, complete sensitivity to BID-dependent MOMP was achieved by in vitro reconstitution of the sphingolipid metabolic pathway. Furthermore, BAX and BAK have different sphingolipid metabolic product requirements for activation and cytochrome c release. Specific pharmacological inhibitors of ER and mitochondrial sphingolipid metabolism also blocked MOMP from intact ER-mitochondrial preparations, but failed to influence cytochrome c release in the presence of sphingolipid reconstituted purified mitochondria. Our studies suggest that BAX/BAK activation and apoptosis coordinate through both the induction of pro-apoptotic BH3-only proteins, and a specific mitochondrial lipid milieu that is actively maintained by the ER.

Keywords: BCL-2 family, mitochondria, cytochrome c, sphingolipids

CD95/Fas triggers the "journey" of GD3 to mitochondria via its association with microtubules

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We analyzed the mechanism of intracytoplasmic movements of GD3, i.e. the "journey" of GD3 from the plasma membrane to mitochondria, in CD95/Fas primed T cells. We found that GD3 association with microtubules was mandatory to obtain a directional movement of GD3 towards mitochondria. Finally, after one hour, GD3 was detectable in mitochondria, after CD95/Fas triggering. In particular, we observed that raft-like microdomains can be detected on mitochondrial membranes. They could represent preferential sites where some key reactions can be catalyzed, contributing to cell death execution steps. For instance, raft-like domains, enriched in gangliosides (GD3, GM3), but with a relatively low content of cholesterol, are detectable on mitochondrial membrane(s), where Bcl-family proteins (i.e. truncated Bid and Bax) are recruited. In addition, the formation of a multimolecular complex that includes VDAC-1, Bcl-2 family and fission proteins, e.g. h-Fis, has been demonstrated.
Mitochondrial lipid raft-like microdomains may thus instruct a sort of mitochondrial "dynamic chamber" where specific reactions can be catalyzed, leading to cell survival or death.

Keywords: apoptosis, mitochondria, lipid raft

Cathepsin D initiates neutrophil apoptosis and its inhibition blocks the resolution of inflammation

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In the resolution of inflammatory responses, neutrophils rapidly undergo apoptosis. We report here a new pro-apoptotic pathway in which cathepsin D directly activates caspase-8. Cathepsin D is released from azurophilic granules in neutrophils in a caspase-independent manner. Under inflammatory conditions, however, the translocation of cathepsin D in the cytosol is blocked. Pharmacological or genetic inhibition of cathepsin D resulted in delayed caspase activation and reduced neutrophil apoptosis. Cathepsin D deficiency or lack of its translocation in the cytosol prolongs innate immune responses in experimental bacterial infection and in septic shock. Thus, we identified a new function of azurophilic granules, which regulate, besides their role in bacterial defense mechanisms, the life-span of neutrophils and therefore the duration of innate immune responses through the release of cathepsin D.

Keywords: Apoptosis, cathepsin D, innate immunity, neutrophils, signal transduction

Molecular effectors of autophagy and apoptosis in ER-photodamaged cells

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Photodynamic therapy (PDT) is an anticancer therapy utilizing cytotoxic reactive oxygen species (ROS) to kill the cancer cells[1]. Photodamage to the sarco(endo)plasmic Ca^{2+}-ATPase (SERCA) pump, due to the local generation of $^1O_2$ in the ER upon hypericin light irradiation, results in loss of ER Ca^{2+} homeostasis and is the most apical event causative for cell death in this paradigm[2]. Downstream of the ER-Ca^{2+} emptying, both caspase-dependent and -independent pathways are activated to ensure cell demise. Apoptosis is dependent on the availability of pro-apopototic Bax and Bak proteins, which are essential effectors of the mitochondrial membrane permeabilization and subsequent caspase activation[2]. Previous investigation has shown that cellular demise in murine embryonic fibroblasts (MEFs) doubly deficient for Bax/Bak (DKO) is due to the induction of an autophagic cell death pathway[2]. However, the exact role of autophagy in apoptosis-competent cells following PDT is not completely understood. To further elucidate the molecular players and the functional role of autophagy in PDT treated cells, we analyzed signalling events underlying cell death in wild type MEFs and in MEFs genetically deprived in mitochondrial apoptosis, such as in Bax/Bak (DKO) or Apaf-$1^{-/-}$ cells, as well as in apoptosis-competent MEFs in which autophagy can be turned off by knocking out essential autophagy genes (atg-genes).
In apoptosis-competent cells the induction of apoptotic cell death occurs concomitantly with the onset of autophagy, suggesting that both pathways are simultaneously propagated in response to ER photodamage. However, both the intensity and kinetics of the autophagic process are enhanced in the absence of caspase signalling, either caused by deficiency of pro-apoptotic Bax and Bak proteins or Apaf-1. Pharmacological inhibition of caspases in human cancer cells enhances PDT-induced autophagy, suggesting that once activated the caspase-signalling may deter autophagy progression. Under conditions of caspase inhibition photosensitized cells are fated to die with the phenotypic manifestations of autophagy. The knock-down of essential atg-genes in apoptosis-competent cells will be used to further define the exact role of autophagy induced by hypericin-PDT and to unravel the potential interplay between apoptosis and autophagy.

[1]Dolmans et al, (2003) Nat Rev Cancer 3:380-7
[2]Buytaert et al, (2006) FASEB J. 20:756-8

Keywords: Photodynamic Therapy, Apoptosis, Autophagy, Signalling, Endoplasmic Reticulum, Oxidative Stress

Place of metacaspases like proteins in Plasmodium falciparum apoptosis

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The ability to undergo apoptosis, previously thought to be exclusive to multicellular organisms, has been demonstrated in unicellular parasites. Concerning *Plasmodium,* apoptosis figures as a key component in life cycle success. Here we report evidence for *Plasmodium falciparum* erythrocytic stage apoptosis upon chloroquine treatment. Cell condensation has been detected with the presence of DNA fragmentation (TUNEL-positive nuclei) and similar mitochondrial depolarization patterns have been observed after incubation with chloroquine and atovaquone or artemether, two well-known depolarizing drugs. Metacaspases have been described as caspase-like proteins in plants and unicellular organisms. Three metacaspases like proteins have already been annotated in *P. falciparum* genome Pf13_0289, PF14_0363 and Pf14_0160 (PfMCA1, PfMCA2 and PfMCA3 respectively). These three metacaspases have been cloned and expressed in several heterologous systems to examine their processing and enzymatic activity. Sequences analysis of PfMCA1 has revealed the presence of catalytic caspase domains and a putative caspase recruitment domain (CARD) belonging to the death domain (DD) superfamily. PfMCA1 revealed also a caspase 3/7 activity and a prodomain cleavage according to the common caspase maturation processing, indicating metacaspases could be involved in *P. falciparum* apoptosis.

Keywords: plasmodium, metacaspase, apoptosis

The mitochondrial TOM complex is required for tBid/Bax-induced cytochrome c release

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Cytochrome c release from mitochondria is a key event in apoptosis signaling that is regulated by Bcl-2 family proteins. Cleavage of the BH3-only protein Bid by multiple proteases leads to the formation of truncated Bid (tBid) which, in turn, promotes the oligomerization/insertion of Bax into the mitochondrial outer membrane and the resultant release of proteins residing in the intermembrane space. Bax, a monomeric protein in the cytosol is targeted by a yet unknown mechanism to the mitochondria. Several hypotheses have been put forward to explain this targeting specificity. Using mitochondria isolated from different mutants of the yeast Saccharomyces cerevisiae and recombinant proteins, we have now investigated components of the mitochondrial outer membrane that might be required for tBid/Bax-induced cytochrome c release. Here, we show that the protein translocase of the outer mitochondrial membrane, the TOM complex, is required for Bax insertion and cytochrome c release.

Keywords: Mitochondria, Bax, tBid, TOM complex, yeast

Bcl-2 Induces Pro-oxidant State by Engaging Mitochondrial Respiration in Tumor Cells

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Mitochondrial respiration, the key process behind cellular energy production, is critical for cell proliferation, growth and survival. However, the regulation of mitochondrial respiratory function in tumor cells is not well understood. In this study, we propose a model whereby tumor cells possess the capacity to fine-tune the balance between energy demands and mitochondrial reactive oxygen species (ROS) status, in order to maintain a milieu optimal for survival. This is achieved through the moderation of mitochondrial respiration, depending on the ROS context within the organelle, with the main players being Bcl-2 and cytochrome c oxidase (COX). We report a higher level of COX activity, oxygen consumption and mitochondrial respiration in tumor cells overexpressing Bcl-2. Transient overexpression, gene silencing and pharmacological inhibition of Bcl-2 corroborate these findings. Interestingly, Bcl-2 is also able to regulate mitochondrial respiration and COX activity in the face of mounting ROS levels, triggered by mitochondrial complex inhibitors. In this respect, it is plausible to suggest that Bcl-2 may be able to create an environment, most suited for survival by adjusting mitochondrial respiration accordingly to meet energy requirements, without incurring an overwhelming, detrimental increase in intracellular ROS.

Note: Part of this work has been accepted for publication in Cell Death and Differentiation and the manuscript was highlighted as a featured article on NPG Cancer website (June 4-10)

Keywords: Bcl-2, COX activity, Superoxide, Mitochondrial Respiration

Phagocytosis of cells dying through autophagy evokes a pro-inflammatory response in macrophages

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In living cells autophagy takes place usually unnoticed to the neighbours. However, its co-ocurrance with cell death may contribute to the clearance of these dying cells by recruited phagocytes. Autophagy associated with programmed cell death is essential for presentation of phoshatidylserine (PS) on the cell surface for clearance of apoptotic cells (Qu *et al.* Cell 2007). Recently, we have demonstrated that when cell death is triggered by autophagy in MCF-7 cells, the corpses were efficiently phagocytosed by both human macrophages and non-dying MCF-7 cells. Death as well as engulfment could be prevented by inhibiting autophagy. Based on our data, two molecular mechanisms have been proposed for the uptake of cells which die through autophagy. A PS-dependent pathway which was exclusively used by the living MCF-7 cells working as non-professional phagocytes. In macrophages, a PS-independent uptake mechanism was also evoked: gene expression data and indirect evidences suggest that calreticulin-mediated recognition, tethering, tickling and engulfment processes were utilized here. LPS-induced production of pro-inflammatory cytokines in macrophages could be prevented by the dying autophagic cells. However, it was also observed that phagocytosis of cells dying through autophagy leads to a pro-inflammatory response in macrophages characterized by the induction and secretion of IL-1β, IL-6, TNFα, IL-8 and IL-10. The IL-1β secretion could be inhibited by preventing autophagy or blocking caspase-1 activation. The results suggest that inside macrophages cells dying through autophagy can activate NLR family protein(s) during the phagocytosis process.

Keywords: autophagy, cell death, phagocytosis, calreticulin, pro-inflammatory response

Positive regulation of apoptosis by HCA66, a new Apaf-1 interacting protein, and its putative role in the physiopathology of NF1 microdeletion syndrome patients

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Apoptosis is an evolutionarily conserved cell suicide process that depends on the activation of caspases, a group of cysteine aspartic acid-specific proteases. As a component of the apoptosome, a caspase-activating complex, Apaf-1 plays a central role in the mitochondrial apoptotic pathway. We report here the identification of a novel Apaf-1 interacting protein, hepatocellular carcinoma antigen 66 (HCA66) that is able to modulate selectively Apaf-1-dependent apoptosis through its direct association with the CED4 domain of Apaf-1. Expression of HCA66 was able to potentiate Apaf-1, but not receptor-mediated apoptosis, by increasing downstream caspase activity following cytochrome c release from the mitochondria. Conversely, cells depleted of HCA66 were severely impaired for apoptosome-dependent apoptosis. Interestingly, expression of the Apaf-1-interacting domain of HCA66 had the opposite effect of the full-length protein, interfering with the Apaf-1 apoptotic pathway. Using a cell-free system, we showed that reduction of HCA66 expression was associated with a diminished amount of caspase-9 in the apoptosome, resulting in a lower ability of the apoptosome to activate caspase-3. *HCA66* maps to chromosome 17q11.2 and is among the genes heterozygously deleted in neurofibromatosis type 1 (NF1) microdeletion syndrome patients. These patients often have a distinct phenotype compared to other NF1 patients, including a more severe tumour burden. Our results suggest that reduced expression of HCA66, owing to haploinsufficiency of *HCA66* gene, could render NF1 microdeleted patients-derived cells less susceptible to apoptosis.

Keywords: Apaf-1; apoptosome; HCA66; NF1; cell death

Differential regulation of pro-survial autophagy by Bcl-2 and Bcl-xL

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Autophagy is a eukaryotic intracellular catabolic process highly conserved throughout evolution. Depending on the model studied, autophagy is described to be involved in homeostasis, development and disease. In most models, this programme is induced under stress conditions, and its execution facilitates survival. In mammals however, autophagy has also been pointed out as a death programme. The mechanisms responsible for its implication both in survival and death have yet to be unravelled. Identification of interrelationships with the apoptotic pathway has begun to shed light on the involvement of autophagy in cell death. Our data show that anti-apoptotic proteins of the Bcl-2 family play a role in regulating survival autophagy. We have observed that an over-expression of Bcl-2 and Bcl-xL is accompanied by an increase in the size of autophagic vesicles and a stimulation of autophagic proteolytic activity. In addition, Bcl-xL seems to exert a tight control on autophagy regulation since its downregulation causes a more potent inhibition of autophagy than Bcl-2. Furthermore, the regulation of autophagy by Bcl-2 and Bcl-xL does not seem to be redundant since, in contrast to Bcl-2, the autophagic function of Bcl-xL is, on the whole, independent of its interaction with Beclin 1. Our work highlights that, in addition to their well-established anti-death functions during apoptosis, Bcl-2 and Bcl-xL have a broader role in survival mechanisms and may stand at the cross-roads between pro-survival and pro-death autophagy.

Keywords: autophagy, Bcl-2 family proteins

Quantitative real time analyses of the signaling dynamics during apoptosis execution

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Apoptotic cell death plays an important role throughout the entire lifetime of multicellular organisms. Following mitochondrial outer membrane permeabilisation (MOMP), the release of cytochrome-c (cyt-c) and Smac into the cytosol is a key requirement for rapid and efficient caspase-3 activation and apoptosis execution in most cell types. Utilizing a systems biology approach of combined computational modelling and live cell imaging, we were able to describe the molecular signalling dynamics during cell death execution in real time. In HeLa cervical cancer and MCF-7 breast cancer cells we identified sharp concentration thresholds for caspase-3 and XIAP that decide on cell death execution or apoptosis resistance. We also found that the release kinetics of the XIAP-inhibitor Smac, and the overall Smac concentration do not significantly influence cell death kinetics and that proteasomal Smac degradation following MOMP is enforced upon caspase inhibition in these scenarios.
MOMP has been found to be impaired in many tumours as a consequence of increased expression of antiapoptotic Bcl-2 family members. We therefore mathematically and experimentally analysed the biochemical network of apoptosis execution in the absence of MOMP. We found that as long as cyt-c is not released, the downstream signalling network remains resistant to cell death execution even upon massive perturbation of enzymatic activity, protein interactions and concentrations of both caspase-3 and XIAP. Aiming to re-sensitize Bcl-2 overexpressing cells to apoptosis we identified a combined treatment with proteasome inhibitors and the caspase activating compound PAC-1 to be a promising new strategy to directly activate the cell death execution network.

Keywords: XIAP, caspase-3, systems biology, live cell imaging

ATM kinase activity modulates Fas-sensitivity through the regulation of Flip in lymphoid cells

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Ataxia Telangiectasia (A-T) is a rare cancer-predisposing genetic disease, caused by the lack of functional ATM kinase, a major actor of the DSB DNA-damage response. A-T patients show a broad and diverse phenotype, which includes an increased rate of lymphoma and leukemia development. Fas-induced apoptosis plays a fundamental role in the homeostasis of the immune system and its defects have been associated with autoimmunity and lymphoma development.
We therefore investigated the role of ATM kinase in Fas-induced apoptosis. Using A-T lymphoid cells we could show that ATM deficiency causes resistance to Fas-induced apoptosis. A-T cells upregulate FLIP protein levels, a well-known inhibitor of Fas-induced apoptosis. Reconstitution of ATM kinase activity was sufficient to decrease FLIP levels and to restore Fas-sensitivity. Conversely, genetic and pharmacological ATM kinase inactivation resulted in FLIP protein upregulation and Fas resistance.
Both ATM and FLIP are aberrantly regulated in Hodgkin lymphoma. Importantly, we found that reconstitution of ATM kinase activity decreases FLIP protein levels and restores Fas-sensitivity in Hodgkin lymphoma derived cells. Overall, these data identify a novel molecular mechanism through which ATM kinase may regulate the immune system homeostasis and impair lymphoma development.

Keywords: ATaxia Telangectasia, ATM kinase,Fas induced apoptosis,FLIP, lymphoma

A novel Bcl-XL peptide inhibitor as a potential anti-leukaemic agent.

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Programmed apoptotic cell death occurs following several kinds of physiological and pathological events. When activated, the extrinsic apoptotic pathway is especially regulated by membrane surface receptors like FasR, TNFR, TRAILR, while the intrinsic apoptotic pathway involves mitochondria, through the interaction network of the Bcl-2 family proteins. Concerning their role, these proteins can be classified in two main subfamilies: anti-apoptotic members (e.g. Bcl-2, Bcl-XL, Bcl-w, Mcl-1), characterized by four Bcl-2 homology domains (BH1-BH4), and pro-apoptotic members, divided into two different groups: Bax-like proteins, with three BH3 domains (e.g. Bak, Bax), and BH3-only proteins (e.g. Bim, Bid, Bad, Bmf, Noxa, Puma). Bcl-XL over-expression is associated with different haematological and solid malignancies, and is also correlated with tumour resistance to common cytotoxic drugs. Recently, Bcl-XL became an intriguing target-signaling-protein for the development of novel anti-cancer agents. A set of peptides, whose sequence was inspired to the human Bim-BH3 domain, was synthesized through the Fmoc-SPPS method. A rational study of the structural interaction between Bcl-XL and the Bim-BH3 wt peptide (aa 83-104) led us to a novel lead molecule (072RB). The replacement of some specific residues with natural and non-natural aminoacids and the C-terminus addition of a 16-mer internalizing sequence, induced a dramatic increase in the affinity for the Bcl-XL target: affinity constants were evaluated using a fluorescence anisotropy assay. Our most active peptide, named 072RB, showed a very efficient Bcl-XL binding (with a Kd value in the low nM range). Biological activity could be drastically reduced changing a single critical aminoacid side-chain. Good serum stability, and excellent cell internalization were observed. Confocal microscopy demonstrated 072RB co-localization with the surface of mitochondria. In addition, 072RB showed a decrease of mitochondrial potential, activation of caspases 9 and 3, strong pro-apoptotic effects on U937, Jurkat and Namalwa tumour cell line (FACS analyses). Total Body Luminescence experiments on NOD/SCID mice injected with U937-luciferase+ cells, showed statistically significant anti-proliferative results, in the treatment of acute myeloid leukaemia (U937). Indeed, 072RB, administered intravenously in PBS (15 mg/Kg, three times, 48 hours intervals starting 48 hours after tumour cell injection), dramatically delayed leukaemic cell growth.

Keywords: Apoptosis, Bcl-XL, Bim, leukaemia

Necrotic cell death, a live experience.

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Necrosis has long time been described as accidental and uncontrolled cell death as a consequence of physico-chemical stress. Recently, it becomes clear that necrotic cell death is as well controlled and programmed as caspase-dependent apoptosis, and that it may be an important cell death mode with important pathological and physiological relevance. Use of a wide variety of inhibitors (ROS scavengers, complex I inhibitors, Ca2+ chelators, calpain inhibitors, cathepsin inhibitors and lipoxygenase inhibitors) has revealed that necrotic cell death involves multiple subcellular compartments such as mitochondria, endoplasmic reticulum and lysosomes. In this study, we analyzed several biochemical and subcellular events in relation to morphological changes and cell membrane permeability (CMP) by means of time laps in TNFR1-induced necrosis. We observed that the incidence of typical necrotic morphology coincides with lysosomal membrane permeability (LMP) occurring ~ 20 min before CMP. These events were preceded by a transient increase in mitochondrial membrane potential (ΔΨm). Generation of reactive oxygen species (ROS) increased in time and was strongly induced upon LMP. In addition, cytosolic calcium strongly increases during LMP. Taken together, these results indicate that accumulation of ROS and a transient increase in ΔΨm precede LMP that acts as a proteolytic bomb resulting in typical necrotic morphological features and concomitant leakage of its cellular contents.

Keywords: Necrosis Time laps

Characterization of the BH3-only protein Bmf in cell death induction and disease

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Bmf is the latest addition to the BH3-only proteins that function as intracellular stress sensors and cell death initiators. Their activation is regulated by mechanisms as diverse as phosphorylation, proteolytic cleavage, ubiquitination, transcriptional regulation or subcellular sequestration.These distinct modes of regulation, together with their cell type specific expression and distinct binding affinities to pro-survival Bcl2-proteins enables exquisite control over apoptosis induction in response to a broad range of stimuli. Although we tend to associate certain BH3-only proteins with distinct cell death stimuli such as e.g. PUMA with DNA-damage, most cell death triggers activates more than one BH3-only protein. Therefore, it is likely that functional overlap exists within this family of proteins, an issue that we currently investigate in animals lacking individual BH3-only proteins or combinations thereof.
Gene targeting of Bmf in mice revealed that, correlating with its expression in the B cell linage, B cells start to accumulate from the early pre-B cell stage onwards leading to hyperplasia of different B cell maturation stages in all hemopoietic organs. Increased B cell numbers are based on reduced cell death sensitivity in response to stress induced apoptosis. Furthermore, we observe that Bmf plays a critical role in glucocorticoid-induced apoptosis in B lymphocytes in vivo. Our analysis of the bmf-/- mice reveals a so far unknown role for Bmf in the regulation of B cell survival and homeostasis in vivo proposing partial redundancy with Bim. To investigate overlapping functions, we have generated mice double-deficient for both, bim and bmf. Preliminary results indicate developmental abnormalities includingmalocclusion, webbed feet and vaginal atresia. Mature B cells, but surprisingly also thymocytes, show increased resistance to cell death induced in response to cytokine withdrawal, certain genotoxic agents or HDAC inhibitors, compared to cells from the single knockouts, confirming non-redundant roles for Bmf and Bim in lymphopoiesis. We are currently conducting reconstitution of lethally irradiated mice with hemopoietic stem cells from bmf-/-bim-/- mice and investigate the role of Bmf in tumorigenesis. Our most recent findings will be discussed.

Keywords: Apoptosis, Cancer, BH3-only proteins

Targeting BCL2 with ABT-737: a promising strategy to kill leukaemia cells

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Resistance of cancer cells towards apoptosis is a major problem in oncology. Despite intensive research chronic lymphocytic leukaemia (CLL) is considered to be an incurable disease. In this study, we investigate the potential use of the BCL2 antagonist ABT-737 for treatment of CLL. While being rather resistant towards some commonly used apoptotic stimuli like etoposide or TRAIL, primary CLL cells isolated from patients are highly sensitive to ABT-737, with an average EC50 in 44 patients of only 6.7 nM upon 4 h of treatment. Notably, sensitivity to ABT-737 is independent of clinical parameters and markers associated with bad prognosis, such as p53 mutation or an unmutated V_H-locus. Taken together, our data indicate that targeting BCL2 seems to be a highly promising strategy for treatment of CLL. In addition to sensitivity to ABT-737 and clinical parameters, we analyzed expression levels of BCL2 and MCL1 in CLL patients, since MCL1 is considered to be the most important factor for resistance towards ABT-737. Interestingly, sensitivity to ABT-737 is independent of BCL2 and MCL1 expression levels, indicating that expression of MCL1 is not sufficient to render CLL cells resistant to ABT-737. In addition, we further induced expression of MCL1 by cytokine treatment, which resulted in a minor delay of apoptosis that was easily overcome by treatment with slightly higher concentrations of ABT-737. Taken together, our data suggest that in contrast to previous reports obtained in a variety of cell lines, MCL1 upregulation is not sufficient to induce resistance to ABT-737 in CLL cells. Further data emphasising the exquisite sensitivity of CLL cells for ABT-737 and its underlying mechanism will be presented.

Keywords: Apoptosis, BCL2, MCL1, CLL, ABT-737

IAP antagonists inhibit tumor growth and induce TNF-dependent apoptosis

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Inhibition of apoptosis enhances the survival of cancer cells and facilitates their escape from immune surveillance and cytotoxic therapies. Inhibitor of apoptosis (IAP) protein family members are anti-apoptotic regulators that block cell death in response to diverse stimuli. IAP proteins are expressed at elevated levels in the majority of human malignancies and because they play an active role in promoting tumor maintenance they are attractive targets for developing a novel class of cancer therapeutics. We demonstrate that both monovalent and bivalent small-molecule IAP antagonists bind with high affinities to select baculovirus IAP repeat (BIR) domains of XIAP or c-IAPs. Binding of IAP antagonists results in dramatic induction of c-IAP auto-ubiquitination activity and rapid proteasomal degradation. Besides neutralizing these anti-apoptotic proteins, the IAP antagonists actively induce cell death that is dependent on TNF signaling and disrupt critical IAP:caspase and IAP:SMAC interactions in a dose dependent manner. The c-IAP1 and c-IAP2 proteins were also found to function as regulators of NF-kB signaling. Through their ubiquitin E3 ligase activities c-IAP1 and c-IAP2 promote proteasomal degradation of critical adaptor proteins in the NF-kB pathways. Finally, our IAP antagonists inhibit tumor growth in vivo as single agents and in combination with a number of standard of care anti-tumor agents. Understanding the significance of protein stability and caspase/SMAC interactions for inhibition of apoptosis by IAP proteins is important for the design of potent IAP-directed compounds for treatment of melanomas and other malignancies in which IAP expression contributes to tumor progression and resistance to conventional chemotherapeutic agents.

Keywords: IAP, apoptosis, TNF

Caspase dependent cleavage of beclin1 during apotosis

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Beclin1 is a key component of the class III phosphatidyl inositol 3-kinase complex, which is essential for the formation of autophagosomes. Mammalian Beclin1 was first identified as an interaction partner of the anti-apoptotic protein Bcl-2. This interaction is likely a point of convergence of the apoptotic and autophagic cell death machinery. Recently, the interaction between autophagy and apoptosis gained a lot of interest since it may be a decision point of stressed cells between survival (recovery by autophagy) and cell death by apoptosis. In the present study we demonstrate that apoptosis induced by IL-3 deprivation in Ba/F3 cells is associated with Beclin1 proteolytic cleavage. The presence of the pan-caspase inhibitor zVAD-fmk prevented Beclin1 cleavage. Furthermore, cotransfection and in vitro transcription/translation experiments show that Beclin1 is cleaved in the presence of caspase 3, 8 and 9 generating a ~20kDa N- and a ~40kDa C-terminal fragment. Using proteasome inhibitors we could demonstrate that the N-terminal fragment is rapidly degraded by the 26kDa proteasome, while the C-terminal fragment retains stable. By generating point mutants of Beclin1, we identified two caspase cleavage sites in the Bcl-2 binding domain. Currently, we are analysing the function of the caspase-dependent cleavage of Beclin1 by overexpressing the generated Beclin1 fragments or cleavage resistant Beclin1 mutants. Our results imply that during apoptosis the autophagic pathway is inhibited by caspases preventing the induction of autophagy.

Keywords: Autophagy, apoptosis, Beclin1, caspases

Poster Abstracts

Abstracts in this section are in **alphabetical** order by the name of the presenting author (underlined).

Supported by EC within the framework of the Marie Curie Conferences and Training Courses

Presenting author: Sonia Alcala

A high-throughput screening for mammalian cell death effectors identifies the mitochondrial phosphate carrier as a regulator of cytochrome c release

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Functional annotation of complex genomes requires the development of novel experimental platforms with increased capacity. Here we describe a high-throughput system designed to identify cDNAs whose overexpression induces morphologically distinct cell death modalities. The methodology incorporates two robotized steps, and relies on co-expression of library clones with GFP to reveal the morphological features presented by the dying cells. By using this system we screened 135.000 cDNA clones and obtained 90 independent molecules. Interestingly, three death categories were identified, namely: apoptotic, vacuolated and autophagic. Among the pro-apoptotic clones we found four members of the mitochondrial carrier family: the phosphate and adenine nucleotide (type 3) transporters, and the mitochondrial carrier homologs 1 and 2. Expression of these molecules induced cytochrome c release and caspase-9-dependent death. One of them, the phosphate carrier, was able to interact with members of the permeability transition pore complex ANT1 and VDAC1, and its binding to ANT1 was stabilized in the presence of apoptotic activators. Depletion of this carrier by siRNA delayed cytochrome c mobilization and apoptosis. These results attribute a previously undescribed apoptotic function to the phosphate carrier and, more generally, suggest that a common property of various mitochondrial transporters was exploited during evolution to regulate apoptosis.

Keywords: high-throughput screening; atypical cell death; permeability transition; phosphate carrier

Presenting author: Carolina Almeida

Rates of phosphatidylserine translocation in ejaculated and testicular human sperm

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We evaluated phosphatidylserine (PS) translocation and compared the rates of apoptosis between ejaculated and testicular sperm from different patient groups. Testicular sperm were obtained during infertility treatments by open testicular biopsy in 19 patients: 5 controls (3 anejaculation, AN; 2 oligozoospermia, OZ), 8 with obstructive azoospermia (OAZ: 4 congenital absence of the vas deferens, CBAVD; 4 inflammatory obstruction of the epididymis, sOAZ) and 6 with non-obstructive azoospermia (SAZ) due to hypospermatogenesis. Ejaculated sperm (neat and swim-up fractions) were studied in 34 cases: 9 with normal semen parameters (controls), 10 with terato-zoospermia, 8 with astheno-terato-zoospermia and 7 with oligo-astheno-teratozoospermia (OAT). Externalization of PS was detected with FITC-conjugated Annexin-V. Dead cells were stained with propidium iodide. Ejaculated sperm: in all groups, significant lower numbers of apoptotic cells were found in the swim-up fraction in comparison with the semen neat fraction; in both semen fractions, significant higher levels of apoptosis were found in the OAT group. Testicular sperm: in controls, the subgroup with OZ showed significant lower rates of apoptosis in comparison with the AN subgroup; significant higher numbers of apoptotic cells were found in the OAZ and SAZ groups regarding controls; however, these differences were towards the OZ subgroup but not to the AN subgroup; no significant differences were found between both OAZ groups (CBAVD/sOAZ) or between OAZ and SAZ. Ejaculated vs testicular sperm: significant higher rates of apoptosis were found in testicular than in ejaculated sperm (neat and swim-up fractions), for both OAZ and SAZ groups. The present data confirms that the swim-up technique is highly efficient in the negative selection of apoptotic sperm and that the group of higher risk in OAT (1). However, and contrary to the current paradigm (2), testicular sperm show higher levels of apoptosis than ejaculated sperm, independently of the azoospermia cause. This unexpected result demonstrates that the use of testicular instead of ejaculated sperm is just valid for cases with severe OAT and that other causes besides apoptosis justifies this approach.

Acknowledgements: FCT (60709/60555/59997/04, UMIB).
References: (1) Almeida et al. Hum Reprod 2005;20:1307-13; (2) Grecco et al. Hum Reprod 2005;20:226-30.

Keywords: phosphatidylserine, testicular sperm, ejaculated sperm

Presenting author: Joana Amaral

Ursodeoxycholic Acid Specifically Targets p53 to Modulate Apoptosis

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p53 plays an important role in regulating expression of genes that mediate cell cycle progression and/or apoptosis. In addition, we have previously shown that the hydrophilic bile acid ursodeoxycholic acid (UDCA) prevents TGF-β1-induced p53 stabilization and apoptosis in primary rat hepatocytes. Therefore, we hypothesized that p53 may represent an important target in bile acid-induced modulation of apoptosis and cell survival. Primary rat hepatocytes were cotransfected with plasmid DNA encoding wild-type or mutant p53 and a reporter gene construct that utilized the *bax* gene promoter to drive transcription of chloramphenicol acetyltransferase (CAT). Twelve hours prior to transfection, hepatocytes were pre-treated with either vehicle or 100 μM of UDCA. At 48 h post-transfection, hepatocytes were harvested for CAT ELISA and luciferase control assays. Cultures were also scored for apoptosis following Hoechst staining, and culture media used for viability assays. General caspase-3-like activity was determined in cytosolic protein extracts by enzymatic cleavage of the substrate. Nuclear, mitochondrial, and cytosolic fractions, or total proteins were also prepared and analyzed by immunoblotting. p53 DNA binding activity was evaluated in parallel experiments. Finally, immunofluorescence and immunoblotting were used to detect p53 cellular distribution. The results showed that UDCA reduced p53 transcriptional activity, thereby preventing its ability to induce Bax expression, mitochondrial translocation, cytochrome *c* release and apoptosis in primary rat hepatocytes. More importantly, bile acid inhibition of p53-induced apoptosis was associated with decreased p53 DNA-binding activity. Subcellular localization of p53 was also altered by UDCA. Both events appear to be related with increased association between p53 and its direct repressor, Mdm-2, as detected by immunoprecipitation analysis. Posttranscriptional silencing of the *mdm-2* gene confirmed its involvement in the antiapoptotic function of UDCA. In conclusion, these results further clarify the antiapoptotic mechanism of UDCA and suggest that modulation of Mdm-2/p53 interaction is a prime target for this bile acid.

(Supported by POCTI/SAU-FCF/62479/04 from FCT, Portugal)

Keywords: apoptosis, bile acids, liver, mdm-2, p53

Presenting author: Nicola Andina

The role of the pro-apoptotic Bcl-2 family member Bim in GM-CSF-regulated neutrophil apoptosis

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Neutrophils are constantly produced in large numbers in the bone marrow. After maturation, they enter into the circulation and undergo apoptosis. However, under inflammatory conditions, neutrophil apoptosis is delayed due to survival factor exposure, and, consequently, cell numbers increase. An important pro-inflammatory cytokine involved in the regulation of neutrophil survival/activation is granulocyte-macrophage colony stimulating factor (GM-CSF). Although GM-CSF mediates anti-apoptotic effects in neutrophils, it does not prevent apoptosis, and the survival effect is both time-dependent and limited. We therefore hypothesised that GM-CSF, in spite of mediating anti-apoptotic effects, allows the activation of pro-apoptotic pathways. Analyzing global gene expression in untreated and GM-CSF – stimulated neutrophils by using oligonucleotide microarrays revealed that GM-CSF induced the expression of pro-apoptotic Bcl-2 family member Bim. To verify these findings, we performed immunoblotting analysis and observed increased Bim protein levels in GM-CSF – stimulated blood neutrophils compared to freshly isolated or cultured control blood neutrophils. Bim upregulation was dependent on de novo transcription and translation as it was blocked by both cycloheximide (translation inhibitor) and actinomycin D (transcription inhibitor). Pharmacological inhibition of PI3K (LY294002) blocked GM-CSF – mediated survival and, surprisingly, decreased Bim expression in neutrophils. The functional role of Bim was investigated using Bim deficient mouse neutrophils. Lack of Bim expression reduced spontaneous neutrophil death. Moreover, in the absence of Bim, both GM-CSF and IL-3 demonstrated much higher efficacy to block neutrophil apoptosis. Taken together, these data demonstrate a functional role for Bim in the regulation of neutrophil apoptosis and suggest that GM-CSF and other neutrophil hematopoietins initiate a pro-apoptotic counter regulation that involves up-regulation of Bim.

Keywords: Bim GM-CSF neutrophils apoptosis

Presenting author: Aintzane Apraiz

Implication of Bcl2-PKC(á) system on the ceramide modulating treatment effectivity applied to Acute Lymphoblastic Leukemia (ALL) cells

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Ceramide and derivatives are well known not only as membrane structure modulators but also as important second messengers on both cell-survival and cell-death activating pathways. While sphingomielin is an important component, molecules as sphingosine-1-phosphate (S1P) are described to be prosurvival pathway activators and accumulation of ceramide, sphingosine or sphingonine is mainly related to cell-death induction. On the other hand, N-(4-hydroxyphenyl)retinamida (4HPR), is a synthetic retinoid and a promising chemopreventive and antineoplasic agent against many cancer types. Previous studies showed an important apoptotic effect on ALL cell lines by inducing an early ceramide accumulation and mitochondrial apoptosis events such as oxidative stress (ROS) increase or mitocondrial membrane potential loss. Bcl2 overexpression induced partial resistance to 4HPR, supporting the importance of the mitochondria in our model.

We observed a general apoptosis increase when combining 4HPR and ceramide metabolism modulators such as PPMP, DHS or safingol (complex treatments). Both, DHS and safingol, are sphingosine kinase (SK) inhibitors but safingol has been also described as a protein kinase C alpha (PKCα) inhibitor. When comparing Bcl2 overexpressing (E1) and not overexpressing (CCRF-CEM) ALL cells lines, E1 cells showed a clearly higher resistance to DHS-based complex treatment as well as a clear PKCα overexpression. In order to study Bcl2 implication on drug resistance, Bcl2 overexpression was supressed what turned E1 modified cells into a more sensitive cell line (E1 doxy500), not only to 4HPR but also to safingol- and DHS-based complex treatments. The differences observed between DHS-based and safingol-based treatment effectivity on both, E1 and E1 doxy500, could be related to the safingol mediated PKCα inhibition. Bcl2 downregulation did not affect PKCα mRNA level and PKC activation by PMA protect (even slightly) cells from DHS-based treatment but not from 4HPR treatment. Bcl2 low-expressing cells (such as CCRF-CEM and E1 doxy500) were also protect by PMA from safingol-based complex treatments. On the whole, our dates suggest activation of different death-pathways when comparing 4HPR and DHS/safingol-based complex treatments as well as PKCα as a key molecule on drug resistance.

Keywords: Bcl2, PKCá, ceramide, ALL

Presenting author: Odeta Arandarcikaite

Estradiol prevents ischemia induced cardiomyocytes death

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Estrogens are known to exert beneficial effects on the cardiovascular system after ischemia/reperfusion. We analyze whether 17-β estradiol can protect cardiomyocytes from cell death during 30 min and 60 min ischemia in mitochondria dependent manner. Results show that 30 min and 60 min ischemia induces release of cytochrome *c* from mitochondria and inhibits mitochondria respiration rate at state 3. Prolonged ischemia also causes activation of caspases and nuclear DNA fragmentation. All these ischemic injury there effectively reduced in presence of estradiol. This suggests that 17β-estradiol protects rat heart mitochondria from loss of cytochrome *c*, increases mitochondrial functional activity and this is may be the mechanism by which estradiol protects cardiomyocytes from apoptotic cell death.

Keywords: cytochrome c, apoptosis,estradiol, ischemia, mitochondria

Presenting author: Marcia Aranha

The apoptosis machinery is required for the differentiation process in mouse neural stem cells

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The concerted integration of apoptosis and survival may ultimately determine whether a precursor cell re-enters the cell cycle or exits and differentiates. Activation of the Akt survival pathway results in up-regulation of inhibitor of differentiation 1 (Id1) through phosphorylation of its repressor FOXO3A. In addition, Akt modulates several apoptosis-related proteins, such as the transcription factor NF-κB and p53. The **aim** of this study was to investigate apoptosis and survival pathways involved in mouse neural stem cell (MNSC) differentiation. Immunocytochemistry analysis of MNSC using neuronal β-III tubulin and astroglial GFAP markers confirmed that neurogenesis and gliogenesis occur at ~ 3 and 8 days, respectively. In addition, immunoblots revealed a significant reduction in total protein levels of p-Akt, p-FOXO3A and Id1 in both neurogenesis and gliogenesis, in association with decreased NF-κB levels. Curiously, p53 expression and caspase-3 processing significantly increased during neuronal and glial differentiation. Nevertheless, the absence of nuclear fragmentation indicates that apoptosis does not reach the endpoint throughout differentiation. Interestingly, when the pan-caspase inhibitor z-VAD.fmk was added to cells during differentiation, p-FOXO3A and Id1 were markedly increased. Moreover, z-VAD.fmk exposure induced a further increase in the pro-apoptotic protein p53. More importantly, the inhibition of caspase activation significantly reduced the number of positive cells for β-III tubulin by ~ 40% and GFAP by ~ 60%, at 3 and 8 days, respectively. In **conclusion**, these results suggest that specific apoptosis-related proteins may play a crucial role in differentiation of MNSC. (Supported by POCTI/SAU-FCF/62479/04 from FCT, Portugal).

Keywords: apoptosis, stem cells, differentiation

Presenting author: Dina Aresvik

5-Fluorouracil Induces Cell Death in Jurkat E6 T cells

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Chemotherapeutic drugs, such as the thymidylate synthase inhibitor 5-Fluorouracil (5-FU), are frequently used to treat cancer and may eliminate responsive cells by inducing apoptosis. Previous studies with different cell lines indicate that the pro-apoptotic protein Bax and the tumor suppressor protein p53 are central actors in this process. The leukemic T-cell line Jurkat E 6 does express transcripts for p53 and Bax but lacks the functional proteins due to mutations. 5-FU may activate alternative death pathways in Jurkat cells.

Jurkat T cells were treated with increasing doses of 5-FU for different time periods. Staurosporine was included as a positive control. In some experiments, cells were pretreated with a general caspase inhibitor, Z-VAD-FMK.
Cell death was analyzed by flow cytometry, after Annexin V-Fluos and propidium iodide staining. Hydroethidine staining was used to determine mitochondrial ROS generation. Gene expression of pro- and anti-apoptotic proteins was examined by real-time PCR.

Flow cytometry analysis indicates that incubation with 5-FU triggers apoptosis in Jurkat cells. The cells die in a dose-dependent manner and a maximal response was observed within 72 hours. Using a pan-caspase inhibitor Z-VAD-FMK, we observed that the death response was attenuated. We also observed increased mitochondrial ROS generation after 48 hours of incubation with 5-FU. Results from real-time PCR suggest that expression of anti-apoptotic proteins is increased.
Conclusion: 5-FU induces cell death in Jurkat cells in a time- and dose-dependent manner. 5-FU signaling is mediated through caspase-dependent and caspase-independent pathways. Further, a slight ROS production suggest mitochondrial involvement.

Keywords: 5-Fluorouracil,cancer, Jurkat, apoptosis

Presenting author: Jane Armstrong

Combining ER stress-inducing agents with protein disulphide inhibitors as a novel therapeutic strategy for metastatic melanoma.

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Malignant melanoma remains largely untreatable due to the notorious resistance of such tumours to apoptosis induced by conventional mechanisms. Currently there is considerable clinical interest in two agents, fenretinide (a synthetic derivative of vitamin A) and Velcade (a 26S proteosome inhibitor) which both induce potent apoptosis of melanoma cells whilst having no effect on normal melanocytes and which we have demonstrated induce cell death by targeting mechanisms culminating in endoplasmic reticulum (ER) stress. Targeting ER-stress-induced apoptosis presents a powerful therapeutic approach but may be counteracted by the defensive homeostatic unfolded protein response (UPR) resulting in the up regulation of protein disulphide isomerase (PDI) family members, key chaperones which assist in the clearance of unfolded proteins and promotion of cell survival. The aim of the current study was to test the hypothesis that the efficacy of ER stress-induced apoptosis in response to fenretinide or velcade would be increased by the combination of these agents with inhibitors of PDI. Dose response and fixed dose ratio experiments of Bacitracin and novel PDI inhibitors were performed in combination with fenretinide or velcade in a panel of human metastatic melanoma cell lines and apoptosis evaluated by flow cytometry. ER-stress-induced apoptosis was confirmed by Western blotting for GADD 153, the key transcription factor regulating the transition from pro-survival to pro-apoptotic signalling during ER stress. Results demonstrated there was no apparent toxicity using PDI inhibitors alone and confirmed a synergistic apoptotic response was induced by the combination of fenretinide or Velcade (eg: for the combination of bacitracin and fenretinide at a fixed dose ratio of 50:1, ED50, CI=0.096). Over expression of wild type PDI (or a mutant control) in A375 cells followed by treatment with fenretinide or velcade in the presence of PDI inhibition confirmed the target activity of individual PDI inhibitors. These data support the novel concept of combining ER stress-inducing agents with PDI inhibition as a more effective therapeutic strategy for metastatic melanoma.

Keywords: Apoptosis, ER stress, Melanoma

Presenting author: Maria Augusta Arruda

Heme, a proinflammatory molecule, inhibits neutrophil apoptosis: NADPH oxidase-derived ROS linking acute and chronic inflammation during hemolytic episodes

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Sickle cell disease, malaria, ischaemia-reperfusion and other pathological states of increased hemolysis result in high levels of free heme in circulation (up to 20 μM). These events are often associated with an acute inflammatory response that usually leads to the development of chronic inflammation. We have previously shown that free heme (1-20 μM) is a proinflammatory molecule, able to promote neutrophil migration both in vivo and in vitro, actin polymerization, NADPH oxidase activation, PKC activation, IL-8 expression. and delays human neutrophil spontaneous apoptosis. These effects depend on protein synthesis, require heme oxygenase (HO) activity, and involve PI3K, ERK and NF-κB pathways, which seem to be coordinated by NADPH oxidase-generated reactive oxygen species (ROS). We have also reported that heme-induced delay of neutrophil apoptosis correlates with the prevention of mitochondria transmembrane potential ($\Delta\psi_m$) dissipation, what occurs in a ROS-dependent mechanism that regulates heme-induced increase on Bcl-X_L / Bad ratio and inhibition of Bax insertion into mitochondria. Taken together, our results point to a prominent role for free heme on the acute and chronic inflammatory responses associated to hemolytic episodes, due to its ability to activate the NADPH oxidase system.

Keywords: neutrophil, apoptosis, heme

Presenting author: Miri Assayag

Mitochondria mediated anti-apoptotic cascade is enhanced in heat acclimated rats.

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Acclimation is a reversible "within lifetime" phenotypic adaptation to long-term environmental stress. Acclimation enhances thermotolerance as well as the ability to cope with novel stressors. Previous studies in our laboratory demonstrated a significant up-regulation in the expression of anti-apoptotic genes in heat acclimated animals. In the current investigation the effects of heat acclimation on mitochondria mediated apoptosis was studied.

The aims of this study were to determine whether heat acclimation decreases apoptosis following heat stress in the heart, to examine the differences in the kinetics of heat shock mediated apoptosis and to investigate the role of the mitochondria in the apoptotic cascade and cytoprotection.

Males Rattus norvegicus were divided into 2 groups: normothermic (C, ambient temperature 24ºC) and heat acclimated (AC, ambient temperature 33ºC±1). Animals from those groups were subjected to 2 hours of heat stress at 41ºC±1 and sacrificed following different recovery periods at normothermic conditions. Protein levels from mitochondrial and cytosolic cellular fractions from rat hearts were measured by Western immunoblotting. mRNA levels were detected by both RT-PCR and qRT-PCR.

Following heat stress there were significantly lower levels of Bad mRNA (pro-apoptotic member of the Bcl-2 family) in the AC vs the C animals. 1 hr post heat stress, the BclXL / Bad protein ratio in the mitochondria fraction was 2.06±0.07 and 1.42±0.12 for AC vs C hearts, respectively $p < 0.05$. Likewise, there were higher cytochrome c levels in the mitochondrial fraction of AC vs C groups (2.09±0.15 vs 1.12±0.17, $p<0.05$, respectively). Significantly lower protein levels of caspase 9 and of the active form of caspase 3 were found in the AC compare to the normothermic animals. In contrast, no change in caspase 8 transcripts (an indicative of FAS ligand apoptotic pathway) was observed in both groups. Tunel analysis of the normothermic and the acclimated hearts before and 10 hours after heat stress showed a significanty lower apoptotic nuclei number in heat acclimated vs normothermic hearts.

Our data indicate that heat acclimation enhances anti-apoptotic pathways in response to heat stress via shifting the balance between pro- and anti-apoptotic Bcl-2 proteins, thus implying mitochondrial mediated cytoprotective activity.

Keywords: Heat Acclimation, apoptosis, Mitochondria

Presenting author: Maria Balakireva

Ral-dependant cell death pathways in Drosophila development

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Drosophila has unique genetic and biological advantages as a model system to study apoptosis, specially since several cell death genes are evolutionary conserved between flies and mammals.
The Ral proteins are GTPases of the Ras family. RalA and RalB collaborate to promote Ras oncogene transformation by serving distinct functions: RalA is crucial for tumor initiation whereas RalB is responsible for transformed cell survival and is important for metastatic progression. Cancer cell lines seem exquisitely sensitive to the depletion of RalB.
Drosophila melanogaster has a single *Ral* gene and the Ral-centered protein network is conserved in human and flies. Reduced Ral expression in hypomorph Ral mutants has no effect on cell division but leads to post-mitotic cell-specific apoptosis during oogenesis and sensory organs development. Ral-dependent apoptosis produced shaft cell death in sensory organ lineage resulting in a loss-of-bristle phenotype and induced a stage-specific death of follicle cells during mid-oogenesis. Genetic analysis and immuno-fluorescence imaging showed that the loss-of-bristle phenotype was caused by an activation of TRAF1/msn–JNK signalling and that the exocyst, one of Ral effectors, executed Ral function in apoptosis. Ral counters apoptotic programs acting as a negative regulator of JNK activity and a positive activator of p38. A pathway from Ral to the exocyst to HGK appears as the molecular basis of Ral action on JNK. The Ral-dependent restriction of JNK activation and the exocyst/HGK relationship are conserved in mammalian cells.
A gain-of-function screen using the Ral apoptotic loss-of-bristle phenotype identified new functional partners of Ral. Fifty genes belonging to five GO groups (cell cycle, programmed cell death, migration, trafficking and adhesion) and/or encoding proteins physically connected to Ral effectors (RLIP and the exocyst complex) were selected for further genetic and cell biology studies in order to gain insight in Ral anti-apoptotic signalling.

Keywords: Ral GTPase, Drosophila, development, exocyst, JNK pathway

Presenting author: Bhavya Balan Chandrika

Significance of Bax and Sub-cellular localization of Bcl2 in Endoplasmic Reticulum Stress Induced Apoptosis

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Endoplasmic Reticulum (ER) is the site of synthesis and folding of secretary proteins. Perturbations of ER homeostasis affect protein folding cause ER stress. ER can sense the stress and respond to it and may either lead to cell survival or apoptosis depending on the strength and duration of ER stress. The mechanism and the factors that mediate ER stress induced apoptosis are still obscure. The Bcl2 family members regulate the programmed cell death either by inhibiting cell death (Bcl2 and BclXL) or by promoting cell death signaling (Bax /Bak). Recent studies suggest that Bcl2 may differ in its functions depending on its sub cellular localizations. Even though the survival functions of Bcl2 is well studied , being a stress confined to ER, we were interested in the functional role of Bcl2 at ER in the regulation of survival , death and cell cycle regulation. In order to study this, we have stable colon cancer HCT cells and corresponding Bax knockout cells over expressing wild type Bcl2 or Bcl2 targeted to ER. Our findings suggest that cells expressing Bcl2 targeted to ER completely prevented ER stress induced apoptosis, in the absence of BAX, whereas wild type Bcl2 over-expression even in the absence of Bax failed to prevent cell death completely. This observation indicates that ER targeted Bcl2 cells are more selectively resistant to ER stress induced apoptosis when compared with wild type Bcl2 overexpressing cells. We have also analyzed possible upstream or downstream survival signaling such as AKT, ERK, p38 and the important apoptotic mediators like caspases and Bak. The results demonstrated the significance of spatilal localization of Bcl2 molecule in the regulation of intracellular calcium and cell cycle.

Keywords: Endoplasmic Reticulum ,Bax, BCL2

Presenting author: Patricia Balsas

Strategies for improving proteasome inhibitor therapy in myeloma: stimulating apoptosis with Apo2L-TRAIL, Nutlin or inhibitors of histone deacetylases (HDACi).

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The proteasome inhibitor bortezomib (Velcade) is currently the drug of choice for treatment of patients with relapsed or refractory multiple myeloma (MM) and elderly patients not suitable for bone marrow transplant. However, there are some patients who do not respond to therapy with bortezomib or who respond briefly and then relapse. This may be due to the up-regulation by myeloma cells of pathways suppressing apoptosis caused by proteasome inhibition. The delineation of these molecular pathways and of the mechanisms to circumvent them are of great importance to improve the therapy of MM and other malignancies. Three different agents are being evaluated in our laboratory in order to strengthen the apoptotic signal triggered by bortezomib: i) Apo2L-TRAIL, a death messenger that induces apoptosis in MM cells by the extrinsic route; ii) Nutlin, a potent and selective small-molecule antagonist of MDM2 that bind MDM2 in the p53-binding pocket and activate the p53 pathway in cells with wild-type p53 (most MM cells) and iii) Valproic acid (VPA) and Depsipeptide (FK228) inhibitors of histone deacetylases (HDACi) of different specificity. Preliminary results indicate that bortezomib-induced apoptosis is strongly potentiated by Apo2L/TRAIL. Co-treatment with Nutlin stabilizes p53 and induces p21 expression leading to an additive apoptotic effect when combined with bortezomib. Pretreatment with HDACi also improved, to different extent, the apoptotic efficiency of bortezomib. Studies are in progress to identify the mechanisms by which these agents improve bortezomib-induced apoptosis.

Keywords: apoptosis, proteasome, Velcade, apo2L, myeloma

Presenting author: Heike Bantel

TRAIL in combination with the histone deacetylase inhibitor depsipeptide induces caspase activation and apoptosis in healthy human liver

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TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in tumor cells but not in most normal cells and has therefore been proposed as a promising antitumor agent. Recent experiments suggested that isolated primary human hepatocytes but not monkey liver cells are susceptible to certain TRAIL agonists, raising concerns about the use of TRAIL in cancer treatment. Whether TRAIL indeed exerts hepatotoxicity *in vivo* and how this is influenced by chemotherapeutic drugs is completely unknown. Employing different forms of recombinant TRAIL, we found that this cytokine can induce pro-apoptotic caspase activity in isolated human hepatocytes. However in marked contrast, these different TRAIL preparations induced little or no cytotoxicity when incubated with tissue explants of fresh healthy liver, an experimental model that may more faithfully mimic the *in vivo* situation. Since about half of the tumor cell lines tested so far are TRAIL resistant, combinatorial regimens are required to sensitize tumor cells for TRAIL induced apoptosis. Among promising new agents for TRAIL sensitization of tumor cells are histone deacetylase inhibitors (HDACi). However, it remains unclear whether TRAIL in combination with HDACi exerts hepatotoxicity. We therefore analyzed caspase activity and apoptosis of healthy liver explants treated with different versions of TRAIL and the HDACi depsipeptide. In contrast to TRAIL treatment alone, the combinatorial treatment of healthy liver with TRAIL and depsipeptide strongly induced caspase activation and apoptosis. This enhanced sensitivity of healthy liver to the combinatorial treatment with TRAIL and depsipeptide was associated with increased expression of agonistic TRAIL receptors and pro-apoptotic Bcl-2 molecules as well as with downregulation of anti-apoptotic modulators of the DISC and mitochondrion.
These results suggest that clinical trials should be performed with great caution, when TRAIL is combined with chemotherapeutic agents.

Keywords: TRAIL, HDAC inhibitors, Apoptosis, Human Liver

Presenting author: Elisa Barbieri

Construction of a mouse model for the conditional ablation of fis1.

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Mitochondria are dynamic organelles that continuously undergo fusion and fission in response to intracellular needs and extracellular signals during life of the cell. Early during apoptosis, mitochondrial fragmentation and remodelling of the cristae occur to ensure complete release of cytochrome c and progression of the apoptotic cascade. Fragmentation depends on the mitochondrial outer membrane protein Fis1, which acts as organellar receptor for the cytosolic mechanoezyme Drp-1. It is currently unclear to which extent mitochondrial fission is required for the progression of apoptosis; moreover, since Fis1 independently regulates fission and apoptosis (Alirol et al., 2006), the two processes could be not necessarily related. In complex, this calls for an in vivo analysis of the role of this protein and of fission in general. In order to address this issue, we are constructing a targeting vector to conditionally knock out Fis1 in the mouse. The targeting vector harbours the mfis1 gene *floxed* between two Lox P sites and followed by an intron sequence, an IRES element, the Lac Z coding sequence and the FRT-flanked hygromcin gene. Notably, the Lac Z allows following the mFis1 promoter during development, adult life and pathologic conditions. In summary, the Fis1 conditional mouse will be an invaluable tool to address the relative contribution of mitochondrial fission in development, apoptosis and pathology.

Keywords: Fis1, mitochondria, conditional KO,apoptosis

Presenting author: Sandra Barisic

Dependence of IL-1 mediated enhancement of UVB-induced apoptosis on p53

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Costimulation of UVB-irradiated cells with IL-1 results in NFκB-dependent enhancement of UVB-induced apoptosis. This enhancing effect is mediated via NFκB-dependent repression of anti-apoptotic proteins cIAP and FLIP as well as of TRAF proteins and coincides with a prolonged and elevated expression of TNF. Secreted TNF then activates TNF-R1 in an autocrine fashion resulting in additive induction of apoptosis, thereby enhancing UVB-induced apoptosis. This seems to be an ubiquitous phenomenon, since it could be verified for a variety of different keratinocyte and epithelial cell lines. Prolonged and elevated TNF production was shown to be due to persistent NFκB activation resulting from disruption of the negative regulatory feedback loop, which involves NFκB-mediated re-synthesis of its inhibitor IκB.

To further elucidate the contribution of UVB-induced DNA damage in this scenario, the effect of p53 was investigated. P53 is the key regulator of DNA damage-induced apoptosis, mediating transcription of cell cycle kinase inhibitors like p21 as well as of proapoptotic genes like bax, finally triggering programmed cell death. The cell line SCL-1 did not respond to physiological doses of UVB with induction of apoptosis. Additionally, this cell line failed to show the enhancing effect of IL-1-mediated NFκB activation on UVB-induced apoptosis coinciding with the failure to express TNF. Further investigations revealed that SCL-1 cells, although bearing a genomic copy, do not express p53. These results first of all question the general role of p53 in DNA damage-induced apoptosis but also imply that p53 might contribute to the constantly high expression level of TNF upon costimulation of cells with UVB and IL-1, e.g. by stabilizing the NFκB induced transcription complex at the TNF promoter.

However, reconstitution of wt p53 and different gain of function mutants of p53 did neither restore the apoptotic response of SCL-1 cells to UVB nor the enhancing effect of IL-1. We therefore took a different approach by knocking down p53 in HeLa cells, which initially show both, response to UVB and enhancement of apoptosis by cotreatment with IL-1. Surprisingly, knock down of p53 by siRNA did not result in reduction of UVB-induced apoptosis itself, but completely inhibited the enhancing effect of IL-1 by significantly reducing TNF secretion. In accordance with this observation, inhibition of the transcriptional activity of p53 by its inhibitor pifitrin also reduced TNF release and IL-1 mediated enhancement of UVB-induced apoptosis. These data indicate a close interrelationship between DNA damage, NFκB and p53 and show that p53 is an important, but not the only player in DNA damage-induced apoptosis.

Keywords: Apoptosis, IL-1, p53, UVB

Immunoreactivity of ubiquitin in the human prostate gland

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BACKGROUND: Ubiquitin is a low molecular weight protein which has been detected in a variety of normal and cancerous tissues. It is involved in many regulatory processes including protein proteolysis. It has been implicated in tumor pathogenesis. The role of ubiquitin in human prostate gland is investigated in this study.

METHODS: In this study, we utilized immunohistochemistry technique to localize ubiquitin in human prostate gland and correlate it with different pathological conditions of the prostate.

RESULTS: Ubiquitin was localized in normal, benign prostatic hyperplasia (BPH) and prostatic adenocarcinoma with variations in the distribution and intensity. In BPH, ubiquitin immunoreactivity was localized mainly in the nuclei while in the adenocarcinoma was localized mainly in the cytoplasm.

CONCLUSION: The presence of ubiquitin mainly in the nuclei and in the cytoplasm of BPH and prostatic adenocarcinoma, respectively, may suggest a role of ubiquitin in the development of the above mentioned conditions. Ubiquitin could be used as a potential marker for the diagnosis and prognosis of pathological conditions of the prostate.

Keywords: Ubiquitin, prostate gland

Presenting author: Veronique Baud

RelB inhibits cell growth and tumorigenesis through p53 expression activation

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The activity of NF-κB is activated by at least two different signaling pathways with different kinetics: the canonical pathway that relies mostly on the IKKß- and IKKγ-dependent IκBα degradation which results in the rapid but transient activation of mainly RelA containing dimers, and the alternative pathway which proceeds via the IKKα-induced p100 processing, thus leading to a late but sustained activation of both RelB/p52 and RelB/p50 dimers. It is well characterized that activation of RelA promotes cell proliferation and antagonizes apoptosis through the activation of pro-survival genes. In contrast, how RelB contributes to the control of cell growth and apoptosis is unknown. We show here that constitutive expression of RelB reduces cell proliferation and tumorigenesis. In addition, RelB induces the expression of the p53 tumor suppressor gene and modulates DNA-damage-induced p53 activity. These findings imply that RelB expression contributes to protection against uncontrolled cell growth and cell transformation in part by functional activation of p53. Our data suggest that inhibition of RelB in tumors that retain wild type p53 may diminish rather than augment a therapeutic response.

Keywords: NF-kB, RelB, p53, cell growth, tumorigenesis

Presenting author: Raphaël Beck

The degradation of the chimerical protein Bcr-Abl by an oxidative stress involves the disruption of molecular chaperone functions of hsp90 and leads to death of chronic myelogenous leukaemia (K562) cells.

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The Philadelphia Chromosome is present inside 95% of the chronic myeloid leukaemia (CML) cells. It leads to the formation of a Bcr-Abl protein which presents an abnormal tyrosine-kinase activity and is responsible for the transformation of normal cells into malignant cells. Imatinib mesylate, a specific inhibitor of the Bcr-Abl tyrosine kinase domain, represents the best treatment for CML. Nevertheless, mutations acquired by the protein causes the appearance of resistance to the treatment with imatinib. Since the association between ascorbate and menadione caused in vitro cell death of K562 cells and inhibited their proliferation in a xenograft tumor mouse model, we studied whether this association is affecting both stability and function of Bcr-Abl protein. The results show that incubation of K562 cells in the presence of ascorbate/menadione causes a cleavage of hsp90 protein and a rapid downregulation of Bcr-Abl. Dephosphorylation of several proteins implicated in mitogen signal transduction pathways like Stat, Erk, Raf, Elk, and MEK 1/2 was also observed. As K562 cell death induced by ascorbate/menadione was suggested to be due to ATP depletion as a consequence of glycolysis inhibition, we studied the impact of the glycolysis inhibitor iodoacetate on Bcr-Abl. We reported no Bcr-Abl degradation when glycolysis was inhibited. We studied the role of the proteasome in Bcr-Abl degradation by using the specific inhibitor MG132. Proteasome inhibition had no effect on Bcr-Abl degradation. We finally reported the disappearance of the Bcr-Abl degradation and hsp90 cleavage induced by ascorbate/menadione when the cells where incubated in the presence of N-acetyl-cysteine (NAC), a well known antioxidant; and an increase in the Bcr-Abl degradation and hsp90 cleavage when the cells were incubated in the presence of aminotriazole (ATA), a catalase inhibitor. Collectively, our results demonstrate that ascorbate/menadione induces a Bcr-Abl degradation in K562 cells which is induced by a mechanism depending on an oxidative stress and not on a glycolysis inhibition or a proteasome-mediated degradation.

(*) R.B. is a FRIA recipient.

Keywords: bcr-abl, hsp90, K562, oxidative stress

Presenting author: Cagri Bodur

HNE (4-hydroxynonenal) induces apoptosis via mitochodrial pathway with the involvement of Bcl-2 family members

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One of the most abundant aldehydic components of ox-LDL is 4-Hydroxynonenal (HNE) which exerts various effects on intracellular and extracellular signaling cascades. HNE was demonstrated to be able to induce apoptosis and ROS formation in a dose dependent manner in a number of studies. Several signaling pathways have been shown to be modulated by HNE, including MAP kinases, PKC isoforms, cell cycle regulators, receptor tyrosine kinases and caspases. We have studied MAP kinase and caspase activation pathways in 3T3 fibroblasts in order to shed light on the mechanisms that HNE induces apoptosis.

HNE induced early activation of JNK and p38 proteins but downregulated the basal activity of ERK 1/2. We have shown that HNE induced caspase-9 and 3 activation and release of cytochrome c from mitochondria. Overexpression studies and utilization of specific kinase inhibitors indicated that HNE induced apoptosis through activation of JNK and c-Jun/AP-1 protein. We also demonstrated that resveratrol, a widely distributed flavone, may protect against HNE-induced apoptosis through modulation of JNK activation upstream of mitochondrial apoptotic pathway. Activation of AP-1 along with increased c-Jun and phospho-c-Jun levels could be inhibited by pretreatment of cells with resveratrol.

Further investigations about the cellular mechanism of apoptotic response to HNE are in progress by a specific focus on the involvement of pro and anti-apoptotic members of Bcl-2 protein family in our laboratory.

Keywords: hne, apoptosis, bcl-2, ox-LDL

Presenting author: Linda Boehme

Double stranded RNA induces apoptosis in host cells infected with *C. trachomatis*

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The gram-negative *Chlamydiae* are obligate intracellular human pathogenic bacteria with a unique biphasic life cycle. To establish an acute or persistent infection, *Chlamydiae* have evolved sophisticated strategies to resist host cell apoptosis until the developmental cycle has completed. We investigated the ability of *C. trachomatis* to inhibit apoptosis mediated by PKR, a protein kinase that recognizes double stranded (ds)RNA. dsRNA is produced by many viruses during their replication within the host cell and functions to activate an anti-viral response. Activation of PKR was induced via transfection with polyI:C, a synthetic dsRNA. We show here that cells infected with *C. trachomatis* are sensitized for apoptosis induced by polyI:C. Activation of procaspase-8 and -3 as well as PARP-cleavage occurred in infected HEp-2 cells. Inhibitor of apoptosis proteins like cIAP-2 and Mcl-1 which are up-regulated during a chlamydial infection, were shown to be down-regulated after transfection with polyI:C. Two possible mechanisms of PKR activity could account for the observed down-regulation and the resulting deficit of apoptosis inhibition by *Chlamydia*: first, dephosphorylation of the p42/p44 MAPKinase ERK1/2 by polyI:C, a kinase that is phosphorylated during infection with *Chlamydia*, and, second, overall shut-down of protein translational via phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α). The importance of ERK dephosphorylation for polyI:C-induced apoptosis was further underlined by the fact that application of U0126, an inhibitor of MEK-1, the direct upstream kinase of ERK, sensitized cells to polyI:C-mediated apoptosis. Elucidating the outcome of PKR activation on chlamydial apoptosis inhibition might allow for new treatments in cancer or anti-*Chlamydia* therapy.

Keywords: Chlamydia, dsRNA, PKR, ERK

Presenting author: Zita Bognar

Development of the prototype of SOD mimetic mPT inhibitors

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In ischemia-reperfusion injuries, elevated calcium and reactive oxigen species (ROS) induce mitochondrial permeability transition (mPT) which plays a pivotal role in mediating damages and cell death. Inhibition of mPT decreases necrotic cell death, however during reperfusion, the continuous production of ROS may contribute to the temporary opening of pore and thus the onset of the delayed apoptotic cell death. Based on amiodarone structure, we developed the first free-radical scavenging mPT inhibitor (HO-3538) that can eliminate ROS in the microenvironment of the permeability pore. In isolated mitochondria, HO-3538 inhibited mPT and the release of pro-apoptotic mitochondrial proteins. It had ROS scavenging effect and anti-apoptotic effect in a cardiomyocyte line and it diminished release of mitochondrial proapoptotic proteins. Furthermore, HO-3538 significantly enhanced the recovery of mitochondrial energy metabolism and functional cardiac parameters, decreased infarct size, lipid peroxidation and protein oxidation, and suppressed necrotic as well as apoptotic cell death pathways in Langendorff-perfused hearts. In these respects it was somewhat superior to its two constituents, amiodarone and a pyrrol deravative free-radical scavenger. These data suggests that the free-radical scavenging mPT inhibitors are ideal candidates for drug development.

Keywords: Mitochondrial permeability transition, ROS, Ischemia-reperfusion, Langendorff-perfuzed hearts, NMR, apoptosis, necrosis

Presenting author: Romain Boidot

Comparison of Survivin and its splice variant Survivin-3B role in response to drugs

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Survivin is one of the Inhibitor of Apoptosis Proteins family members. *Survivin* gene encodes four protein variants: Survivin-ΔEx3, Survivin-3B, Survivin-2B and Survivin-2α. Some studies have indicated that Survivin and Survivin-ΔEx3 have anti-apoptotic activities, whereas Survivin-2B and Survivin-2α have pro-apoptotic properties. Up to now, no study has explored the role of Survivin-3B, and has compared its action with Survivin's one.

In this study, we analyzed the role and the location of Survivin and Survivin-3B in response to chemotherapeutic drugs. Each GFP-coupled protein was stably transfected in MCF-7 mammary cancer cell line. The expression of transfected protein was checked by western blot with an anti-GFP antibody. Cells were treated with cis-platin, 5-fluorouracil, epirubicin, docetaxel or vinblastine. The treatment-induced apoptosis was assessed by flow cytometry after 48 hour treatment. The Student t test was used for statistical analysis. Only $p<0.05$ was considered significant. Fluorescent microscopy was performed after 18 hour treatment to avoid artifacts induced by massive cell death.

After 48 hour treatment, Survivin and Survivin-3B overexpressing cells did not show statistically significant difference in their apoptotic rate in response to 5-fluorouracil (37% *versus* 26%, respectively), cis-platin (62% *vs* 62%), and epirubicin (28% *vs* 25%). In presence of the mitotic spindle poisons docetaxel or vinblastine, cells transfected with Survivin were less sensitive than those transfected with Survivin-3B: 7% of apoptosis *versus* 21% with docetaxel, and 6% *versus* 14% with vinblastine.

Without treatment, Survivin was located in cytoplasm and, in some cells, in mitochondria, whereas Survivin-3B were exclusively cytoplasmic. After 17 hour treatment, Survivin and Survivin-3B were still and exclusively cytoplasmic, except for treatment with 5-fluorouracil that increased Survivin accumulation in mitochondria.

These results showed that Survivin and Survivin-3B had the same action in response to DNA damaging agents, whereas the anti-apoptotic role with spindle poison of Survivin-3B is weaker than Survivin's in MCF-7 cells. Moreover, their localization seemed to be not involved in their function.
These results will be confirmed in HBL100 cell line. The study of possible interactions between both proteins will be also performed after drug exposure.

Keywords: survivin, survivin-3B, localization, chemotherapeutic drugs, apoptosis

Presenting author: Lea Bojic

Apoptosis induced by selective lysosome membrane permeabilization proceeds through mitochondrial destabilization and is largely dependent on cathepsins B and L

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There is increasing evidence that lysosomal proteases, in particular cysteine cathepsins, play a role in certain apoptotic pathways, especially during aging and in several pathological conditions (1). Lysosomal destabilization was often found to be an early event in apoptotic cascade, preceding other hallmarks of apoptosis including mitochondrial outer membrane permeabilization and caspase activation. *In vitro* and cellular studies demonstrated that lysosomal cysteine cathepsins cleave proapoptotic homologue Bid thereby inducing cytochrome c release from mitochondria and subsequent caspase activation (2,3). We found that mouse embryonic fibroblasts (MEFs) from Bid-deficient animals exhibit reduced mitochondrial dysfunction in the presence of lysosomotropic agent LeuLeuOMe as compared to wt MEFs. Moreover, phosphatidylserine exposure and caspase activity were also diminished in Bid-deficient MEFs compared to wt MEFs. Furthermore, Bax/Bak-deficient MEFs were found to be completely resistant to apoptosis whereas mitochondria remained largely intact following LeuLeuOMe treatment. This suggests that cysteine cathepsins predominantly trigger apoptosis through MMP and subsequent caspase activation and not through caspase-independent pathways.
In order to investigate, which of the cathepsins is responsible for this apoptosis, primary fibroblasts from various cathepsin-deficient animals were exposed to LeuLeuOMe. Apoptosis and Bid cleavage were diminished in cathepsin B and L – deficient cells and completely abolished in cathepsin B/L double-deficient cells, suggesting that cathepsins B and L play a major role in LMP-mediated apoptosis.

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Keywords: apoptosis, cysteine cathepsins

Presenting author: Pedro Borralho

microRNA-143 overexpression decreases colon cancer cell proliferation and alters the response to 5-Fluorouracil

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Apoptosis is decreased throughout progression from normal colonic epithelium to carcinoma, and metastasis. However, loss of ability of colon cancer cells to undergo apoptosis and underlying molecular pathways are far from completely understood. MicroRNAs are a recently discovered class of noncoding RNA molecules that can modulate gene expression at the post-transcriptional level. Several evidences attribute microRNAs an important role in cancer genesis and progression. Furthermore, microRNA-143 (miR-143) was reported to be down-regulated in colorectal neoplasia. Here, we used HCT116 human colorectal cancer cells and investigated the biological role of miR-143. Cells were transiently transfected with miR-143 expression vector coding for miR-143 precursor, miR-143 sensor vector comprising two sequences complementary to mature miR-143, and pRL-SV40 for luciferase based transfection efficiency and normalization. miR-143 expression levels were evaluated by qRT-PCR and luciferase activity assays. General cytotoxicity and cell proliferation were evaluated by lactate dehydrogenase activity and metabolism of MTS to soluble formazan assays, respectively. The results showed that miR-143 was almost undetectable in human colon cancer cells. In addition, miR-143 overexpression resulted in decreased cell proliferation at 48 h after transfection. Further, HCT116 cells stably expressing higher levels of miR-143 were selected with G418 and then incubated with the apoptosis-inducing drug, 5-Fluorouracil. miR-143 expressing clones were significantly more sensitive to the cytotoxic drug. In conclusion, miR-143 may play a putative pro-apoptotic role. Its reduced levels in colon cancer cells possibly contributes to apoptosis evasion. Collectively, our results underline the relevance of miR-143 as a potential target for gene modulation-based therapeutic strategies in colon cancer.

(Supported by Fundação Calouste Gulbenkian, Portugal)

Keywords: microRNA; proliferation; 5-FU

Presenting author: Maria Grazia Bottone

Relationship between centromeric proteins and PARP-1 during mitosis and apoptosis

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The kinetochore is made of a large complex of proteins associated with the centromeric DNA (called CENP) and is responsible for mediating the segregation of sister chromatids to daughter cells *via* its interactions with the mitotic spindle. The cell cycle-related expression of CENP-E and CENP-F is is required for the transition from metaphase to anaphase. We analyzed the distribution of PARP-1 throughout the cell cycle and its interaction with different CENP. Immunofluorescence analysis demonstrated the colocalization of PARP-1 with CENP-B and CENP-A during the whole cell cycle, while PARP-1 colocalizes with CENP-E and CENP-F only in some phases. The observation of poly (ADP-ribosylation) as a possible regulator of kinetochore proteins adds a further level of complexity to the mechanisms underlying centromere assembly and function. It is known that some centromeric proteins are powerful autoantigens in patients affected by autoimmune diseases. Since defects in the apoptotic process have been hypothesized to be involved in the etiology of autoimmune diseases, we have also analyzed the distribution of CEN proteins during apoptosis. The p89 fragment of PARP-1 in late apoptotic steps proved to move into the cytoplasm, as it occurs for other nuclear and nucleolar proteins. We found a similar behaviour for CEN proteins: during apoptosis, and in particular in late apoptotic steps, they were extruded in the cytoplasm in a still immunodetectable form, thus demonstrating that their degradation, if any, is incomplete. This makes CEN proteins and PARP-1 potential triggers for the production of autoantibodies in the presence of a defective clearance of apoptotic bodies.

Keywords: centromeric proteins, PARP-1, apoptosis

Presenting author: Catherine Brenner

The permeability transition pore in apoptosis: cellular and molecular mechanisms and therapeutic perspectives

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Mitochondrial membrane permeabilization can be a rate limiting step of apoptotic or necrotic cell death (1, 2). In numerous physiopathological models, the permeabilization of the outer mitochondrial membrane and the inner membrane is mediated by the opening of the permeability transition pore complex (PTPC), a polyprotein complex (3, 4). PTPC is formed at the mitochondrial membranes contact site and contains the most abundant protein of the inner membrane, the adenine nucleotide translocator (ANT). ANT exhibits two opposite functions. On the one hand, ANT is a vital, specific antiporter, which accounts for the exchange of ATP and ADP. On the other hand, ANT can form a non-specific lethal pore, as shown by electrophysiological characterization of purified ANT or by measuring the permeabilization of ANT-containing proteoliposomes (3, 4). Pore formation by ANT can be induced by a variety of chemotherapeutic agents (Lonidamine, Arsenite, CD437, Verteporfin) and is enhanced by Bax and inhibited by Bcl-2 (6, 7, 8). ANT pore formation would result from conformational changes of ANT, as well as changes in its interactome. Thus, using co-immunoprecipitation and proteomic analysis, we identified some of the interacting partners of ANT in several normal tissues and human cancer cell lines (8). During chemotherapy-induced apoptosis, some of these interactions were constant (e.g. ANT-VDAC), whereas others changed strongly concomitantly with the dissipation of the mitochondrial transmembrane potential and until nuclear degradation occurred (e.g. Bax, Bcl-2, subunits of the respiratory chain, a subunit of the phosphatase PP2A, phospholipase PLC beta 4 and IP3 receptor). Recently, we identified two novel regulators of PTPC, a glutathione-S-transferase (GST) that interacts with ANT and GAPDH that interacts with VDAC (9). We characterized the molecular mechanisms of PTPC regulation in normal tissue, in tumoral cells and in vitro, and showed that GST behaves as an endogenous repressor of PTPC and GAPDH as an activator of PTPC. Thus, the function of PTPC in apoptosis is modulated, positively or negatively, by mitochondrial and cytosolic proteins. These results have important implications for the design of potential therapeutic peptides that can target mitochondrial proteins and kill cancer cells (e.g. Team-Vp) (10).

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Acknowledgments : ARC, INCa.

Keywords: apoptosis, mitochondria, ANT, VDAC, oncogene

Presenting author: Diana Brust

Characterization of putative apoptosis-inducing factor (AIF)-homologs of the fungal aging model *Podospora anserina*

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Wild-type strains of the ascomycete Podospora anserina are characterized by a limited lifespan. After about 25 days of linear growth, the growth rate of the wild-type strain s declines and the morphology and physiology of cultures changes dramatically, ending up with the death of the hyphal tips. This senescence syndrome has a clear mitochondrial etiology raising the question whether or not apoptosis-like processes are involved in the final stage of development of this fungus. An in-silico analysis identified several putative apoptosis factors, e. g. two metacaspases whose involvement in apoptosis-like processes could be verified (Hamann A., Brust D., Osiewacz H.D., Mol Microbiol, in press). However, the role of AIF (apoptosis-inducing factor) and AMID (AIF homologous mitochondrion-associated inducer of death) homologs of P. anserina has not been investigated so far. Since the mammalian AIF and AMID proteins are involved in a mitochondial pathway of apoptosis, the corresponding P. anserina proteins may be of special relevance for aging. The PaAIF1/GFP fusion protein was found to be localized in the cytoplasm, while the PaAMID2/GFP and the PaPRG3/GFP fusion proteins localize to mitochondria suggesting a role in a mitochondria-dependent and caspase-independent signalling pathway. The localization of PaAMID1 and PaAIF2 is under investigation. Deletion of PaAmid1 results in a moderate lifespan extension on corn meal medium (59 %), while the PaAif1::ble strain is characterized by a mean lifespan of 150 days (340 % increase). Interestingly, PaAmid2::ble is strongly impaired in fertility suggesting a vital role of PaAMID2 in the mitochondria. Currently, we investigate the age-dependent translocation of the P. anserina AIF and AMID homologs and their mitochondrial release after an apoptotic stimulus achieved by chemical inductors.

Acknowledgement: Financial support is provided by the European Commission (LSHL-CT-2004-512020, Acronym: MiMage) and by grants of the Deutsche Forschungsgemeinschaft.

Keywords: P. anserina, AIF

Presenting author: Esther Buytaert

Molecular effectors of hypericin-PDT induced stress response pathways in bladder cancer cells

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Photodynamic therapy (PDT) is an anticancer modality based on the combination of a light-absorbing photosensitizing agent and visible light irradiation to generate, in the presence of molecular oxygen, cytotoxic reactive oxygen species (ROS) which cause tumor destruction[1]. Given that the photosensitizer hypericin is under consideration for the PDT treatment of bladder cancer, we performed an oligonucleotide microarray analysis in the T24 bladder cancer cell line after PDT treatment to identify differentially expressed genes with therapeutic potential[2].
This study reveals that the expression of several genes involved in a variety of metabolic processes, stress-induced cell death, autophagy, proliferation, inflammation and carcinogenesis is strongly affected by PDT with hypericin. Hypericin-photogenerated ROS act proximal to the endoplasmic reticulum (ER) to induce a rapid loss of ER Ca^{2+} homeostasis, whose persistence is causative for cell death[3]. In consistency with this notion, the microarray data revealed an increased expression of ER stress-induced unfolded protein response (UPR) target genes, including PERK-eIF2alpha and CHOP/GADD153. Furthermore, hypericin-PDT also activates rescuing responses which are governed by the activation of the stress-induced mitogen-activated protein kinase (MAPK) p38. $p38^{MAPK}$ inhibition or silencing by RNA interference during PDT treatment unravelled that the induction of an important subset of differentially expressed genes regulating growth and invasion, as well as adaptive mechanisms against oxidative stress, is governed by this stress-activated kinase. ROS-mediated activation of $p38^{MAPK}$ is required for the induction of a cluster of Nrf2-regulated targets, including heme oxygenase 1 (HO-1) with cytoprotective function[4], and for the up-regulation of COX-2, which is essential to support autonomous re-growth and migration of cancer cells escaping PDT-mediated cell death. These studies identify new molecular effectors of the cancer cell response to PDT-induced photodamage and open attractive avenues to improve the therapeutic efficacy of PDT of bladder cancer.

1. Dolmans DE *et al. (*2003) Nat Rev Cancer 3: 380-387
2. Buytaert E et al. *Oncogene*, manuscript under revision
3. Buytaert E *et al.* (2006) FASEB J, 20: 756-758
4. Kocanova *et al.* (2007) Apoptosis, 12 : 731-741

Keywords: photodynamic therapy, p38MAPK, microarrays

Presenting author: Nadine Camougrand

Regulation of mitophagy by redox pathways in *Saccharomyces cerevisiae.*

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Autophagy is a general non-selective process of intracellular degradation. It plays a crucial role in the survival against nitrogen starvation in yeast, mammals and plants. The selective degradation of mitochondria termed “mitophagy” has not been proven, but a selective degradation of altered mitochondria may play a role in cell maintenance.
Lactate-grown yeast cells subject to nitrogen-starvation, have showed the existence of both, a selective and a non selective process for mitochondria degradation. This selective degradation was absent from Δuth1 mutant.
So we investigated the possible mitochondrial signals regulating mitophagy process. Studies in stationary phase have showed that Δuth1 mutant exhibited higher levels of ROS productions as well as mitochondrial proteins carbonylation. Moreover the fatty-acid composition of phospholipids was different.
The induction of mitophagy by nitrogen starvation in yeast wild type cells was specifically delayed by N-acetylcysteine (but not by others ROS scavengers).This data suggest a possible role of glutathion redox potential in the regulation of mitophagy. This is consistent with proposals by Lemasters and others authors suggesting that mitophagy may play a key role in delaying the accumulation of damaged mitochondria induced by oxidation events associated with aging.

Keywords: autophagy, mitochondria, redox state

Presenting author: Enrica Cannizzaro

Influence of the HTLV-1 p13 protein on mitochondrial function and cell turnover

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p13 is a small accessory protein of human T-cell leukemia virus type 1 (HTLV-1) that accumulates in the inner mitochondria membrane. p13 reduces cell proliferation rates *in vitro* and exerts a suppressive effect in *in vivo* models of tumor growth.
Our studies are aimed at defining the mechanistic links between the effects of p13 on cell turnover and mitochondrial function. Results showed that p13 induces a K+-influx into the matrix, causing depolarization and increased oxygen consumption. The increase in respiratory chain activity induced by p13 is associated with a dose-dependent increase in production of reactive oxygen species by mitochondria. p13 lowers the threshold for PTP opening in isolated mitochondria and sensitises cells to apoptosis mediated by ceramide and treatments affecting redox balance. In addition, p13 influences cellular energy production, favoring oxidative metabolism over glycolysis, an effect that is consistent with its anti-tumor effect *in vivo*. These findings provide clues into the function of p13 as a negative regulator of cell growth, and underscore a link between mitochondria and tumorigenicity. In the context of the HTLV-1 life cycle, the negative effects of p13 on cell growth might favor persistence of the virus and adaptation to the host.

Keywords: mitochondria, apoptosis, leukemia

Presenting author: Andrea Caporali

Identification of the anti-apoptotic activity of Nerve Growth Factor (NGF) on cardiac myocytes

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Neurotrophins (NTs) control the survival and regeneration of neurons. Recent research showed that the potential of neurotrophins expands beyond the nervous system to include cardiovascular actions. In this study, we investigated the hypothesis that the NT nerve growth factor (NGF) prevents cardiomyocyte apoptosis. We demonstrated that cultured rat neonatal cardiomyocytes (RNCMs) produce NGF and express its trkA receptor. RNCMs given a neutralizing antibody for NGF or the trkA inhibitor K252a underwent apoptosis, thus suggesting that NGF is an endogenous pro-survival factor for cardiomyocytes. Adenovirus (Ad)-mediated NGF over-expression protected RNCMs from apoptosis induced by either hypoxia/reoxygenation or angiotensin II (AngII). Similarly, recombinant NGF inhibited AngII-induced apoptosis in isolated adult murine cardiomyocytes. In RNCMs, recombinant NGF induced trkA phosphorylation, followed by Ser473-phosphorylation and nuclear translocation of Akt. In response to Akt activation, Forkhead transcription factors Foxo–3a and Foxo-1 were phosphorylated and excluded from the nucleus. The pro-survival effect of Ad.NGF was inhibited in vitro by K252a, LY294002 (a pan-phosphatidyl inositol 3-kinase ¬-PI3K- inhibitor), and adenoviruses carrying a dominant negative mutant form of Akt (Ad.DN.Akt) or an Akt-resistant Foxo-3a (Ad.AAA-Foxo-3a). Finally, in a murine model of myocardial infarction, NGF gene transfer promoted cardiomyocyte survival. These results newly demonstrate the cardiac pro-survival action of NGF and provide mechanistic information on the signaling pathway, which encompasses trkA, PI3K-Akt, and Foxo.

Keywords: NGF, cardiomyocytes, TrkA, Apoptosis, Akt, Foxo, Hypoxia/Reoxygenation, Myocardial Infarction

Presenting author: Sara Caprodossi

TRPV1 induces a p53-dependent apoptosis of human bladder cancer cells.

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Carcinoma of the urinary bladder is the fifthy most common neoplasm and the twelfth leading cause of cancer death. It is known that capsaicin (CPS), the pungent compound of red pepper, inhibits in vivo and in vitro the growth and promotes programmed cell death of various tumor cells such as adenocarcinoma, melanoma, leukaemia and also glioblastoma cells. Many of the vanilloid-mediated anti-tumor effects are mediated by TRPV1, a non-selective cation channel structurally related to members of the transient receptor potential family of ion channels.
The present study was undertaken to analyze the expression and role of TRPV1 in human normal urothelial (HUC) and RT4 bladder cancer cell lines, expressing p53 wild-type.TRPV1 mRNA expression was evaluated by quantitative real time PCR showing an up-regulation of TRPV1 in RT-4 cells compared to HUC, used as control. The analysis of TRPV1 protein levels, performed by cytofluorimetric and western blot analyses confirmed this data, indicating a high TRPV1 expression in RT4 cells, localized both in cytoplasm and plasma membrane.
The TRPV1 channel is functional in RT4, as evaluated by its ability to induce increase of $[Ca^{2+}]_I$ in response to capsaicin (CPS), a specific TRPV1 agonist.
CPS treatment also significantly reduces in a dose-dependent manner RT4 cell viability. Moreover, CPS exposure induces PS externalization increase, caspase-3 activation, Go/G1 subdiploid oligonucleosomal DNA fragmentation and apoptosis in RT4. The CPS-induced apoptosis of RT4 cells is associated to a time-dependent increase of p53 protein levels, and to the p53 phosphorylation at the Ser-15, -20 and -392 residues.
Since p53 phosphorilation is mediated by ATM protein signaling, we investigated CPS-induced ATM activation by western blotting analysis. Data showed that ATM phosphorilation in Ser-1981 was induced by CPS in a time-dependent manner.
Overall, these findings suggest that p53 wild-type and TRPV1 expressing RT4 bladder cancer cells are highly susceptible to CPS-induced apoptosis.

Keywords: Bladder cancer cells, capsaicin, TRPV1, p53.

Presenting author: Jessy Cartier

cIAP1 regulates cell proliferation

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cIAP1 *(cellular Inhibitor of Apoptosis Protein 1*) belongs to the IAP family of proteins (*Inhibitors of Apoptosis Proteins*) which have been first characterized by their capacity to inhibit apoptosis. However, other functions have also been described such as regulation of signaling pathways, cell division or immune response. In a previous work, we identified a relocalisation of cIAP1 from the nucleus to the golgi apparatus in cell undergoing macrophagic differentiation.[1] To get insight into the role of cIAP1 on cell differentiation, we analyse the influence of cIAP1 overexpression or downregulation on cell differentiation and proliferation.

We showed that retroviral-mediated overexpression of cIAP1 inhibits the apparition of some differentiation markers such as CD11b expression and cell growth arrest in THP1 and U937 monocytic cell lines induced to differentiate into macrophage-like cells by phorbol ester exposure. Moreover, an overexpression of cIAP1 stimulates cell proliferation. On contrary, a downregulation of cIAP1, by antisens or RNA interference strategies, induces a spontaneous expression of some differentiation markers in monocytic THP1 and colic epithelial HT29 cells and decreases cell proliferation of THP1 and Hela cells, independently from caspases activation. cIAP1 does not significantly modify the cell cycle distribution but alters the level of expression of some cell cycle regulators such as cyclins. To end, we showed that cIAP1 can stimulate the transcriptional expression of cyclin A and B. All together, our data showed that cIAP1 is a regulator of cell proliferation. The nuclear export of cIAP1 during differentiation process might allow the arrest of proliferation required for the commitment of differentiation. cIAP1 is overexpressed in a large range of cancer cells and its nuclear expression appeared to be a bad prognostic.[2] Recently, BIRC2/cIAP1 has been demonstrated to display oncogenic properties in mouse hepatocarcinoma model.[3] This study opens new insights into the role of cIAP1 in the development and evolution of these diseases.

[1]Plenchette et al. Blood 1004, 104: 2035-2043.
[2]Zender et al. Cell 2006, 125: 1253-1267.
[3]Tanimoto et al. Cancer Letters 2005, 224: 141-145

Keywords: IAP, proliferation, differentiation, cyclins

Presenting author: Gisela Ceballos

Regulation of mitochondrial Smac/DIABLO-selective release by survivin

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The intrinsic apoptotic pathway is characterized by the release of several mitochondrial intermembrane proteins into the cytosol, like cytochrome c, Smac/DIABLO, Omi/HtrA2, Endonuclease G and AIF. Smac/DIABLO (second mitochondria-derived activator of caspase/ direct IAP binding protein of low pI) potentiates apoptosis by binding IAP's (inhibitor of apoptosis proteins) and neutralizing their inhibitory activity on caspases. The release of Smac into the cytosol is an important regulation mechanism for this protein. Although it is known that the Bcl-2 family proteins, ERK1/2 and JNK regulate this process, the precise mechanism still remains unclear. It has been reported that Smac is released together with cytochrome c as well as independently. This discrepancy might be the result of the utilization of different apoptotic stimuli that might retain Smac in the mitochondria. To gain more insights into the Smac release during apoptosis, we chose two cell death stimuli that engage the mitochondrial pathway. HeLa cells were exposed to LD50 of staurosporine and etoposide, and the Smac release was analyzed. We showed that during etoposide induced apoptosis, survivin and, to a lesser extent, the survivin splice variant survivin DeltaEx3 regulate the specific liberation of Smac/DIABLO. Etoposide induced posttranscriptional upregulation of survivin and survivin DeltaEx3. Mitochondrial survivin associated with Smac/DIABLO, delaying its release. In addition, cytosolic survivin also stabilized the cytosolic levels of released Smac/DIABLO. These results could explain the observed differences in the release of mitochondrial proteins in various apoptotic models and present a new mechanism for the anti-apoptotic effects of survivin in cancer cells.

Keywords: cancer; DNA damage; inhibitor of apoptosis proteins; mitochondria

Presenting author: Chiara Cenciarelli

Mitotic catastrophe and pericentriolar matrix dismantling are induced by the tubulin-depolymerising agent combretastatin-4 in lung cancer cells H460

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The relationship between microtubular dynamics, dismantling of pericentriolar components and induction of apoptosis was analysed after exposure of H460 non-small lung cancer cells to antimitotic drugs. The microtubule destabilising agent, combretastatin-A4 (CA-4) led to microtubular array disorganization, aberrant mitosis, accompanied by the presence of numerous "star-like" structures containing pericentrosomal matrix components like γ-tubulin, pericentrin and ninein, whereas the structural integrity of centrioles was not affected by treatment. Alphãacetylated-, drug-resistant short microtubules connected aggregates of the pericentrosomal material to kinetochores. Prolonged exposure to CA-4 or high concentrations of the drug caused the disappearance of such aggregates, whereas exposure to paclitaxel, which stabilises mitotic microtubules, led to multiple α-tubulin aggregates lacking of pericentriolar components. Treatment with 7,5 nM CA-4, which produced a high frequency "star-like" mitosis, was accompanied by mitotic catastrophe commitment characterized by activation of caspases-3/9 and DNA fragmentation.
Overall, our findings suggest that the maintenance of microtubular integrity plays a relevant role in stabilising the pericentriolar matrix, whose dismantling can be associated with apoptosis via mitotic catastophe mechanism after exposure to microtubule depolymerising agents.
Financially supported by SIGMA-TAU

Keywords: mitotic catastrophe, pericentriolar matrix, tubulin depolymerising agents

Presenting author: Cristina Cerqua

Trichoplein, a Keratin-binding protein, participates in mitochondrial morphology and dynamics

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Cytoskeleton is crucial for the maintenance of mitochondrial morphology, motility and distribution. In yeast, mitochondrial transport depends on actin, while in higher eukaryotes mitochondria primarily move along microtubules. It has been proposed that intermediate filaments could contribute to anchor mitochondria at specific cellular sites. Trichoplein is a novel protein that interacts in vitro with keratin and contains a trichohyalin/plectin homology domain (TPHD); it displays a punctuate distribution reminiscent of fragmented mitochondria. We explored the possibility that trichoplein participates in mitochondrial dynamics and morphology. Fractionation experiments indicated that a large fraction of trichoplein is retrieved on mitochondria. Proteinase K and carbonate extraction assays demonstrated that trichoplein is a peripheral protein of the outer mitochondrial membrane. Given the lack of an obvious mitochondrial targeting sequence, we investigated the requirements for its mitochondrial localization. Fusion to GFP of different fragments of trichoplein showed that the first 111 aa are sufficient for a punctuate distribution that partially overlaps with mitochondria.

Levels of trichoplein influence mitochondrial morphology, as its overexpression causes fragmentation of the mitochondrial network, which is independent from Drp-1, a protein that regulates mitochondrial fission. Mitochondrial fragmentation induced by trichoplein was not caused by mitochondrial dysfunction, yet it was conversely dependent on the TPHD of trichoplein, suggesting an involvement of IF in regulating mitochondrial morphology. Finally, real time confocal microscopy experiments demonstrated that mitochondrial motility was inversely related to levels of trichoplein. In conclusion, our data show the involvement of an IF-binding protein in the regulation of mitochondrial morphology and movement, suggesting that IF can play a role in scaffolding and shaping this organelle.

Keywords: Keratin, cytoskeleton, mitochondria, movement

Iridovirus infection blocks apoptosis triggered by UV irradiation

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Iridoviruses are enveloped, icosahedral, large double stranded DNA viruses, belong to the family Iridoviridae. Grouper iridovirus (GIV) has been isolated from spleen tissue of diseased grouper *Epinephelus awoara.* Our previous study on complete genomic sequencing and analyses of GIV revealed the presence of an open reading frame (ORF) for anti-apoptotic B-Cell Lymphoma (Bcl)-2-like gene. The sequence analysis of Bcl-2 showed 30% similarity to that of human and four viruses, and possessed all the four putative domains, BH1, BH2, BH3, BH4 and a transmembrane domain (TM). So, this study was carried out to understand whether the GIV blocks apoptosis in the host cells and the influence of Bcl-2 in this anti-apoptotic process. The UV-irradiated grouper kidney (GK) cells produced apoptosis. However, the DNA fragmentation assay for the DNA extracted from the UV-exposed GK cells after GIV infection revealed an inhibition in the apoptosis. This was also proved by morphological features as well as TUNEL assay. To further confirm the ability of GIV on apoptosis inhibition, we have repeated the experiment using UV or heat –inactivated GIV. Northern blot hybridization showed that the transcriptional expression of GIV Bcl-2 starts at 2 hours post infection. The expression of Bcl-2 increased significantly in the presence of cycloheximide indicating that this Bcl-2 is an immediate-early gene. Western blot results revealed that this Bcl-2 protein is translated earlier post infection and also assembled into viron particle. Co-transfection studies showed that this protein is localized on the mitochondrial membrane. By fluorescent staining, it has been proved that the Bcl-2 expressing cells can effectively inhibit apoptosis. Taken together, these results demonstrate that GIV may inhibit GK cells to promote apoptosis. It is worth mentioning that the ability of an iridovirus to inhibit apoptosis is herein reported for the first time.

Keywords: iridovirus, apoptosis, bcl-2, UV

Presenting author: Boris Chernyak

Interaction of mitochondria-targeted antioxidants with cells in culture

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Quinones conjugated via C10 linker with triphenyl phosphonium cation (MitoQ and SkQ1) are powerful antioxidants in isolated mitochondria at 1-100nM. SkQ1 has much higher antioxidant activity than MitoQ. Fluorescent analog of SkQ1 accumulates in HeLa cells reaching maximum at 2h and selectively stains mitochondria. Uncoupler FCCP suppresses such an accumulation. Incubation with 1-100nM MitoQ or SkQ1 for 2h, 24h, or 48h does not result in increase of resistance of HeLa cells and human fibroblasts to H2O2 or other prooxidants (menadione, paraquat). No decrease in ROS accumulation is observed. Higher concentrations of antioxidants are toxic in the presence of H2O2.
However, 5-6 d incubation with 1-20nM SkQ1 causes strong resistance against oxidative stress. MitoQ was 100 times less effective. Simultaneously significant morphological changes and appearance of myofibroblasts (smooth muscle actin positive) are observed. All the effects are prevented by uncoupler. The changes in fibroblasts persist for 30-40 passages after removal of the agents. It is shown that MitoQ and SkQ1 stimulate production of TGFb by fibroblasts which is self-supported and causes morphological changes. Protective antioxidant effect of MitoQ and SkQ1 is not related to production of TGFb. Slow development of the protection is probably related to slow redistribution of these compounds in the cell, resulting in accumulation of these cations in mitochondria.

Keywords: mitochondria, antioxidants

Presenting author: Jun Hong Chng

Plasmodium Programmed Cell Death: The Suicide of a Killer

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The causative pathogen of malaria is the unicellular apicomplexan *Plasmodium*, of which *Plasmodium falciparum* is the most lethal species accounting for over 90% of fatalities. Despite advances in healthcare, the disease situation has been exacerbated with the development of resistance to commonly used antimalarials like chloroquine. It is imperative that new treatments are designed to keep the disease in check and better understanding of the parasite will be integral in aiding such design.

Despite chloroquine's profound impact in combating malaria, little is known about its mechanism of action. It had been shown previously that chloroquine treatment results in DNA laddering, a typical apoptotic hallmark. Yet till today, the existence of programmed cell death (PCD) pathways in the *Plasmodium* has remained controversial although evidence of apoptosis in other unicellular organisms has been mounting.

This study on *P. falciparum* observed for classical apoptotic features including mitochondrial dysregulation, the activation of caspase-like proteases and the *in situ* fragmentation of DNA after chloroquine or staurosporine treatment. Three conventional assays were used to detect these apoptotic features: a mitochondrial outer-membrane potential sensing dye, a fluorescent pan-caspase inhibitor CaspaTag and DNA nicked-end labeling using TUNEL. Results were then quantified using flow cytometry to show not only the existence of the hallmarks but also their prevalence in the chloroquine or staurosporine treated populations.

Additionally, the use of selective apoptotic inhibitors (cyclosporin A and zVAD-fmk) allowed for the characterization of the speculated PCD pathway. Cyclosporin A is known to prevent the loss of mitochondrial membrane potential while zVAD-fmk is able to bind irreversibly to active caspase heterodimers at their active site thereby nullifying its activity. The varying ability of these inhibitors to limit apoptotic features suggested the existence of a single linear pathway linking mitochondrial dysregulation and caspase-like activity to DNA fragmentation. This is the first time the existence of an apoptotic pathway has been delineated in *P. falciparum* and posits a novel approach in the design of future anti-malarial drugs.

Keywords: malaria, Plasmidium falciparum, caspase, MOMP, DNA fragmentation, programmed cell death

Presenting author: Sara Cipolat

The role of OPA1 in differentiation of embryonic stem cell to neurons and cardiomyocytes

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OPA1 is a dynamin-related protein located in the inner mitochondrial membrane and mutated in dominant optic atrophy. OPA1 plays two genetically distinct roles in promoting mitochondrial fusion by cooperating with MFN1, a large GTPase of the outer mitochondrial membrane; and in regulating apoptosis, controlled by the mitochondrial rhomboid protease PARL. PARL operates upstream of OPA1, by participating in the production of a soluble form of OPA1 required to keep in check the pathway of cristae remodeling and cytochrome *c* redistribution. Thus, OPA1 likely affects complex functions, as substantiated in overexpression studies that show a role for this protein in lymphocyte movement and dendritic spine formation. We therefore reasoned that levels of OPA1 are likely to affect development and function of multiple organs, by regulating mitochondrial fusion or apoptosis. To dissect the roles of OPA1 we decided to generate an OPA1 conditional knock-out mouse model, required to study the function of OPA1 in specific tissues and at different development stages. At the same time, we decided to study whether ablation of OPA1 influenced differentiation of embryonic stem (ES) cells in vitro using a hanging-drop differentiation system. To this end, we analyzed an ES cell line where Opa1 had been gene trapped ($Opa1^{gt}$), resulting in an Opa1+/- genotype. We compared the differentiation potential into cardiomyocytes and neurons of this $Opa1^{gt}$ ES cell line to its relative wt ES cell line. $Opa1^{gt}$ ES cells display a decreased capacity to differentiate into beating cardiomyocytes, while they retained an almost normal neuronal differentiation potential. We aim at understanding the molecular mechanism by which levels of Opa1 influence differentiation into cardiomyocytes.

Keywords: OPA1, embryonic stem cells, cardiomyocytes, neurons

Presenting author: Clay Clark

Folding and assembly of procaspase-3: The role of the dimer interface in active site formation

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We have examined the assembly of the procaspase-3 subunits into the native dimer and the roles of several amino acids in the dimer interface in active site formation. Our objectives are to understand the role of dimerization in stability and active site formation of caspases. Using stopped-flow circular dichroism and fluorescence emission studies, we show that the monomer folds rapidly upon dilution from urea (<1 msec), and the species contains approximately 60% of the total secondary structure and thermodynamic stability of the monomer. Longer time course studies suggest that the monomeric intermediate may be misfolded since dimers form only after about forty seconds of refolding. By using a catalytically active, yet uncleavable, mutant of procaspase-3, we show that a late folding reaction correlates to formation of the active site. The protein is fully folded only after about 30 minutes. Following dimerization, several residues in the interface are involved in stabilizing the active site. We show that a mutation of V266H abolishes activity in the (pro)caspase, further demonstrating that residues in the dimer interface affect proper active site formation. In contrast, a mutation of V266E resulted in an enzymatically active procaspase. Importantly, the gain of activity for V266E was not dependent on cleavage of the polypeptide chain. We have determined the high resolution structure of V266E, and the data show that the side chain is positioned to interact with R164 from the active site, similar to interactions found in caspase-1, and biochemical data suggest that the intersubunit linker is displaced such that the active site loop bundle partially forms. In addition, the V266E mutation disrupts a network of water molecules that connects the two active sites of caspase-3. Likewise, the crystal structure of V266H shows the mechanism of inactivation of the caspase-3 active site through the dimer interface. Collectively, the data show that the dimer interface of caspase-3 should prove to be a fruitful target for the binding of small molecules that affect procaspase-3 activity, either for activation or for inactivation. This work was supported by a grant from the National Institutes of Health (GM065970).

Keywords: dimer assembly, crystallography, folding

Presenting author: Marco Corazzari

Combining homeostatic mechanisms of endoplasmic reticulum stress to increase susceptibility of cancer cell to ER stress-induced apoptosis: The role of stress proteins ERdj5 and ERp57.

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Endoplasmic Reticulum (ER) malfunction, leading to ER stress, can be a consequence of genome instability and hypoxic tissue environments. Cancer cells survive by acquiring or enhancing survival mechanisms to counter the effects of ER stress and these homeostatic responses may be new therapeutic targets. Understanding the links between ER stress and apoptosis may be approached using drugs to specifically target ER stress responses in cancer cells. Currently there is considerable clinical interest in the use of two ER stress inducing agents, fenretinide (a synthetic retinoid) and velcade (a 26S proteasome inhibitor) for the treatment of neuroectodermal tumours. Both agents induce potent apoptosis of both neuroblastoma and melanoma cells together with inducing the ER stress response genes ERdj5, ERp57, GRP78, calreticulin and calnexin. Since ERdj5 and ERp57 are induced as a consequence of ER stress, the aim of this study was to test the hypothesis that knockdown of these homeostatic response genes would increase the efficacy of either fenretinide or velcade. RNAi mediated knockdown of ERdj5 and ERp57 was carried out in SH SY 5Y neuroblastoma cells and A375 melanoma cells and verified by real time PCR and western blotting. In contrast to apoptosis-induced in response to the chemotherapeutic drugs vincristine and temozolomide, both fenretinide and velcade-induced apoptosis of melanoma or neuroblastoma cells was significantly increased through abrogation of either genes. These data suggest that down-regulating homeostatic ER stress responses may enhance apoptosis induced by ER stress-inducing agents. Therefore, ER-resident proteins such as ERdj5 and ERp57 may represent novel chemotherapeutic targets for neuroectodermal tumours.

Keywords: ER Stress, Apoptosis, Cancer

Presenting author: Veronica Costa

Exploring the role of the OPA1-dependent cristae remodelling pathway in the mitochondrial alterations of Huntington's Disease

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Huntington's disease (HD) is caused by an abnormal expanded polyglutamine (polyQ) repeat in the huntingtin (Htt) protein, whose function remain unclear. Alterations of mitochondrial function have been suggested to play a central role in the pathogenesis of HD. The underlying molecular mechanisms has not been yet elucidated; however, recent reports indicate a dramatic mitochondrial ultrastructural reorganization, resembling cristae remodelling, in lymphoblasts from HD patients.
Lymphoblasts from heterozygous HD patients were characterized by polarized and coalescent mitochondria, while mitochondrial network in homozygous cells was highly fragmented and distributed through the whole cell volume, albeit expression levels of mitochondria-shaping proteins were comparable to wt. Morphological changes were accompanied by latent mitochondrial dysfunction in situ and by faster release of cytochrome c induced by recombinant BID in vitro. Oligomers of OPA1 that correlate with the maintenance of cristae structure, appear less represented and disrupt faster following cBID in HD mitochondria, suggesting that by a yet unidentified mechanism mutated Htt interferes with oligomerization of OPA1. These results open the possibility that in the course of HD mitochondria are hypersensitive to apoptotic stimulation and call for a deeper investigation of the molecular interplay between Htt and the inner mitochondrial membrane.

Keywords: Huntington's Disease, OPA1, cyt c release, mitochondria, apoptosis

Presenting author: Valerie Croons

The protein synthesis inhibitor cycloheximide selectively depletes macrophages in rabbit atherosclerotic plaques

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Atherosclerotic plaque stabilization is an important pharmacological objective since unstable atherosclerotic plaques mainly contribute to cardiovascular mortality. Macrophages, which are an essential component of unstable atherosclerotic plaques, play a pivotal role in the destabilization process, whereas smooth muscle cells (SMCs) promote plaque stability. Recently, we demonstrated that local delivery of the mammalian target of rapamycin (mTOR) inhibitor everolimus selectively cleared macrophages in rabbit atherosclerotic plaques. Because mTOR controls mRNA translation, inhibition of protein synthesis might induce selective macrophage cell death. We therefore investigated in the present study whether the protein synthesis inhibitor cycloheximide can selectively deplete macrophages in atherosclerotic plaques. In vitro studies with cultured macrophages and SMCs showed that cycloheximide induced selective apoptosis of macrophages in a concentration- and time-dependent manner. Moreover, macrophages could be selectively depleted in rabbit carotid artery rings with collar-induced atherosclerotic plaques after in vitro treatment with 30 µg/ml cycloheximide. Local in vivo administration of cycloheximide via osmotic minipumps (10 µg/ml, 10 µl/h) to rabbit carotid arteries with collar-induced atherosclerotic plaques significantly reduced the macrophage but not the SMC content. Cycloheximide-treated plaques showed signs of apoptosis (increased terminal deoxynucleotidyl transferase end labeling and FITC-Val-Ala-DL-Asp(O-methyl)-fluoromethylketone labeling) that did not colocalize with SMCs. Vascular reactivity studies demonstrated that the functionality of the SMCs and the endothelium was not influenced by cycloheximide treatment. In summary, our data demonstrate that cycloheximide decreases the macrophage load in atherosclerotic plaques via apoptotic cell death without changing SMC content or contractility. These findings are important in terms of atherosclerotic plaque stabilization.

Keywords: apoptosis, cycloheximide, atherosclerosis

Presenting author: Hrvoje Cvija

The role of Fas/Fas ligand system in estrogen deficiency-induced osteoporosis

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Fas is a death receptor whose major function is mediation of T cell cytotoxicity and regulation of immune response. It is also ubiquitously expressed on various cell types where it is involved in regulation of differentiation and survival. Fas ligation induces apoptosis, but there is increasing evidence of non-apoptotic functions of Fas mediated by distinct signaling mechanisms dependent on the tissue type and other regulatory factors including cytokines, chemokines and growth factors. Fas is expressed on osteoblastic and osteoclastic cells, where it may, under specific circumstances, induce apoptosis. Fas may also inhibit osteoblast differentiation via caspase 8 dependent mechanism. It is hypothesized that estrogen deficiency up-regulates Fas on osteoblasts, which may lead to increased osteoblast apoptosis and/or their decreased differentiation and contribute to bone loss. The aim of this study was to estimate importance of the proposed pathogenic mechanism in vivo
We first analyzed the expression of Fas gene four weeks after the ovariectomy in bones and bone cell cultures from wild-type mice, and confirmed increased Fas expression in total bone tissue and osteoblasts from ovariectomized compared to sham operated animals.
Four weeks after ovariectomy in mice deficient for Fas gene (Fas -/-) we analyzed bone histomorphometric parameters and osteoblast and osteoclast differentiation in vitro. Bone volume was generally higher in Fas -/- mice than in wild-type controls, and significantly decreased after ovariectomy in wild-type mice, whereas it was unaltered in Fas -/- mice. Number of osteoclasts in vivo and osteoclastogenesis in vitro were increased after ovariectomy in wild-type mice, but unchanged in Fas -/- mice. Osteoblastogenesis in vitro was stimulated by ovariectomy in both mouse strains, and this effect was more pronounced in Fas -/- mice. Fas -/- osteoblasts expressed higher levels of osteoblast specific genes than the control osteoblasts. Osteoblast differentiation genes had similar expression patterns in sham operated and ovariectomized mice.
Our findings show that Fas/Fas ligand system may have an important role in the pathogenesis of postmenopausal osteoporosis. Modulation of its effects on bone cells may contribute to the development of new strategies for osteoporosis treatment.

Keywords: Fas, Stromal Cells, Osteoblasts, Osteoporosis, Transgenic/Knockout Mice

Presenting author: Fabrizio D'anselmi

Apoptosis and growth-inhibition induced by embryonic Zebrafish proteins in colon cancer cells

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In a previous research we showed that a set of regulatory proteins extracted from zebrafish embryo during differentiating processes, exerted inhibitory properties on several cancer cell lines. An experimental study was carried out in order to ascertain the pro-apoptotic effects of this protein network on colon cancer cells. Whole cell-count, flow-cytometry analysis and apoptotic parameters were recorded in human colon cancer cells line (Caco2) treated with Zebrafish embryonic proteins (ZEP, 3 µg/ml) in association or not with 5-Fluorouracil in the subpharmacological therapeutic range (0.01 mg/ml, FU). Cancer cell proliferation was significantly inhibited in a dose-dependent manner by embryonic extracts and an additive enhancing effect was evidenced in association with 5FU. At 48h the inhibition rate was 47,9% (ZEP), 68,5% (FU alone) and 74,3% in ZEP+FU-treated samples. Zebrafish proteins exerted a significant and increasing apoptotic effect at both 24h and 72h (36% and 17.3% respectively vs 7% in the control condition). Is note worthy that the association with FU leads to an enhanced additive apoptosis rate; in fact, programmed cell death increases from 41,4% (FU) to 60% (ZEP+FU) at 24h; at 72h, in FU-treated cells apoptosis account for 19,9% and raises to 24,2% in ZEP+FU-treated cells. ZEP alone and in association with FU triggered both the extrinsic and the intrinsic apoptotic pathways: in fact, in both conditions, we were able to detect an increase in the activation of caspase-8 and also of caspase-3 with respect to control condition. Western blot analysis of several factors (c-FLIP, c-Myc, Rb, E2F1, Bcl-2 and Bax) corroborate these results. These preliminary promising data suggest that zebrafish embryo factors could improve chemotherapy efficacy, exerting autonomous and complex interference on cancer cell viability.

Keywords: apoptosis embryonic Zebrafish proteins

Presenting author: Peter Daniel

Role of p21, Bax and Bak in TRAIL -induced apoptosis of colon carcinoma cells

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The death ligand TRAIL has been suggested as a suitable biological agent for the selective induction of cell death in cancer cells. Moreover, TRAIL synergizes with DNA damaging therapies such as chemotherapeutic drugs or ionising irradiation (IR). Here, we show that knockout of the endogenous CDK inhibitor p21 (CIP/WAF-1) sensitizes HCT116 colon carcinoma cells for TRAIL-induced apoptosis. This results in mitochondrial membrane permeabilisation, caspase-3 activation and cell death by apoptosis. Synergy of TRAIL and IR, i.e. cross-sensitization between TRAIL and IR for induction of apoptosis, entirely depends on Bax proficiency in HCT116 cells. Thus, human carcinoma cells are type II cells that entirely depend on the mitochondrial death pathway for the execution of TRAIL induced cell death pathways. Loss of Bax impaired TRAIL induced cell death and abrogated synergistic tumor cell killing by TRAIL and IR in both HCT116 colon and DU145 prostate carcinoma cells. Notably, Bax deficient HCT116 and DU145 cells still express significant levels of the multidomain proapoptotic Bcl-2 homolog Bak. This indicates that Bak is not sufficient to mediate cross-sensitization and synergism between IR and TRAIL. A molecular rationale for this functional impairment of Bak in TRAIL mediated apoptosis pathways is proposed. These data establish distinct roles for Bax and Bak in linking the TRAIL death receptor pathway to the mitochondrial apoptosis signaling cascade upon DNA damage by IR.

Keywords: TRAIL, p21, Bax, apoptosis

Presenting author: Anne-Zélie Decrion-Barthod

Potentiation of staurosporine effect by histone deacetylase inhibitors in restoring apoptotic pathways in uterine cervix-derived cancer cell lines.

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Carcinoma of the uterine cervix represents a major health problem worldwide. These tumors induced by persistent infection with high-risk human papillomaviruses (HPV), especially HPV16 and 18, are often resistent to conventional therapies. The E6 and E7 viral oncoproteins expressed in infected cells bypass cell death pathways. Thus, the development of novel cancer therapies that can specifically restore these pathways is an important task for the clinical treatment of HPV-induced cervical cancer.

In our laboratory, several pieces of evidence support that staurosporine (ST) is able to activate apoptosis of HeLa and CaSki cells which is associated with an inhibition of E6 and E7 expression and higher levels of p53 and its target products $p21^{WAF1}$ and Bax. p53 as well as Bax are then localized in the mitochondria and promote loss of membrane potential and cytochrome *c* release thereby activating the APAF1/ caspase 9 cascade. Thus ST restores p53-dependent apoptosis *via* intrinsic pathway.

On the other hand, histone deacetylase inhibitors (HDis) are able to block cell cycle of HeLa and CaSki cells in the G1 phase independently of the viral oncogene expression and HPV type. Moreover, a prolonged incubation of HeLa cells with HDIs induces apoptosis *via* the E2F/p73 pathway, and especially through the expression of the pro-apoptotic isoforms p73alpha and p73beta.

Therefore the combined use of ST and HDI was tested to highlight a synergistic apoptotic effect in HeLa and CaSki cells. Time-courses and dose-responses were performed with different combinations of drugs. Our results show that co-treatment of HeLa and CaSki cells by ST and HDIs (sodium butyrate or Trichostatine A) clearly blocks cell cycle progression in the G1 phase whatever the staurosporine concentration and increases in a synergistic manner the proportion of cells with depolarized outer mitochondria membrane and subsequently the number of apoptotic cells. Different proteins depending on the apoptotic stimulus and intracellular pathways utilized are currently studied: p53, p73, Bax, and Bcl-2.

Keywords: cancer, HR-HPV, therapy, apoptosis

Presenting author: Livia Di Renzo

p38 MAPK activation and COX-2 induction mediate the survival of human leukemic cells.

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Cells sense various forms of injury and insult by biochemical and molecular machinery and execute appropriate programs of response: they undergo cell cycle arrest to allow adequate time for repair of damage or undergo programmed cell death, if the damage is too severe. In tumor cells surviving to cytotoxic/apoptotic stimuli a network integrating several pathways may involve different survival pathways, as mitogen-activated protein kinase (MAPK) signaling cascades, the phosphoinositide 3-kinase (PI3K)/Akt pathway, the nuclear factor (NF)-kB signalling system and the heat shock response.
In this study, we selected monensin as a drug provoking, in a receptor independent and in a concentration and time-dependent manner, pH stress, cell cycle arrest or apoptosis of several tumor cell lines. Our observations that 20% of human leukemic U937 cells, upon 24 h of treatment with a low dose of monensin, undergo apoptosis, prompted us to investigate the signals allowing 80% of the cells to survive to the alkalinization stress.
Our results show that the delay of death of U937 cells, growth arrested in G1 cell cycle phase, was allowed by the activation of p38 MAPK, as its inhibition with SB203580, but not the inhibition of ERK with PD98059 or of PI3K with wortmannin, caused a significant cell death increase. The activation of p38 MAPK was confirmed by Western blotting detection of the phosphorylated protein.
COX enzymes form prostaglandins and thromboxane from Arachidonic acid. COX-2 is almost undetectable in normal tissues and it is promptly expressed upon the action of oncogenes, cytokines and mitogens. Accordingly, we observed COX-2 in monensin treated U937 cells, but not in untreated cells and, furthermore, by using the inhibitors celecoxib and NS398, we detected that this enzyme contributes to realize the survival pathway activated by monensin.
Overall, the here reported p38 MAPK-COX-2 survival pathway, activated upon stress conditions in human leukemic cells, may be important in order to establish more effective antineoplastic therapy.

Keywords: MAPK, p38, COX-2, arachidonic acid, survival pathway, leukemic cells

Presenting author: Luciana Dini

Pathways involved in Rose Bengal-induced apoptosis in HeLa cells

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Photodynamic therapy (PDT) is a novel treatment for cancer and certain non-cancerous conditions. The procedure requires exposure of cells or tissue to photosensitizing drug followed by irradiation with visible light of the appropriate wavelength. Several studies demonstrated PDT-mediated apoptotic/necrotic outcomes in various cell types; in many cases PDT is highly efficient in inducing apoptosis. We recently showed that the fluorogenic substrate Rose Bengal acetate (RBAc), , induced cell death in HeLa cells, whose morphological modifications and mitochondria membrane potential loss were typical of apoptotic death. In this present study we investigated whether apoptosis was induced through intrinsic or estrinsic apoptosis pathways upon PDT treatment. Time course of recovery in fresh medium from one hour to 72 hours of HeLa cells treated with PDT using RBAc, irradiated with a green LED DPL 305, emitting at 530±15 mm in order to obtain 1.6 J/cm^2 as total light dose, was investigated. 4/12hours following treatment 40% of the cells exhibited typical apoptotic features. Caspases 3, 8 and 9 and bcl2, bax and bid product genes were evaluated by Western blots in subcellular fractionated samples. Bax and bid proteins were increased soon after the PDT treatment while bcl2 proteins dropted. Release of cytochrome c from mitochondria followed by the cleavage of caspeses 9 and 3 was observed. A high increase of the cleaved form of caspase 9 and cytochrom c release was observed between 4 and 12 h from the PDT treatment. The last caspase to be cleaved (between 12 and 72 h) was caspase 8. Alterazione lisosomi e reticolo.
These results demonstrate that caspase-independent and caspase-dependent pathways were both activated but in different times. The intrinsic pathway of apoptotic induction of PDT was induced earlier than the extrinsic pathway.

Keywords: caspases 3-8-9; cytochrome c; PDT

Presenting author: Lidia Domnina

Antioxidant Mito Q changes the morphology of transformed cells to normal state and protects the cells from H2O2 induced apoptosis

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We investigated the effect of antioxidant Mito Q (mitochondrial targeted antioxidant) which in very small concentrations protects the cells from death, induced by H_2O_2. We used human and rat epithelial (HeLa, IAR-2) and fibroblastic (10/3, 10/3-ras, rat-1,L) cell lines and primary cultures of human subcutaneous fibroblasts. Morphometry method, confocal microscope, immunofluorescence staining were used to investigate cell morphology, cytoskeleton and mitochondrial system. It was shown that after antioxidant treatment the area of spread cells increased and the cells shifted from fibroblastic to epithelioid shape. After antioxidant treatment transformed 10/3-ras cells became more spread like control 10/3 fibroblast and actin cytoskeleton normalized also. All cell types demonstrated increasing and thickening of actin microfilament bundles (stress fibers) as well as increasing in amount of actin-connected myosin.
Areas and lengths of focal contacts substantially increased.
After antioxidant treatment large number of differentiated human fibroblast, containing α-smooth actin appeared. The antioxidant deleted effect of oxidative stress of H_2O_2: mitochondria did not fragment and the cells escaped H_2O_2-induced apoptosis. Formation of mitochondrial network under MitoQ-influence was shown in experiments with laser beam point-like damage of HeLa cells and of human fibroblast in contrast to control cells where the single mitochondria were present. Possible mechanisms of observed alterations will be discussed.

Keywords: antioxidant apoptosis cells mitochondria hydrogen peroxide

Presenting author: Valerio Donato

The Glucocorticoid-Induced Leucine Zipper (GILZ) inhibit skeletal myogenesis by counteracting MyoD Activity

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Myogenesis is characterized, at a molecular level, by the activation of transcription factors named muscle related factors (MRFs) and myocyte enhancer factors-2 (MEF-2), which promote myoblast exit from cell cycle and fusion of myoblast into myotubes. This process is self-sustained by the release of insulin-like growth factor-1, while other molecules, such as myostatin, limit muscle formation and help to keep a pool of reserve cells (satellite cells) in an undifferentiated state. However, the intracellular mechanisms that regulate the fine tuning of myoblast differentiation are not completely understood.
GILZ is a small molecule known to be a glucocorticoid effector in T cells, where it counters activation signals, regulates cell survival and exerts anti-inflammatory effects. Its expression is not restricted to T cells and comprehends mesenchymal derived tissues such as skeletal muscle.
Preliminary data indicate that GILZ can interact with histone-deacetylase (HDAC)-1, which is an important myogenic regulator. MyoD, the best known MRF, is HDAC1-bound when inactive, and myogenic stimuli promote HDAC1 removal and MyoD acetylation: this allows MyoD association with chromatin regulators, such as CBP/p300 and SWI/SNF, and consequent induction of myogenin, the main inducer of terminal differentiation.
GILZ expression is developmentally regulated being reduced during early differentiation stages and successively re-induced. We found that GILZ inhibits myogenesis when over-expressed in C2C12 myoblast cell line. Our data indicate that GILZ suppresses myogenin induction and, consequently, the myogenin target gene myosin heavy chain, a late differentiation marker. Moreover, morphological analyses revealed a significant inhibition of myotube formation. We investigated MyoD activity on myogenin promoter and found that GILZ suppresses MyoD transcriptional activity in a dose dependent manner; intriguingly, GILZ can directly interact with MyoD.
Based on these observations, we hypothesize that GILZ recruits HDAC1 to MyoD target loci and speculate that this process physiologically happens in later stages of myogenesis, thus contributing to the fine tuning of differentiation. Therefore, GILZ might represent a pharmacological target to sustain an effective recovery after muscle injury.

Keywords: GILZ, differentiation, myogenesis

Presenting author: Linde Duprez

The intermediate domain of RIP1 is necessary but not sufficient to mediate death receptor-induced necrotic cell death

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Receptor-interacting protein 1 (RIP1) is a crucial adaptor kinase on the crossroad of a cell's decision to live or die as it can differentially signal to apoptosis, necrosis, or activation of NF-κB and MAPKs. RIP1 consists of three different domains: an N-terminal kinase domain (KD), an intermediate domain (ID) and a C-terminal death domain (DD). It is obvious that these divergent activities of RIP1 could arise from different properties harboured in one of its three domains (reviewed by Festjens et al. CDD 2007).
Studies in RIP1-deficient Jurkat cells have shown that RIP1 kinase activity is necessary to initiate death receptor-induced caspase-independent necrotic cell death. To further examine the role of the three different domains of RIP1 in necrotic signalling processes, we performed a detailed structure-function analysis of RIP1. We therefore retrovirally transduced L929sAhFas cells with either single domain mutants of RIP1 (DD, KD, ID) or deletion mutants of RIP1 (delta DD, delta KD, delta ID). Neither a domain alone was sufficient to induce caspase-independent cell death, the latter being confirmed in the presence of zVAD-fmk or in L929sACrmA cells. Overexpression of delta DD or delta KD induces caspase-independent death, whereas this is not the case for the delta ID mutant. Moreover, we show that RIP1 delta ID performs dominant negative effects on death receptor-induced necrotic signalling pathways in the presence of caspase inhibitors whereas it induces a shift to apoptosis in the absence of caspase inhibitors.
Altogether these results suggest that the RIP1 ID is necessary but not sufficient to mediate death receptor-induced necrosis, arguing for an ID regulatory role, in addition to the crucial role of the RIP1 kinase activity in necrotic signalling pathways.

Keywords: RIP1, necrotic cell death

Presenting author: María Beatriz Durán Alonso

Effect of extracellular matrix proteins on resistance to cell death induction and cathepsin expression

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Gliomas are the most common cancer of the central nervous system and patients with glioblastoma (GBM), the most malignant form, have a life expectancy of under a year. Main obstacles to therapy are the highly infiltrative nature of this cancer, which precludes the complete resection of the tumour and the resistance of a proportion of the infiltrating cells to available therapeutic agents. These are thought to be responsible for the tumour recurrences that follow shortly after treatment, leading to the death of these patients. Elucidation of the mechanisms that lead to drug-resistance of glioma cells is therefore critical if this cancer is to be treated successfully.

It is now widely accepted that one of the main players in the appearance of drug-resistant phenotypes is the extracellular matrix (ECM). Our aims were (a) to test the effect of various matrix proteins, namely vitronectin, fibronectin and collagen, on the resistance of U87 glioblastoma cells to cell death induction, and (b) to establish whether differences in the resistance of these cells correlate with a differential regulation of cathepsin B (Cat B) and/or cathepsin L (Cat L) expression. These two lysosomal cysteine proteases are over-expressed in various cancers and are involved in tumour progression.

Here we address the possible function of these cathepsins in ECM-mediated drug-resistance mechanisms in view of their known roles in cell death and reported effects of extracellular matrix proteins on the expression of these proteinases. We have identified vitronectin and collagen as substrates that promote the survival of U87 glioma cells exposed to the cell death-inducer staurosporine. This is of interest as vitronectin is abundant at the tumour edge, where most of the recurrences appear, while collagen is a preferred migratory substrate for glioma cells. We do not observe increased resistance of U87 cells to cell death induction on poly-L-lysine; this phenomenon is thus likely mediated by integrins. Regarding cathepsin expression, we have identified differences at the mRNA level, pointing to a possible role of these proteases in ECM-mediated drug-resistance. These data may be relevant to the treatment of glioblastoma, offering a route to improve the efficacy of chemotherapy.

Keywords: Extracellular matrix, cell death, glioma, cathepsin

Presenting author: Jürgen Eberle

Preparing the basis for efficient targeting of melanoma by proapoptotic genes in conditional replication-competent, oncolytic adenoviral vectors

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Melanoma incidence has been increasing worldwide in the last decades, while mortality remained on a high level. There is no suitable therapy for metastasized melanoma by now, which could lead to a significant increase in overall survival.
Due to the pronounced therapy resistance of melanoma, apoptosis deficiency appears as a most critical factor for melanoma malignancy. Our previous work has characterized the basic mechanisms of apoptosis regulation in melanoma. Thus the mitochondrial pathway appeared most important, also for extrinsic induction of apoptosis, and overexpression of Bcl-2 or Bcl-xL was able to largely prevent any apoptosis induction in melanoma cells. However, genes and strategies suitable for efficient induction of apoptosis in melanoma cells could be identified, which are based on proapoptotic Bcl-2 proteins (Bcl-xS, Bik/Nbk and Bax) as well as on TNF-related death ligands (CD95L/FasL and TRAIL). In conditional expression systems, these genes induced apoptosis and enhanced chemosensitivity of cultured melanoma cells. In appropriate nude mouse models, they could prevent melanoma growth in vivo. The related signal cascades were thoroughly analyzed giving indications for well described apoptosis pathways as caspase activation and mitochondrial activation, but also indications of new cell death pathways largely independent of any caspase activation. Proapoptotic genes may be employed in improved gene therapeutic strategies, based on conditional replication-competent, oncolytic adenoviral vectors. For melanoma, these vectors are based on selective, tyrosinase promoter-driven expression of adenoviral replication genes and on inducible expression of the proapoptotic factors by improved tetracycline-responsive promoters.

Keywords: cancer - melanoma - Bcl-2 proteins - death ligands - adonoviral vectors

Presenting author: Leopold Eckhart

Phylogenetic analysis of mammalian caspases

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Proteases of the caspase family play central roles in apoptosis, inflammation, and other processes. The different functional properties of caspases have evolved by repeated duplications of a common ancestral caspase gene and subsequent sequence modifications. To establish the phylogenetic basis for tests of caspase structure-function relationships, we have analyzed mammalian caspases by comparative genomics using a broad panel of genome sequences. We identified a novel caspase gene, tentatively named caspase-16, that has been conserved in most descendents of the last common ancestor of marsupials and placental mammals, including humans. Lineage-specific gene duplications were found for caspase-3 in opossum and caspase-4/5 in primates, whereas lineage-specific gene inactivations were found for caspase-10 in muroid rodents, caspase-12 in human, rabbit and cow, and caspase-15 in Afrotheria (including elephant) and Euarchontoglires (including mouse and human). Caspase-1 and 4 genes have undergone fusion to form a single gene in carnivores (dog and cat). Other caspases were generally conserved in all mammalian species investigated. Comparison of exon-intron structures showed that there are three phylogenetic groups of caspase genes: caspase-1/2/4/5/9/12/14/15/16 (group I), caspase-3/6/7 (group II), and caspase-8/10/CFLAR (group III). Our analysis suggests that gene segments encoding a caspase recruitment domain (CARD) and a tandem of death effector domains (DED) were fused to the ancestral genes of group I and group III caspases, respectively. The results of this study reveal a complex phylogenetic history of mammalian caspases and suggest that differences in the pool of caspases limit the usefulness of several animal models of caspase-dependent physiological processes.

Keywords: caspases, evolution, genomics

Presenting author: Anna-Mart Engelbrecht

Proanthocyanidin from grape seeds inactivates the PI3-Kinase/PKB pathway and induces apoptosis in a colon cancer cell line.

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The aim of this investigation was to evaluate the potential chemopreventative/antiproliferative potential of a grape seed proanthocyanidin extract (GSPE) against colon cancer cells (CaCo2 cells) and to investigate its mechanism of action. GSPE (10-200 μg/ml) significantly inhibited cell viability in a dose-dependent manner in CaCo2 cells, but did not alter viability in the normal colon cell line (NCM460). GSPE also significantly increased apoptosis in CaCo2 cells as indicated by significant increases in caspase-3 activation and PARP cleavage. Furthermore, our results also indicate that GSPE could be a potent chemotherapeutic agent for colon cancer targeting the phosphatidylinositide 3-kinase (PI3-K)/PKB signaling pathway.

Keywords: PI3-Kinase, caspase-3, PARP

Presenting author: Mari Enoksson

Detection of protein cleavage sites by N-terminal specific labeling

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We report a strategy for the identification of the exact location of proteolytic cleavage events in biological samples. This proteomics method takes advantage of specific N-terminal labeling of protein samples, followed by affinity enrichment and conventional liquid chromatography (LC)-MS/MS analysis. The method is simple, uses conventional and easily obtainable reagents, and is applicable to most proteomics facilities. As proof of principle we demonstrate profiles of proteolytic events that reveal exquisite in vivo specificity of methionine aminopeptidase in E. coli, and unexpected processing of mitochondrial transit peptides in yeast, mice and human samples. We have further developed the strategy using iTRAQ technology to provide quantitative measurements of proteolysis of natural substrates. We were able to identify proteolytic cleavage products associated with the induction of cell-free apoptosis. Taken together, we demonstrate how to rapidly distinguish real proteolysis that occurs in vivo from the predictions based on in vitro experiments.

Keywords: protease, substrate cleavage site, proteomics, N-terminal labeling

Presenting author: Natalia Ermak

Oxidized low density lipoprotein-induced apoptosis in human monocytic cells proceeds through a reactive oxygen species and Bax-dependent process

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This study investigated the proapoptotic effects of oxidized low density lipoprotein (oxLDL), which plays a key role in atherogenesis, on normal fresh human monocytes isolated from peripheral blood (PBMs), on human monocyte-derived macrophages, and on U937 monocytic cell line.
OxLDL were generated by hypochlorous acid (HOCl) treatment of native LDL. We analysed apoptosis using Hoechst coloration, annexin V-7AAD double labelling by flow cytometry, mitochondrial membrane potential ($\Delta\psi m$) using the fluorochrome $DiOC_6$, the expression of caspase-9, caspase-3, and cytochrome c and the modulation of Bcl-2 family proteins by western blotting.
We demonstrated that HOCl-oxLDL (200 µg/ml) induced apoptosis in PBMs and U937 cells, via the mitochondrial pathway, whereas it failed to induce apoptosis in human monocyte-derived macrophages. OxLDL-induced U937 cells apoptosis involved ROS generation, mitochondrial Bax translocation with a disruption of mitochondrial membrane potential, cytosolic liberation of cytochrome c and subsequently activation of caspases-9 and -3. The interference of ROS scavengers N-acetylcysteine and catalase with HOCl-oxLDL-induced apoptosis further supports the importance of mitochondrial ROS production in this process. Bcl-2 overexpression prevented Bax translocation whereas it failed to prevent ROS generation indicating that ROS is an upstream signal for inducing mitochondrial apoptotic damages. Because monocyte apoptosis could limit early atheroma formation, it will be interesting to identify the signaling pathway(s) induced by HOCl-oxLDL leading to ROS generation. In contrast, monocyte-derived macrophages, which resist to HOCl-oxLDL induced oxidative stress, may promote atherosclerosis.

Keywords: Apoptosis, ROS, Bax, mitochondria, oxidized LDL

Presenting author: Diego Esposito

Probing the structure of the death inducing signalling complex formed at the CD95/Fas receptor.

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The interaction between the death domains (DD) of CD95/Fas and the Fas-associated death domain adaptor protein FADD is a key event in the initiation of Fas ligand (FasL)-induced apoptosis. Disruption of this interaction allows cells to become resistant to FasL. In patients with autoimmune lymphoproliferative syndrome (ALPS) type Ia, death domain mutations can lead to expanded T cell populations. In the canonical apoptotic signalling pathway, procaspase-8/10 is recruited, via homotypic association with the death effector domain (DED) of FADD, to form a high molecular weight multi-protein complex known as the death-inducing signalling complex (DISC).

Despite the availability of three-dimensional structures of several of the DISC components, there are few details of the molecular mechanisms that underlie the process of apoptotic signal transduction.

In our work, the CD95/Fas-DD - FADD-DD interaction has been studied using a combination of biophysical, biochemical and functional studies. Nuclear magnetic resonance spectroscopy was utilised to monitor the molecular interaction between the two domains in solution and a mammalian cell-based assay was developed in which CD95/Fas wild-type and mutant forms were tested for their ability to induce apoptosis in a FasL-stimulated human cell line.

Although it has been previously reported that the DD of FADD by itself is insufficient for interaction with CD95/Fas, a specific and direct interaction was observed between the two domains in solution.

Our data suggest that the two DDs can form of a large multimeric complex with a defined stoichiometry and strong affinity that can be disrupted by the ALPS-associated mutation D244A or A241D in CD95/Fas. Size exclusion chromatography and analytical ultracentrifugation data have demonstrated the presence of a *ca.*160 kDa entity in CD95/Fas-DD:FADD-DD mixtures maintained at concentrations equivalent to the NMR measurements. An extensive mutational analysis of the Fas-DD identified broad regions of this domain that are crucial for interaction with FADD-DD.

Together these observations are consistent with a model of DISC formation that implicates a high order clustering of CD95/Fas receptors in the cell membrane in the initiation of the Fas-induced apoptotic signal.

Keywords: CD95, Fas, FADD, DISC, NMR

Presenting author: Roberta Fajka-Boja

Acid sphingomyelinase mediated release of ceramide is essential to trigger the mitochondrial pathway of apoptosis by galectin-1

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Human galectin-1 (Gal-1), the member of the galectin superfamily has a strong immunomodulatory effect, causing apoptosis of thymocytes, activated peripheral T cells and tumor T cell lines. We have previously shown that human recombinant Gal-1 induces protein tyrosine phosphorylation in Jurkat T cells, exposure of membrane phosphatidyl serine (PS), release of intracellular ceramide, decrease of mitochondrial membrane potential ($\Delta\psi m$) and caspase activation. Gal-1 induces the mitochondrial pathway of apoptosis in which caspase-8 is not involved. **The aim** of this study was to clarify the order of the signaling events, the enzyme participating in ceramide generation and the role of $p56^{lck}$, ZAP70 and ceramide in the apoptotic steps. **Results: 1/** Genistein, a tyrosine kinase inhibitor blocked the Gal-1 induced protein tyrosine phosphorylation, ceramide release and DNA degradation. The cells lacking $p56^{lck}$ or ZAP70 protein tyrosine kinase were not sensible to Gal-1. Re-expression of these kinases in cells restored the PS exposure, ceramide generation and decrease of $\Delta\psi m$ upon Gal-1 treatment. Moreover, Gal-1 stimulated the autophosphorylation of $p56^{lck}$ and ZAP70. **2/** Blocking of ceramide synthase activity with fumonisin B1 did not affect the Gal-1 cytotoxicity. In contrast, inhibitors of acidic sphingomyelinase (aSMase), desipramine and imipramine, abolished the increase of ceramide level. Inhibition of aMSase activity prevented the further apoptotic steps: the decrease of Bcl-2 anti-apoptotic protein, the loss of $\Delta\psi m$ and the activation of caspase-9 and -3. **3/** Inhibition of raft formation by the cholesterol-chelating agent, β-cyclodextrin attenuated the ceramide generation, depolarisation of mitochondrial membrane and the caspase-9/-3 activity. Desipramine did not prevent the formation of rafts, and the ceramide generated upon Gal-1 stimulation was not accumulated in rafts. **Summary:** Based on our overall results we propose a modell for the molecular mechanism of Gal-1 induced apoptosis on Jurkat T cells. The early tyrosine phosphorylation events are mediated by tyrosine kinases $p56^{lck}$ and ZAP70, which are responsible for activation of aSMase and elevation of ceramide level. Ceramide is not localized in membrane rafts, but probably acts as a second messenger, transmitting the apoptotic signal to mitochondria, leading to the downstream activation of effector caspases and breakdown of nuclear DNA.

Keywords: galectin-1, T-cells, p65lck, ZAP70, ceramide

Presenting author: Dean Fennell

BH3 domain of BID interacts with VDAC1/Prohibitin Complex and Depolarizes Mitochondria in the absence of Cristae Remodelling

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The multidomain proapoptotic BCL-2 family proteins BAX and BAK are pivotal regulators of cell death activated by BH3 proteins, and mediate outer mitochondrial membrane permeabilization (MOMP). Complete release of cytochrome C activates caspases via the apoptosome, and uncouples respiration by inhibiting electron transport between complex III and IV. Remodelling of cristae junctions by BID mobilizes the majority of mitochondrial cytochrome C in a cyclosporin A inhibitable and BH3 independent manner. We have observed that cell permeable $BH3^{BID}$ triggers rapid mitochondrial depolarization (MD) prior to permeability transition, and kills in a caspase and calcium independent manner. L90A mutation or BAX/BAK double siRNA knockdown abolished MD in isolated state IV mitochondria. Although endogenous BID, BIM localized to the outer mitochondrial membrane, neither double siRNA knockdown nor a combination of BAD and NOXA BH3 peptides were sufficient to inhibit MD. Depolarized mitochondria retained cytochrome C after MOMP with OPA1 remaining in a high molecular weight complex consistent with an absence of cristae remodelling. Artificially induced MOMP by C2 ceramide failed to induce MD. $BH3^{BID}$ interactions at the outer mitochondrial membrane were therefore explored by crosslinking, coimmunoprecipitation, and peptide mass fingerprinting, and identified VDAC1/prohibitin 1/prohibitin 2 complex as a primary target, spanning the outer and inner membranes. BID has been reported to reduce ADP permeability of the outer membrane which we propose may be due to a BH3 dependent interaction with VDAC1. Furthermore, VDAC1/PHB complex provides a possible link between $BH3^{BID}$ and disruption of electron transport independent of BAX/BAK.

Keywords:

Presenting author: Peter Ferenc

Hypericin induced phototoxicity is enhanced by tyrosine kinase inhibitor genistein

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Photodynamic therapy (PDT) is a relatively new method suitable for treatment of various tumors. PDT uses photosensitizing agents, which selectively accumulates in target cells. Illumination of such agents causes sequence of photochemical events that generate reactive oxygen species. These induce oxidative damage, which acts by killing of cancer cells. Hypericin (HY) is a photosensitizing pigment isolated from the plant *Hypericum perforatum* which displays inhibition of proliferation and induction of apoptosis after illumination. GE is a soybean isoflavone with various biological activities, e.g. it is a potent antioxidant, specific inhibitor of protein tyrosine kinase and phytoestrogene. The illumination is not essential for the cytotoxic effect of genistein (GE). Due to similar antiphosphorylation of protein tyrosine kinase activities of HY and GE we decided to investigate the effect of PDT-HY combined with chemotherapy of GE on human breast adenocarcinoma cell line MCF-7. The response of the cells was evaluated using different methods: total cell number counting, MTT assay, floating cells quantification, cell cycle analysis, test of clonogenic survival, and integrity membrane assay. Based on MTT assay the concentrations of both agents (GE and HY) with relatively slight cytotoxic effects for combined treatment were selected. Additionally we evaluated apoptosis on the base detection morphological changes using Hoechst/PI staining, PARP fragmentation, and detecting sub-G0/G1 cell population. We have observed a significant and synergistic increase in the percentage of apoptotic cells and PARP cleavage which correlated with a degradation of Bcl-2, Mcl-1 proteins, decrease of phosphorylated Ser 470 of Akt. Furthermore increased level of apoptotic protein Bad and increased activity of caspase 7, 8, 9 in PDT-HY+GE treatment vs. GE or HY alone. On the other hand, flowcytometric analysis showed reversible accumulation of MCF-7 cells in G2 phase of the cell cycle again in combination of PDT-HY +GE. These results indicate that combined treatment of PDT-HY and tyrosine kinase inhibitor GE may improve the therapeutic effectiveness of PDT.

Acknowledgment. This work was supported by Slovak Science and Technology Assistance Agency under the contract No. APVT-20-012104 and VEGA 1/3253/06

Keywords: photodynamic therapy, hypericin, genistein, apoptosis

Presenting author: Pablo Nicolas Fernandez Larrosa

Apoptosis resistance in HIV-1 chronicaly infected cells is not associated to viral replication or *tat* expression

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HIV triggers the decline of CD4+ T cells and leads to progressive disfunction of cell-mediated immunity, mainly during the acute phase of HIV infection. On the other hand, chronically-infected macrophages and memory T-cells seem to be resistant to cell death, representing a potential viral reservoir. In order to analyse the effects of HIV-1 over reservoir cells, persistently infected cells were treated with different apoptosis inducers and results were compared with those of uninfected cells.
Uninfected cell lines(H9, Jurkat, U937) and their HIV-1 persistently infected counterparts (H9/HTLVIIIB, J1.1, U1) were treated with H_2O_2 or staurosporine (STS) and collected 24 hours post treatment. Cells were treated with medium as controls (Ctrl). *Tat*-transfected Jurkat cells (Jurkat-*tat*) were also treated with inducers in order to evaluate the effect of *tat* over apoptosis susceptibility. Cell death parameters were evaluated by annexin-V/PI or APO-BRDU staining and FACS. P24 antigen production was quantified. Western Blot analyses were performed to evaluate apoptosis proteins.
When treated with both inducers, persistently infected cells showed significantly lower apoptosis levels than uninfected cells. Infected cells showed significant decrease in the levels of p24 production when subjected to both treatments. Neither the induction of viral production in J1.1 with TNF-α and U1 with PMA, nor expression of *tat*, marked significant differences in cell viability with respect to uninduced cells. Bax levels were higher decreased (H_2O_2: 40%; STS: 70%) in H9/HTLVIIIB cells treated with apoptosis inducers than in H9 cells (H_2O_2: 20%; STS: 40%), while no difference in Bcl-2 expression was observed in both cell lines. Besides, H9 cells treated with STS showed an important decrease of procaspase-3 (70%), indicating high levels of cleavage; compared to H9/HTLVIIIB (40%).
Our results suggest that, when treated with H_2O_2 and STS, persistently infected cell lines were more resistant to dying by apoptosis compared with uninfected cell lines, and this effect was independent of active viral replication or *tat* expression. This resistance could be regulated at the mitochondrial level via Bcl-2/Bax balance. This *in vitro* resistance could help to understand the *in vivo* HIV reservoir persistence.

Keywords: Apoptosis, Bcl-2, Bax, Tat, Chronic HIV infection

Presenting author: Elisabetta Ferraro

Transcriptional and post-translational regulation of the apoptosome

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In order to define the role of each component of the apoptosome in mediating apoptosis, we are comparing Apaf1$^{-/-}$ cells with cells overexpressing a dominant negative form of caspase-9 (caspase-9-DN), which binds the apoptosome but does not cleave effector caspases. We found that both Apaf1$^{-/-}$ cells and caspase-9-DN cells are strongly resistant to apoptosis. We have demonstrated that if these two apoptosome-deficient cell models were induced to die by apoptotic stimuli, they were still able to produce ATP and they were metabollically active for at least six days after the treatment. ATP production was supported by glycolysis and not by mitochondrial respiration. We also propose that both systems undergo autophagy, which can allow them to survive. In fact, if we block autophagy the cells can't produce ATP and die. As for this aspect, the absence of Apaf1 and the overexpression of caspase-9-DN have the same effects on cell survival.
By contrast, We found that only overexpression of caspase-9-DN protects cells against the release of cytochrome *c* from mitochondria; this protection might be due to the overexpression of caspase-9-DN itself, to the presence of Apaf1 or to the presence of a mutated apoptosome (on its caspase-9 component).
To study the transcriptional regulation of the *Apaf1* gene we have cloned a region of about 7 Kb including the murine *Apaf1* gene promoter upstream of the firefly luciferase cDNA. By means of an in silico analysis we looked for transcriptional factors able to bind and regulate *Apaf1* promoter. We found several putative factors and we decided to focus on Pax4, a factor involved in pancreas and neural tube differentiation. Co-expressing Pax4 cDNA with the complete *Apaf1* promoter–luciferase construct we observed an increase in luciferase activity. Moreover, we generated and transfected in several cell types a series of promoter deletions, observing an increased luciferase activity using one of them compared to the entire promoter region. This finding suggests the existence of a recognition site for a transcriptional repressor factor which we defined in a fragment of about 150 bp.

Keywords: Apaf1, ATP, autophagy, beclin 1, glycolysis, Pax4

Presenting author: Gian Maria Fimia

Regulation of autophagy during neural development: dissecting the *Ambra1* molecular network.

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Autophagy is a self-degradative process involved both in basal turnover of cellular components and in response to nutrient starvation or organelle damage in a wide range of eukaryotes. In vertebrates, this process plays a role in different physiological and pathological conditions, such as neurodegeneration and cancer; however, the roles of autophagy during embryonic development are still largely uncharacterized. Ambra1, a WD40-containing protein, is a novel activating molecule in Beclin 1-regulated autophagy and plays a role in the development of the nervous system. Ambra1 binds to Beclin 1 and favours Beclin 1/Vps34 interaction. At variance with these factors, Ambra1 is highly conserved among vertebrates only, and its expression is mostly confined to the neuroepithelium during early neurogenesis. Ambra1 functional inactivation in mouse led to lethality in utero (starting from embryonic day 14.5), characterized by severe neural tube defects associated with autophagy impairment, unbalanced cell proliferation, accumulation of ubiquitinated proteins, and excessive apoptosis. We demonstrated that hyperproliferation was the earliest detectable abnormality in the developing neuroepithelium, followed by a wave of caspase-dependent cell death. Accordingly with these findings, we showed that the modulation of Ambra1 expression results in the alteration of cell proliferation in vitro, and this function is also dependent on Beclin1 activity. Finally, by using a yeast two hybrid approach, we found that Ambra1 interacts with components of the cytoskeleton and this interaction regulates its autophagic activity.

Keywords: Autophagy, Ambra1, Beclin 1, Neural Development

Presenting author: Aarne Fleischer

Identification of critical signaling pathways activated during IL-2 deprivation-induced cell death

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Apoptosis of mature T lymphocytes is an essential process for maintaining immune system homeostasis. However, the details of the molecular signaling pathways leading to T cell apoptosis are poorly understood. We used cDNA microarrays containing 15,630 murine genes to study the gene expression profile in T lymphocytes at different time points of IL-2 withdrawal. Comparison of the gene expression profiles revealed that 2% of the genes were affected by cytokine starvation. Interestingly, the apoptotic program rather seems to activate gene expression in the early phase of cell death. On the contrary, transcription was strongly repressed in later stages of apoptosis. Self-organizing map clustering of the 270 differentially expressed transcripts revealed specific temporal expression patterns supporting the idea that IL-2 deprivation triggers a tightly regulated transcriptional program to induce cell death. To validate microarray results, changes in gene expression following IL-2 deprivation were confirmed for selected genes by Northern blot. In addition, the signaling pathways created can explain the molecular events leading to T cell apoptosis, even if the T cell line used in this study might not reflect individual T cell subpopulations expressing different level of IL-2 receptor or IL-2 dependence. Taken together, these results provide novel insights into the temporal regulation of gene expression during T lymphocyte death.

Keywords: IL-2, gene expression profiling, pathways

Presenting author: Alessandra Fontanini

Comparing the cell death properties of bortezomib and G5, two different inhibitors of the ubiquitin-proteasome system

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Inhibitors of the ubiquitin-proteasome system (UPSi) include a large cohort of compounds that can target not only the proteolytic chamber of the proteasome but also other components of the UPS such as deubiquitinating enzymes. Molecules that show the ability to modulate the ubiquitin turnover are emerging as new candidates for anti-tumor treatments. Bortezomib is a well known UPSi used in the treatment of multiple myeloma, whereas G5 is an inhibitor of deubiquitinating enzymes recently identified in our laboratory. The multidomains proapoptotic Bcl-2 family members Bax and Bak are fundamental and redundant regulators of the intrinsic/mitochondrial death pathway in response to a plethora of signals. Bax and Bak are ubiquitously expressed and represent the master regulators of mitochondrial outer membrane (MOM) permeability.
To investigate the killer properties of G5 and bortezomib we decided to use murine embryonic fibroblasts (MEFs) obtained from mice double-deficient for Bax and Bak (DKO) in comparison with WT MEFs. Higher doses of G5, but not of bortezomib, were able to kill not only WT, but also DKO cells. Further cellular and biochemical studies suggested that the cell death induced by high doses of G5 in DKO is characterized by caspases activation, appearance of autophagy and finally by necrosis. Using DKO cells stably expressing crmA we demonstrated that in this cells G5 was able to activate the extrinsic pathway, while bortezomib was not. However cell death occurred also when caspases activation was inhibited. Autophagy, scored by LCII generation and EM imaging, was observed in WT and DKO cells in response to both G5 and bortezomib. Inhibition of autophagy did not alter the cell death phenotype. We also noted that G5 is able to induced cell death by activating a necrotic response, as scored by release of HMGB1, uptake of propidium iodide and collapse of mitochondrial potential in absence of outer mitochondrial membrane permeabilization. Further studies are now ongoing to define the necrotic response induced by the isopeptidase inhibitor G5

Keywords: G5, necrosis, caspases

Exploring the molecular composition of OPA1 complexes

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Optic Atrophy 1 (OPA1) is a pro-fusion dynamin-related protein of the inner mitochondrial membrane mutated in dominant optic atrophy. It protects from apoptosis by preventing cytochrome c release independently from mitochondrial fusion. This function correlates with the oligomerization of two forms of OPA1, a soluble, intermembrane space and an inner membrane integral one. During apoptosis, the pro-apoptotic BCL-2 family member BID disrupts OPA1 oligomers, while high levels of OPA1 stabilize them and prevent mobilization of cytochrome c. We are investigating the composition of OPA1-containing complexes taking advantage of blue native (BN) electrophoresis. We were able to detect supramolecular assemblies of OPA1 spanning from ≈300 KDa up to ≈1000 KDa. BID results in a decrease of OPA1 oligomers and in the parallel increase of monomeric OPA1. Complexes of OPA1 retained in the native dimension were dissociated using an orthogonal denaturing SDS electrophoresis (2D BN/SDS electrophoresis). We studied the interaction partners of OPA1 and their stoichiometry using mass spectroscopy analysis. Our preliminary data indicate that the stress induced protein HSP70 seems to associate with OPA1 in normal and apoptotic mitochondria. Moreover, this analysis confirmed that in response to BID the amount of high molecular weight complexes are quantitatively reduced.
These data indicate that 2D BN/SDS electrophoresis can be a useful approach to analyze in details composition and function of OPA1 complexes in normal and apoptotic mitochondria.

Keywords: OPA1, apoptosis, cytochrome c release, cristae remodelling

Presenting author: Gerhard Fritz

The HMG-CoA reductase inhibitor lovastatin protects human endothelial cells (HUVEC) from ionizing radiation- and doxorubicin-induced stress responses and apoptotic death

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Apart from their clinical application as lipid-lowering drugs, HMG-CoA reductase inhibitors (statins) are known to impact various stress-induced signaling mechanims, probably by inhibiting the function of Ras/Rho GTPases. In the present study, we investigated the effect of lovastatin on ionizing radiation (IR)- and doxorubicin-induced signaling and apoptosis in primary human endothelial cells (HUVEC). Low dose pre-treatment with lovastatin shields HUVEC from IR- and doxorubicin-induced cytotoxicity, as measured by cell viability, cell proliferation and FACS-based apoptosis assays[1,2]. IR- and doxorubicin-provoked increase in CD95L and CD95R mRNA expression was partially blocked by lovastatin. Activation of caspases (i.e. caspase-3, -7, -8 and -9) was not detected 48-72h after IR exposure (up to 20 Gy). Nevertheless, IR-stimulated apoptosis was blocked by the pan-caspase inhibitor Z-VAD. Similar results were obtained with doxorubicin (up to 5 µg/ml). The anticancer drug cisplatin caused cleavage of procaspase-3 and -7, showing that HUVEC are not generally compromised in the activation of executor caspases. Having in mind that doxorubicin and IR severely damage DNA, we speculated that lovastatin might protect HUVEC from DNA damage-triggered apoptotic death. Examining the effect of lovastatin on DNA strand break induction (using the comet assay) and ATM/ATR-catalyzed H2AX phosphorylation (γ-H2AX), we found radioprotection by lovastatin to be independent of the formation and repair of DNA damage. In contrast, doxorubicin-triggered DNA strand break induction was attenuated by lovastatin. This effect is not due to alterations in doxorubicin uptake or efflux. Genotoxin-inducible DNA damage-related stress responses, including accumulation of p53 and p21 protein as well as activation of checkpoint kinase (Chk-1), stress kinases (SAPK/JNK) and NF-κB, were impaired by lovastatin. Overall, the data show that the HMG-CoA reductase inhibitor lovastatin has pleiotropic inhibitory effects on IR- and doxorubicin-induced stress responses in HUVEC and eventually operates in an anti-apoptotic manner[1,2]. The molecular mechanisms underlying lovastatin-mediated radio- and doxorubicin resistance appear to be different. Based on the data we suggest that lovastatin might be clinically useful in attenuating side effects of IR- and doxorubicin-based antitumor therapy on normal tissue.

[1]Nübel et al. (2006) Clin. Cancer Res. 12, 933-39; [2]Damrot et al. (2006) Brit. J. Pharmacology 149, 988-97.

Keywords: Genotoxic stress, DNA damage response, statins, endothelial cells, apoptosis

Presenting author: Simona Frosali

Role of intracellular calcium ions and glutathione in apoptosis induced by mixture of isothiazolinones in HL60 cells.

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The combination of 5-chloro-2-methyl-4-isothiazolin-3-one (CMI) and 2-methyl-4-isothiazolin-3-one (MI), is commoly used as biocide to inhibit the microrganism's growth in industrial products. The molecular mechanism of this biocide appears very interesting and not yet fully understood. We showed that brief exposure of HL60 cells to CMI/MI induces apoptosis at low concentrations (0.001%, 0.01%) and necrosis at higher concentrations (0.05%, 0.1%). Glutathione (GSH) depletion, reactive oxygen species (ROS) generation, hyperpolarization of mitochondrial transmembrane potential ($\Delta\Psi m$ ($\Delta\Psi m$) and formation of protein-GSH mixed disulphides (S-glutathionylation) were early molecular events that preceded the induction of cell death by CMI/MI (Di Stefano et al, 2006). In this study we investigated the role of calcium and of glutathione level modulation in the molecular mechanism of apoptosis and necrosis induced by CMI/MI in HL60 cells. By flow cytometric analysis we show that CMI/MI treatment at 0.01% and 0.05% doses causes a rapid and sustained increase of calcium level in a calcium free medium in a dose-dependent manner. Experiments performed to evaluate the relative contribution of extracellular Ca^{2+} entry and store Ca^{2+} release, have shown that the primary source of calcium is most likely intracellular stores.. Pre-incubation of cells with the calcium chelator BAPTA-AM reduces necrosis cell death and secondary necrosis, but does not protect from apoptosis. Conversely pre-treatment with the permeable agent 10 mM glutathione monoethylester, which substantially increases intracellular GSH, provides protection against the depletion of the glutathione and cytotoxic effects of CMI/MI. These observations taken together with previous study (Di Stefano et al, 2006) highlight the importance of GSH in CMI/MI induced cell death

A.Di Stefano, S.Frosali, A.Leonini, A.Ettorre, R.Priora, F.Cherubini Di Simplicio, P.Di Simplicio (2006), "GSH depletion, protein S-glutathionylation and mitochondrial transmembrane potential hyperpolarization are early events in initiation of cell death induced by a mixture of isothiazolinones in HL60 cells", *Biochimica et Biophysica Acta*,1763: (214-225).

Keywords: apoptosis, calcium ,glutathione, flow cytometry

Presenting author: Konrad Gabrusiewicz

Inhibition of intracranial glioma growth by a systemic administration of cyclosporin A

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Gliomas, the most common primary brain tumors, which account for more than 40% of all central nervous system neoplasms are highly resistant to current therapeutic approaches. We have previously demonstrated that cyclosporin A (CsA) affects growth/survival of cultured rat and human glioblastoma cells and impairs tumorigenicity of intracranial gliomas (Sliwa et al. 2007). Our studies suggested that microglial cells which are attracted by glioma, support growth, migration and invasiveness of glioma cells. Counteracting the pro-tumorigenic activity of microglial cells with a clinically relevant doses of CsA could affect glioma invasion. CsA, Tacrolimus (FK506) and Rapamycin are immunosuppressive drugs. We have demonstrated their anti-inflammatory activity and ability to block LPS-induced activation of primary microglial cultures. The aim of present study was to investigate the efficiency of CsA and FK506 in reduction of glioma growth and to evaluate their impact on density and morphology of microglia.

EGFP-GL261 glioma cells were stereotactically injected into the striatum of C57BL/6 mice. Afterwards, mice were randomly assigned into two groups: the first group received i.p. CsA (2 or 10 mg/kg) or FK506 (1 or 5 mg/kg) every 2 days starting from the 2nd day after cells implantation; the second group received treatment from 8th day after glioma inoculation. 14 days after glioma implantation, animals were perfused and tumor volumes were calculated using Image-Pro Plus. CsA-treated mice had significantly smaller tumors than control mice. Both doses of CsA reduced tumor of ~45 and 70% respectively, only the higher dose of FK506 was able to impair glioma growth. When the treatment was postponed to 8th day, only the higher dose of CsA was effective causing ~64% tumor regression. To evaluate mechanisms of anti-tumor activity of immunosuppressants, the drug effects on glioma proliferation and cell death were evaluated proliferation antigen Ki-67 immunodetection and TUNEL staining, respectively. Isolectin B_4 immunohistochemistry was used to evaluate a number of tumor-infiltrating activated microglia.

Keywords: glioma, cyclosporin A

Presenting author: Malgorzata Gajewska

Role of hormones and growth factors in the regulation of autophagy in bovine mammary epithelial cells

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Autophagy is a process responsible for degradation and recycling of cytoplasmic content by lysosomes. It is thought to facilitate cell survival during periods of nutrient starvation, but it can also be involved in other physiological processes including regulation of protein homeostasis, degrading intracellular bacteria, tumor suppression and regulation of programmed cell death. We were the first group to report autophagy in bovine mammary epithelial cells, both *in vitro*, on BME-UV1 mammary epithelial cell line and *in vivo*. The highest intensity of autophagy in bovine mammary gland was noted during dry period, when the gland undergoes intensive remodeling and the deprivation of bioactive compounds (hormones, growth factors, cytokines) and nutrients occur. The present study was focused on the role of hormones and growth factors in the regulation of autophagy in bovine mammary epithelial cells (MEC). We have used a model of bovine mammary gland involution *in vitro*, dependent on FBS deficiency in the culture of bovine BME-UV1 cells. The effects of IGF-I, EGF, sex steroids and rapamycin (inhibitor of mTOR) on autophagy in bovine MEC have been investigated. Immunofluorescence and laser scanning cytometry were used for analysis of lysosomes activation and expression of MAP1LC3 and active caspase-3 as biochemical markers of autophagy and apoptosis, respectively.

The results of our study showed that IGF-I completely abolished autophagy induced by FBS deficiency. A similar but less pronounced effect of autophagy suppression was observed in the case of EGF. Conversely to IGF-I and EGF, 17β-estradiol and progesterone exerted stimulatory effects on autophagy in bovine MEC. In conclusion, autophagy in bovine MEC undergoes complex regulation, where the principal function belongs to survival pathways dependent on IGF-I and EGF, which are involved in suppression of autophagy as well as pregnancy steroids which are inducers of this process.

Keywords: autophagy, IGF, EGF, sex steroids, bovine MEC

Presenting author: Tatiana Gartner

Immunogenicity and protective efficacy of a tuberculosis DNA vaccine co-expressing pro-apoptotic caspase-3

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DNA vaccination is a potent means for inducing strong $CD4^+$ (Th1) and $CD8^+$ mediated immune responses and protective immunity against a variety of viral, bacterial and parasite pathogens in small rodents. In an attempt to increase cross-priming induced by a prototype tuberculosis DNA vaccine through apoptosis, the sequence for wild-type or catalytically inactive mutated caspase-3 was inserted into plasmid DNA encoding the 32 kDa mycolyl-transferase (Ag85A) from *Mycobacterium tuberculosis*. Transient transfection experiments showed that the plasmid encoding mutated caspase induced slow apoptosis, normal Ag85A expression levels and NF-κB activation. Transfection with plasmid encoding wild-type caspase led to rapid apoptosis, lower expression and no NF-κB activation. Ag85A specific antibody production in DNA vaccinated $(B6D2)F_1$ mice was increased by the inclusion of the mutated caspase gene and decreased by the inclusion of wild-type caspase gene. Vaccination with both pro-apoptotic plasmids increased Ag85A specific IFN-γ production after vaccination and triggered more efficiently IL-2 and IFN-γ producing memory cells in spleen and lungs after *M. tuberculosis* challenge. Finally, mice vaccinated with Ag85A pDNA co-expressing wild type caspase showed increased protection against subsequent infection with *M. tuberculosis* (delayed cachexia, prolonged survival and reduced bacterial replication in lungs) as compared to mice vaccinated with Ag85A pDNA. Plasmid co-expressing mutated caspase was not protective, possibly due to of the induction of IL-6, IL-10 and IL-17 production.

Keywords: DNA vaccination, tuberculosis, caspase

Presenting author: Georg Gdynia

Basal caspase activity promotes migration and invasiveness of glioblastoma cells

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Glioblastomas, the most malignant brain tumors, are characterized by cellular resistance to apoptosis and a highly invasive growth pattern. These factors contribute to the poor response of glioblastomas to radiochemotherapy and prevent their complete neurosurgical resection. However, the driving force behind the distinct motility of glioma cells is only partly understood. Here, we report that in the absence of cellular stress and pro-apoptotic stimuli, human glioblastoma cells exhibit a constitutive activation of caspases *in vivo* and *in vitro*. Inhibition of caspases by various peptide inhibitors decrease the migration of cells in scrape motility assays and the invasiveness of cells in spheroid assays. Similarly, specific siRNA- or antisense-mediated down-regulation of caspase 3 and caspase 8 results in an inhibition of the migratory capability of glioma cells. The constitutive caspase-dependent motility of glioblastoma cells is independent of CD95 activation and is not mediated by MAP/ERK kinase signaling. The basal caspase activity is accompanied by a constant cleavage of the motility-associated gelsolin protein which may contribute to the caspase-mediated promotion of migration and invasiveness of glioblastoma cells. Our results suggest that the administration of low dosages of caspase inhibitors that block glioma cell motility without affecting the execution of apoptotic cell death might be a novel strategy for the treatment of glioblastomas.

Keywords: migration, invasion, brain tumors, caspases, gliomas, apoptosis

Presenting author: Barbara Geering

Cell death-associated and -independent functions of DAPK2 in primary human neutrophils

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Death associated protein kinases (DAPKs) are Ca^{2+}/Calmodulin-dependent serine/threonine kinases involved in programmed cell death. Three closely related members of this family have been identified, named DAPK1, DAPK2 and ZIPK. These proteins display a high degree of homology in the catalytic domain and variable extra-catalytic regulatory elements, leading to different subcellular localisation and protein-protein interactions. DAPKs phosphorylate a diverse set of substrates, including myosin light chain, calmodulin-regulated kinase kinase, syntaxin-1A and S6 kinase. There is increasing evidence for DAPK functions independent of cell death. Indeed, DAPK1 overexpression has been shown to reduce cell adhesion to extracellular matrix proteins, due to inactivation of the integrin signalling pathway. Furthermore, ectopic DAPK2 expression enhanced cellular differentiation of human NB4 promyelocytic leukemia cells treated with all-*trans* retinoic acid. While these studies were carried out in cell lines, we are interested in assessing cell death-associated and -independent functions of DAPK2 in *primary* granulocytes.
Primary immature neutrophils contained little DAPK2. Upon *in vitro* stimulation of these cells with the neutrophil hematopoietins G-CSF and GM-CSF, which concomitantly induced cellular maturation, DAPK2 expression was markedly enhanced. Hence, primary mature neutrophils displayed largely elevated DAPK2 levels, which could be further induced by stimulation with G-CSF and GM-CSF. Treatment of mature neutrophils with LY294002, but not with inhibitors of MAPK or JAK/STAT pathways, led to reduced DAPK2 expression implying a role of the PI3K signalling pathway in the regulation of DAPK2 expression. In order to assess DAPK2 function(s) in mature neutrophils, the protein was ectopically expressed using a lentiviral vector system. To our surprise, DAPK2 did not increase apoptosis. However, ectopic expression of DAPK2 induced changes in neutrophil functions associated with the cytoskeleton, including degranulation and adhesion. In summary, our data support a role of DAPK2 in cell-death independent neutrophil functions that are critical for innate immunity.

Keywords: DAPK, neutrophil, differentiation, cytoskeleton

Comet Assay – a Novel Approach for Quantification of Cell Death in Glioblastoma multiforme

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Human *Glioblastoma multiforme* (GBM) is the most common malignant astrocytoma, associated with a dismal prognosis leading to a median survival rate of less than 1 year. *Glioblastoma multiforme* cells possess a highly invasive nature. Extensive histo-morphological heterogeneity with great variability in the extent of invasiveness, angiogenesis and cell death are distinctive to this type of human brain tumors.

Two main forms of cell death are encountered in biology: apoptosis (i.e. the programmed cell death) and necrosis (i.e. an accidental cell death). Apoptosis is a complicated process driven by complex regulatory pathways, whereas necrosis is mostly a passive process followed by inflammatory reaction in the surrounding tissues. Both processes lead to genomic DNA fragmentation: apoptosis - to inter-nucleosomal DNA cleavage, while necrosis manifests random DNA degradation.

Comet assay is an elaborate technique for detection of dead cell populations in a variety of clinical samples, including biopsies, blood, etc. It is a sensitive method for detection of DNA fragmentation at the level of a single cell and offers a significant improvement in monitoring the kinetics of DNA fragmentation during the process of cell death.

The process of cell death in *Glioblastoma mutiforme* is questionable and yet poorly understood. Thus certain part of cancer research is orientated toward investigating the mechanisms that trigger cell death in tumors.

Here, we present our data, obtained by the method of Comet assay, applied on tumor samples from *Gliobastoma multiforme*. Our results reveal that the Comet assay is a very sensitive approach for quantification of cell death in tumor samples.

A detailed evaluation of tumor cell death is quite prerequisite for neurooncologic studies of *Gliobastoma multiforme*. This will greatly help designing a more successful therapy of these tumors in the future.

Keywords: Glioblastoma multiforme, cell death, Comet assay

Presenting author: Saeid Ghavami

Back to The Nature: Brevinin-2R, a Rana Ridubunda Skin Extract, Kills Semi-selectively Cancer Cells via Lysosom al-Mitochondrial Cell Death Pathway

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Granular glands present in the skin of certain anurans (frogs and toads) synthesize and store polypeptides belonging to the superfamily of defensins that have a broad-spectrum of antimicrobial activity. Frogs of the genus *Rana*, a successful group with at least 250 species distributed worldwide, are a valuable source of antimicrobial defensins and their peptide sequences are unique among different *Rana* species. Based on the limited structural similarities, these *Rana* peptides can be grouped into families that are believed to share similar evolutionary traits. There are no consensus amino acid sequences that can be directly associated with biological activity, but the peptides are almost invariably cationic, relatively hydrophobic, and have the propensity to form an amphipathic helix in a membrane-mimetic environment.

Brevinin-2R a novel 25-amino acid polypeptide was recently isolated from the skin of the frog *Rana ridibunda*. Contrary to the majority of defensins, Brevinin-2R does not exhibit the typical hemolytic activity but semi-selectively kills cancer cells. This preferential tumor cytotoxic activity was observed in a variety of malignant cells, including Jurkat (T-cell leukemia), BJAB (B-cell lymphoma), HT29/219, SW742 (colon carcinoma), L929 (fibrosarcoma), and MCF-7 (breast adenocarcinoma). Malignant hematopoietic cell lines were more sensitive towards Brevinin-2R than primary cells, including peripheral blood mononuclear cells (PBMC), human T-cells and B-cells. In addition, human lung carcinoma cells (A549) were more sensitive towards Brevinin-2R than normal human lung fibroblasts. Jurkat and MCF-7 tumor cells stably over-expressing Bcl2 and stable transfectants of L929 and MCF-7 over-expressing a dominant-negative mutant of a pro-apoptotic Bcl2-family member BNIP3 (ΔTM-BNIP3) were significantly more resistant towards Brevinin-2R-triggered cell death.We did not observe an activation of caspase family of proteases or a release of Apoptosis Inducing Factor (AIF) and endonuclease G (Endo G) from mitochondria during Brevinin-2R-induced cell death. The decrease in mitochondrial membrane potential ($\Delta\Psi m$) and cellular total ATP levels and the increase in reactive oxygen species (ROS) were early indicators of Brevinin-2R triggered death. Lysosomal membrane permeabilization inhibitors and irreversible inhibitors of cathepsin B and cathepsin L prevented Brevinin-2R-induced cell death. Our results show that Brevinin-2R activates the lysosomal-mitochondrial death pathway. The semi-selective anticancer activity of Brevinin-2R makes it a promising new lead for cancer therapy development.

Keywords: Antibacterial Peptide; Defensin; Brevinin-2R; Cell Death; Caspase Activation; Lysosomotrophic agent, late and early endosome, Reactive Oxygen Species, Mitochondrial Pathway.

Presenting author: Bernhard Gillissen

Mcl-1 mediates Bax dependency of Nbk/Bik-induced apoptosis

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The pro- and anti- apoptotic members of the Bcl-2 family are critical regulators of programmed cell death. The pro-apoptotic subgroup of so called BH3-only proteins share homology in only one of four conserved regions termed Bcl-2 homology (BH) domains 1 to 4. Activated by a variety of stimuli, these BH3-only proteins are essential initiators of apoptosis whereas multidomain pro-apoptotic Bax and Bak are executioners of death orders relayed by the BH3-only proteins. We have shown that Bax-deficiency protects cells from apoptosis induction by the BH3-only protein Nbk/Bik. Nevertheless, it has been assumed that life-death decisions rest simply on the balance of the abundance of all proapoptotic versus antiapoptotic family members. Thus, we could not rule out that loss of Bax protects cells just by decreasing the amount of Bax/Bak-like molecules under a critical threshold necessary for Nbk to induce apoptosis. We therefore studied the influence of Bak knock-down in the Bax-proficient cell line HCT116. Bak knock-down by shRNA did, however, fail to protect these cells from Nbk-induced apoptosis, whereas Bax knock-out resulted in complete resistance to Nbk enforced cell death. Additionally, re-expression of Bax in Bax-deficient and Nbk-resistant DU145 cells markedly sensitized DU145 cells to Nbk-induced apoptosis whereas Bax negative, Bak-overexpressing DU145 cells remained resistant. Expression of Nbk induced clustering of GFP-Bax but not of GFP-Bak fusion in DU145 cells. These results indicate that Nbk acts via an entirely Bax-dependent pathway as opposed to Bcl-x_S that triggers cell death via a Bax-independent, Bak-dependent pathway. Notably, Nbk increases the expression level of the antiapoptotic Bcl-2 family protein Mcl-1, which in turn sequesters and inactivates Bak. Binding of Mcl-1 to Bak persists after Nbk expression and inhibits Nbk-induced apoptosis in Bax deficient cells. Thus, Nbk is held in check by Mcl-1 that interferes with activation of Bak. In contrast, the BH3-only protein Puma disrupts Mcl-1/Bak interaction and triggers cell death via both Bax and Bak. The finding that different BH3-only proteins rely specifically on Bax, Bak or both has important implications for the design of anticancer drugs targeting Bcl-2.

Keywords: BH3-only, Nbk, Bik, Mcl-1

Presenting author: Zoltan Giricz

Role of PERK in ER Stress-induced Apoptosis

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Mechanisms underlying the death receptor-mediated and the mitochondrial pathways of apoptosis are well characterized. However, processes contributing to endoplasmatic reticulum (ER) stress-mediated apoptosis are not fully understood. Although, ER stress-mediated apoptotic signal has been shown to converge with the mitochondrial pathway, but the role of the three ER stress sensors, PERK, ATF6 and Ire1 and their downstream mediators has not been studied extensively in triggering apoptosis. In this study we have investigated the role of PERK in ER stress-induced apoptosis. $PERK^{+/+}$ and $PERK^{-/-}$ mouse embryonic fibroblasts (MEFs) were subjected to 48 h treatment with thapsigargin (a sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor) or tunicamycin (an inhibitor of protein N-glycosylation) to induce ER stress and a subsequent apoptosis. We have found that the $PERK^{-/-}$ MEFs are more susceptible to ER stress-induced apoptosis, which was assessed by Annexin-V staining and by cell morphology analysis following to a hematoxylin-eosin staining. The enhanced cell death was associated with an earlier cleavage of caspase-3 and of its substrate, PARP. In contrast, induction of CHOP, which has been thought to play a direct role in mediating ER stress-induced apoptosis, has not been observed in $PERK^{-/-}$ cells. Our results clearly show that ER stress-induced apoptosis is independent of PERK. Since we still do not have convincing evidence on the role of ER stress pathways in triggering apoptosis, further experiments are necessary to elucidate the exact mechanisms by which the ER stress is connected to the apoptotic machinery.

Keywords: PERK, ER stress, apoptosis

Exploring the role of mitochondrial fission and Fis1 in autophagy and apoptosis

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Mitochondria are highly dynamic organelles that continuously fuse and divide during life of the cell. It was recently established that mitochondrial fragmentation occurs early during apoptosis. Increasing evidence indicates that the machinery of mitochondrial fission participates in autophagy, a program of organelle elimination and death. Known regulators of fission include Drp-1, a cytosolic dynamin-related protein, and Fis1, the mitochondrial adaptor for Drp-1. Both proteins seem to be involved in programmed cell death. Interestingly, the role hFis1 plays in cell death is independent from its function in mitochondrial fission, raising the question of the physiological role of hFis1. We decided to investigate the role of mitochondrial fission and, in particular, of Fis1 in autophagy and dissect it from its role in apoptosis. To this end we evaluated autophagy and apoptosis in the genetically defined cell models expressing different levels and mutants of hFis1. Here we will present data on the role of hFis1 in autophagy as opposed to its function in apoptosis.

Keywords: mitochondria, autophagy, apoptosis

Mitogaligin, a new barrier breaking cytotoxic protein.

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Galig is a new human gene embedded in galectin-3 gene. This gene, specifically expressed in leucocytes, allows the production of a mRNA encoding two different proteins, cytogaligin and mitogaligin, located in cytoplasm or mitochondria respectively. These proteins have no homology with galectin-3. Although *galig* function is not yet defined, its overexpression induces cell death. Previous studies showed that this cytotoxic process is characterised by a leakage of cytochrome c from mitochondria, and is inhibited by Bcl-2 and Bcl-XL, thus arguing for the commitment of mitochondria in the cell death induced by *galig*. Since mitogaligin is targeted to this subcellular compartment, further investigations were performed to define the implication of this protein in *galig* cytotoxicity. Studies showed that mitogaligin overexpresssion lead to mitochondrial membranes destabilization, since cytochrome c is released from mitochondria. Whether this activity is mediated by Bcl-2 family members such as Bax, or is directly dependent on mitogaligin is here investigated. We focus more specially on the effects of mitogaligin on the lipidic part of membranes. We show that peptides derived from mitogaligin are able to interact with several types of anionic membranes, but display a more pronounced specificity for cardiolipin, an anionic phospholipid specific of mitochondrial membranes. Moreover, as a consequence of the interaction, the peptides permeabilize and aggregate these membranes. These results suggest that, by homology with tBid, mitogaligin may induce cytochrome c release from mitochondria independently of other proteins through its interaction with cardiolipin.

Keywords: mitogaligin, cardiolipin, apoptosis, cytochrome c,

Presenting author: Ales Goropevsek

Monitoring anti-apoptotic cytokine signalling in subsets of SLE T cells

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Systemic lupus erythematosus (SLE) is a prototype autoimmune disease characterized by a break of tolerance to nuclear components and profound alterations of the immune system . The typical SLE PBMCs IFN-αβ signature cannot be found in other autoimmune diseases tested to date. Still only a fraction of the patients display detectable circulating IFN-αβ. Increased expression in SLE MN cells of IFN-regulated genes could also be due to hightened response to IFN-alpha in efector cells. Both, faulty regulation of apoptosis and the inappropriate expression of several cytokines have been considered important defects of lymphocytes in SLE.

To study multiple activated signaling pathways in complex populations of cells, flow cytometric–based biochemical analysis at the single-cell level for kinase and phosphoprotein profiling have been developed. We used a novel multiparametric flow cytometry assay to measure basal and cytokine (IFN α)-induced phosphorylation of various STAT proteins in specific subsets of immune cells. In addition intracellular bcl-2 expression was measured .

Our first results show higher responses of SLE leukocytes to IFN-alpha. Subsequently, we tested in vitro effect of recombinant interleukins and interferons on peripheral blood mononuclear cells from patients with SLE and from healthy volunteers.

We found higher bcl-2 expression in T cells of lupus patients, that correlated with previous findings, as were higher levels of some cytokines in SLE serum .

Flow cytometry data simultaneously collected on one phospho-protein and its regulated gene product are correlated in our studies and show signaling differences that suggest T cells are composed of different subsets of cells with distinct signaling modes.In SLE T cells, a subset with highest bcl-2 expression also shows highest response to IFN-alpha in terms of STAT phosphorylation.

Anti-apoptotic cytokine signaling may significantly influence the deregulation of cell death in SLE lymphocytes.Moreover, single-cell signal analysis can be efficently used to monitor patients and may be useful for understanding mechanisms of disease progression, treatment resistance, and development of diagnostic indicators.

Keywords: SLE, cytokine signalling ,T cells, bcl-2, IFN-alpha

Presenting author: Francesca Grespi

Investigating the function of different isoforms of Bmf, pro-apoptotic members of the Bcl-2 family

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Bmf is a proapoptotic BH3-only protein belonging to the Bcl-2 family. Its mRNA has been detected in primary and secondary lymphatic organs, colon and lung and throughout early developmental stages of the mouse embryo. Bmf expression and protein function was reported to be controlled transcriptionally by growth factor deprivation, pharmacological inhibition of histone deacetylases (HDACs) as well as post-translationally by binding to actin via dynein light chain 2 (DLC2), a component of the actin-based myosin V motor complex. Biochemical studies suggest that Bmf binds preferentially to the pro-survival molecules Bcl-2, Bcl-x, Bcl-w but not others and that different isoforms of Bmf exist.

In vitro translation experiments and site directed mutagenesis revealed that the two major isoforms of Bmf, found in lymphatic tissues, arise from the usage of alternative START sites located in exon 2 and 3 of mouse *bmf*, respectively. Interestingly, the longest isoform appears to arise from the usage of an unconventional CUG start site. To investigate possible functional differences between Bmf isoforms, we started to compare their protein stability in response to different cell death stimuli in NIH-3T3 fibroblasts and in HC11 mammary epithelial cells. Western blotting analysis revealed an increase in Bmf protein expression after Cytochalasin D application or upon serum deprivation. Blockage of mRNA translation using Actinomycin D prevented this increase, confirming that de novo protein synthesis is required. So far no differences between the two major isoforms have been observed regarding protein stability or induction during apoptosis. Knock-down of both Bmf-isoforms by RNAi conferred partial resistance to apoptosis caused by Cytochalasin D treatment or serum deprivation.

Currently we are investigating whether the different isoforms of Bmf possess distinct affinities to anti-apoptotic Bcl-2 family members and compare their relative cell death-inducing properties as well as subcellular localization.

Keywords: apoptosis, BH3-only proteins

Presenting author: Georg Gruber

A delicate balance between glucocorticoid receptor expression and its ligand determines sensitivity and kinetics of glucocorticoid-induced apoptosis

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Glucocorticoid (GC)–induced apoptosis is an essential component in the treatment of acute lymphoblastic leukemia (ALL) and related malignancies. Although the pathway leading to cell death is not well understood, it is clear that it is initiated by the GC receptor (GR), a ligand activated transcription factor of the nuclear receptor family, and that the response somehow depends on expression of the GR and its ligand. Since GR expression differs between patients and the amount of GC administered varies between treatment protocols, the details of this receptor-ligand interplay are clinically relevant. Therefore we decided to address the effects of GR expression and GC concentration on kinetics and extend of GC-induced cell death. To this end, we generated a childhood acute lymphoblastic leukemia cell line (CCRF-CEM) expressing a tetracycline-responsive transactivator but lacking functional endogenous GR and stably transfected it with a GR^{wt}-ires-GFP construct. In the resulting cell line, GR levels can be modulated at will by varying the exposure to doxycycline and be readily monitored by fluorimetric analysis of GFP expression. Moreover, this cell lines allowed us to mimic, and study the effects of, GR autoinduction that is observed in GC-sensitive (but not GC-resistant) ALL cell lines and some T-ALL patients. Our results show a dramatic effect of the investigated parameters on “therapy outcome” in this model, i.e., GC resistance in cells with low GR levels could be overcome by exposure to high GC concentrations and cells expressing high levels required very low amounts of GC to undergo high degree of apoptosis. GR induction during GC exposure (mimicking GR autoinduction) resulted in a more delayed cell death. Very high GR levels were associated with a paradox response, i.e. high GC induced less apoptosis than low GC. Our data suggest that monitoring GR expression and auto-regulation in ALL patients might have relevance for selecting appropriate GC doses in future “individualized medicine” protocols.

Keywords: glucocorticoid-induced apoptosis, resistance, glucocorticoid receptor autoinduction, lymphatic leukemia, CCRF-CEM

Presenting author: Paolo Grumati

Molecular mechanism of muscle apoptosis caused by the absence of collagen VI

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Collagen VI (ColVI) is an extracellullar matrix protein forming a microfilamentous network in skeletal muscle and other organs. Inherited mutations of genes encoding ColVI in humans cause two muscle diseases, Bethlem myopathy and Ullrich congenital muscular dystrophy. *Col6a1*$^{-/-}$ mice display a myopatic phenotype affecting diaphragm and other skeletal muscles. *Col6a1*$^{-/-}$ muscle fibers have a loss of contractile strengh associated with ultrastructural alterations of sarcoplasmic reticulum, mitochondrial dysfunction and spontaneous apoptosis.
Signaling pathways regulated by ColVI are largely unknown. Cell-matrix interactions are important for promoting survival in different ways. Therefore, the apoptotic phenotype found in ColVI-deficient muscles might be due to molecular defects of protein kinases and/or other mediators involved in cell survival pathways. Primary myoblasts dissociated from *Col6a1*$^{-/-}$ diaphragm display ultrastructural alterations, mitochondrial dysfunction and spontaneous apoptosis, similarly to *Col6a1*$^{-/-}$ muscle fibers. When compared to wild-type cells, *Col6a1*$^{-/-}$ myoblasts show also an increased sensitivity to apoptotic stimuli, such as staurosporin or oligomycin (an inhibitor of mitochondrial ATP synthase).
Biochemical studies reveal a likely correlation between cell death in ColVI-deficient muscles and the Akt/protein kinase B pathway. In *Col6a1*$^{-/-}$ myoblasts, Akt protein levels are apparently normal, but the kinase is less active, as indicated by the markedly decreased Akt phosphorylation when compared to wild-type cells. Growth onto a purified ColVI substrate normalizes Akt phosphorylation and protects cells from apoptosis. A similar effect is displayed after treatment of *Col6a1*$^{-/-}$ myoblasts with insulin. In agreement with these observations, different targets of Akt, such as Bad and Gsk-3, are also deregulated in *Col6a1*$^{-/-}$ myoblasts. This leads us to suppose that the Akt pathway may play a key role in transducing ColVI survival signals in muscle cells.
Moreover, lack of ColVI impinges on the Bcl2 pro-/anti-apoptotic family of proteins, an essential gateway to mitochondrial dysfunction and cell death. In fact, *Col6a1*$^{-/-}$ myoblasts show a marked decrease of Bcl2 protein levels, which appears to be due to a down-regulation of mRNA synthesis. Therefore, Bcl2 transcription seems to be regulated by ColVI.

Keywords: collagen VI, muscle, apoptosis

c-FLIP-mediated resistance to death receptor-induced apoptosis in pancreatic cancer cells

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Pancreatic cancer cells are naturally resistant against current chemotherapy and radiation therapy. Furthermore they show a high resistance against death receptor mediated apoptosis although almost all pancreatic cancer cell lines express proapoptotic members of the TNF-receptor superfamily. Depending on the cellular context the protein FLIP might act as a caspase inhibitor or activator at the DISC (death inducing signaling complex), displaying either a pro- or antiapoptotic function. In this study we investigated the role of the c-FLIP isoforms in pancreatic carcinoma cells.
We either downregulated c-FLIP on translational level by incubation with Cylcloheximide (CHX) or on transcriptional level by incubation with the chemotherapeutic agent 5-FU. Both substances lead to a preferential downregulation of c-FLIP(S) compared to c-FLIP(L). Pretreatment with either CHX or 5-FU and subsequent TRAIL stimulation or stimulation with an agonistic anti-CD95 antibody leads to increased cell death compared to non-pretreated but stimulated cells. Western Blot analysis shows that downregulation of c-FLIP occurs concurrently with cleavage of procaspase-8, procaspase-3, Bid and RIP after stimulation with TRAIL suggesting death receptor-mediated apoptosis.
Simultaneous downregulation of c-FLIP(L) and c-FLIP(S) by retroviral RNAi leads to increased cell death after TRAIL stimulation as well as after treatment with an agonistic anti-CD95 antibody compared to control cell lines. Western Blot analysis after TRAIL stimulation shows a strong upregulation of the FLIP(L) cleavage fragment p43 and c-FLIP(S) at early timepoints in the FLIP knockdown cell lines cell lines as well as in control cell lines. Interestingly procaspase-8 is cleaved at early timepoints after TRAIL treatment, but upregulated at later timepoints in FLIP knockdown cell lines. Specific downregulation of c-FLIP(S) leads to increased cell death after TRAIL- or anti-CD95 treatment compared to controls, while specific downregulation of c-FLIP(L) leads to increased cell death only after anti-CD95 treatment, but not after TRAIL treatment.
Taken together, our data indicate that downregulation of c-FLIP is sufficient to overcome resistance to death receptor mediated apoptosis in pancreatic cancer cells.

Keywords: c-FLIP, pancreatic cancer, apoptosis

Presenting author: Jakub Hanus

Major apoptotic endonuclease DFF40/CAD is a deoxyribose-specific and double-strand-specific DNase

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DFF40/CAD endonuclease is primarily responsible for internucleosomal DNA cleavage during the terminal stages of apoptosis. DFF40/CAD nuclease is specific for double-stranded DNA. Single-stranded RNA, although not a substrate for the enzyme, competes with DNA and inhibits its cleavage by the nuclease. Here we performed more detailed study on specificity of the nuclease using single-stranded (ss) and double-stranded (ds) DNA, ssRNA, dsRNA and RNA-DNA heteroduplexes of the same sequences as substrates of the enzyme. We have found that neither ssDNA, ssRNA, dsRNA nor RNA-DNA heteroduplexes are cleaved by the DFF40/CAD nuclease. Importantly, all types of oligonucleotides that are not cleaved by the nuclease (ssRNA, ssDNA and dsRNA) inhibit cleavage of dsDNA.

Knowing that both ssRNA and dsRNA inhibited activity of DFF40/CAD in vitro, we aimed to investigate whether degradation of DNA and degradation of RNA were temporally correlated in apoptotic cells in vivo. One could postulate that apoptotic chromatin breakdown might be co-ordinated with RNA degradation, and DFF40/CAD activity would be kept in check until RNA levels fall below an inhibitory threshold. However, our preliminary data indicated that degradation of RNA did not precede DNA cleavage in cells undergoing apoptosis.

Keywords: DFF40/CAD, DNA fragmentation, RNA fragmentation

Presenting author: Mohammad Hawat

Initiation caspases suppress the formation of reactive oxygen species in a model of L-Glutamate-induced oxidative neurotoxicity

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Acute cerebral ischemic injury results in increased L-glutamate release that can cause cell death via L-glutamate receptor- and non-receptor-mediated mechanisms. HT22 murine hippocampal cells (Elphick et al., 2006) lack ionotropic glutamate receptors and represent a model for non-receptor-mediated cell injury by L-glutamate. L-glutamate-induced toxicity was characterised by nuclear shrinkage and chromatin condensation, yet occurred in the absence of DNA fragmentation and mitochondrial cytochrome c release. In addition, no activation of execution caspases-3 and -7 was detected. Instead, the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD) significantly increased L-glutamate-induced cell death with a corresponding increase in the number of cells showing nuclear shrinkage and chromatin condensation. In order to identify the caspases responsible for enhancement of L-glutamate-induced cell death, HT22 cell were pre-treated with a range of different caspase inhibitors. Inhibition caspases-3 and -7 by Ac-DEVD-CHO failed to significantly alter L-glutamate-induced cell death. In contrast, both Ac-LEHD-CHO and Ac-IETD-CHO, caspase-9 and -8 inhibitors, respectively, enhanced L-glutamate-induced cell death significantly. The enhancement of glutamate toxicity by caspase inhibition correlated with an increase in L-glutamate-dependent production of reactive oxygen species (ROS), measured using CM-H2DCFDA. Caspase inhibition by itself did not affect basal ROS production. The increase in ROS production was detected as early as 6h after exposure to L-glutamate and several hours before a loss of cell viability was observed.

We conclude that oxidative L-glutamate-induced HT22 cell death is not mediated by caspases. Instead, caspases-8 and -9 may protect from L-glutamate-induced oxidative neuronal damage by decreasing L-glutamate-induced oxidative stress.

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Keywords: Glutamate, Caspases

Presenting author: Nastaran Heidari

Mitochondrial events are involved in noscapine-induced Apoptosis

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Monitoring apoptosis is becoming increasingly important in finding new chemoprevention drugs and their mechanism. Previously, the microtubule opium alkaloid noscapine was discovered as a microtubule destabilizing agent that arrests mammalian cells at mitosis and induces apoptosis. Noscapine is water-soluble drug that can be absorbed very fast after oral administration with little toxicity to normal tissues and no inhibition of immune responses. We selected P53-null myelogenous leukemia cells; apoptosis-prominent HL60 and apoptosis-resistance k562 cells to monitor apoptosis and study of noscapine-induced apoptosis mechanism. K562 cells showed delayed but effective responses to noscapine treatment in comparison with HL60. We monitor apoptosis by detecting the exposure of phosphatidylserine, increase the activity of caspase-2,-3,-6,-8, and-9, PARP cleavage, DNA fragmentation, and finally nuclear morphological changes. Using the inhibitory approach help us to elucidate caspases cascade. We proved caspase-8 activity in a Fas-independent manner, downstream of caspase-9 activation. The increasing of Bax/Bcl-2 ratio in the first hours of apoptosis induction accompanied with Bcl-2 phosphorylation confirmed mitochondrial events involved in the noscapine induced apoptosis.

Keywords: noscapine apoptosis Bcl-2 Bax

Presenting author: Christian Hellwig

TRAIL-induced Caspase-8/-10 activity is a dose-dependent response which is temporally integrated into a strict cell death decision at the single cell level

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TRAIL is a potent cytotoxic ligand inducing apoptosis preferentially in tumour cells. TRAIL binding to its cognate receptors is followed by the formation of the death inducing signalling complex and subsequent activation of caspases-8 and -10. In type II cells, caspases-8/-10 initiate apoptosis by cleaving the protein Bid. Cleaved Bid induces mitochondrial outer membrane permeabilisation (MOMP), resulting in cytochrome-c release, mitochondrial depolarisation, and apoptosis execution.

Utilisation of a highly sensitive fluorescence resonance energy transfer (FRET) substrate allowed us to measure caspase-8/-10 activation and activity in single living HeLa cells. Importantly, we could exclude any feedback contribution on our measurements from downstream effector caspases.

On the single cell level we determined (i) how death receptor stimulation temporally and quantitatively translates into intracellular caspase-8/-10 activity and (ii) how this activity feeds into triggering apoptosis execution by MOMP. Dose-dependently (10 to 1000 ng/ml TRAIL + 1 µg/ml CHX), caspases-8/-10 were activated between ~45 min and ~600 min after stimulus addition. At low TRAIL doses, low Caspase-8 activity persisted for ~60 min until eventually inducing MOMP. At high TRAIL doses, MOMP was induced after ~15 min of high caspase-8/-10 activity. Independent of the TRAIL concentrations used or the resulting caspase-8/-10 activities, MOMP was initiated as soon as ~10-15 % of the FRET substrate was cleaved.

Our results demonstrate that (i) in contrast to the largely dose-independent kinetics of apoptotic signalling downstream of MOMP, caspase-8/-10 activation and activity are dose-dependent responses at the single cell level and (ii) that this variability is translated into a strict death decision (MOMP) at a critical threshold.

Keywords: Caspase-8, TRAIL, single-cell analysis

Presenting author: David Hill

Defining the role of B-RAF induced ERK kinase in the resistance of metastatic melanoma to apoptosis

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Malignant melanoma, the most aggressive form of skin cancer is largely unresponsive to current chemotherapy due to the notorious resistance of such tumours to apoptosis. During melanoma progression melanocytes become transformed due to DNA mutations which frequently involve the B-RAF kinase. Activated mutants of B-RAF (eg B-RAFV600E) occurring in 70% of all melanomas result in enhanced activation of extracellular signal-regulated kinase (ERK) pathways resulting in tumour survival and progression. The mechanism(s) by which the downstream effects of B-RAF induced ERK activation result in the resistance of melanoma cells to apoptosis however, remain entirely unclear but likely involve the activation of Inhibitor of Apoptosis Proteins (IAP's) which block caspase activation. The aim of the current study was to test the hypothesis that mutation of B-RAF increases IAP expression and that blocking B-RAF-induced ERK signalling sensitizes melanoma cells to drug induced apoptosis in response to fenretinide (a synthetic analogue of vitamin A) or velcade (a 26 S proteosome inhibitor) both agents which are able to target apoptosis via endoplasmic reticulum (ER) stress. Treatment of cells with a MEK specific inhibitors increased inhibition of melanoma cell viability in response to both fenretinide and velcade, whilst increasing fenretinide-induced apoptosis. Both RNAi mediated knockdown of B-RAFV600E and MEK inhibition also resulted in at least a two fold down regulation of XIAP and survivin expression. These results suggest that blocking B-RAF induced ERK signalling increases the sensitivity of melanoma cells to ER stress-induced apoptosis through the down regulation of IAP's and defines a novel therapeutic strategy for B-RAF mutated melanoma in which the combination of agents to abrogate B-RAF-induced ERK with agents to target the IAP family member most crucial for melanoma cell survival will overcome the apoptotic resistance of such tumours.

Keywords: melanoma, apoptosis, ER stress, B-RAF kinase

Presenting author: Julia C. Hoffmann

Procaspase-8 processing at the CD95 DISC

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Apoptosis can be triggered by a number of factors including UV or γ-irradiation, chemotherapeutic drugs and signalling from death receptors (DRs), such as CD95. Stimulation of CD95 causes oligomerization of the receptor and formation of the intracellular death-inducing signalling complex (DISC). Upon recruitment to the CD95 DISC procaspase-8 is autocatalytically activated by sequential cleavages at defined aspartate (Asp) residues. This process has been described in a two-cleavage-step model.

Activation of procaspase-8 at the DISC plays a central role in the regulation of CD95-mediated apoptosis. Recently, we showed that the active caspase-8 heterotetramer is formed at the DISC. Subsequently, the mature caspase-8 heterotetramer is released into the cytosol to trigger apoptotic processes. In addition, CAP3 was identified as an intermediate cleavage product of procaspase-8a. Furthermore, yet other procaspase-8 cleavage products are generated upon stimulation of CD95. One of these proteins, the protein p30, comprises the C-terminus of procaspase-8 and is generated upon CD95 stimulation by cleavage of procaspase-8 at Asp210. P30 can be further processed to form the active caspase-8 heterotetramer. The identification and characterization of this and other cleavage products of procaspase-8 upon CD95 stimulation provides new insights into the mechanism of procaspase-8 activation at the CD95 DISC. Further analysis of the generation, the processing and the functional role of these proteins will help to clarify the mechanism of procaspase-8 activation and CD95 signalling.

Keywords: Procaspase-8, CD95

Presenting author: Andrea Holme

The Use of Laser Scanning Slide Base Cytometry in the Assessment of Cell Death

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The Laser-Scanning-Cytometer (LSC) is a microscope-based cytofluorometer which combines both flow and image cytometry, and automatically scores fluorescence signals. No one specific method exists for assessing cell death, thus there this a need to combine different methods/fluochromes, and if possible dissect the heterogeneity of the response. Using the adherent HCT116 and A549 cells the study aimed to dissect the response to a novel drug, MPO-Zn using the LSC technology. This allowed the assessment of the cell death process, per cell in real time using combinations of: 1) measurements of mitochondrial membrane potential, 2) degree of nuclear fragmentation, 3) surface membrane alterations using Annexin V-FITC 4) plasma membrane permeability, and 5) measurement of caspase activation in real time within a 96 well plate format. Thus, the LSC afford the flexibility in technique and analysis to investigate the temporal progression of cell death in response to the drug effect within the whole cell population and sub-populations.

Keywords: laser scanning cytometry, cell death, drug

Presenting author: Li-Jin Hsu

The role of WW domain-containing oxidoreductase in methotrexate-induced cell death in human squamous cell carcinoma

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Fragile *WWOX* gene encodes a candidate tumor suppressor WW domain-containing oxidoreductase (designated WWOX, FOR or WOX1). Overexpressed WOX1 induces apoptosis in numerous cancer cells and suppresses tumor growth both *in vitro* and *in vivo*. Previous study showed significant reduction of WOX1 and its family proteins in poorly differentiated cutaneous squamous cell carcinomas (SCC) without down-regulation of *WWOX* mRNA, indicating a translational blockade of *WWOX* mRNA to protein. Verrucous carcinoma is a distinctive variant of SCC. Continuous intra-arterial infusion of methotrexate (MTX), a folate antagonist, significantly upregulates WOX1 protein expression in the SCC cells and results in complete tumor regression in patients with verrucous carcinoma. These observations imply a role of WOX1 in the chemotherapeutic potential. In this study, we determined that MTX induced apoptotic cell death in SCC-4 and SCC-15, but not SCC-9, in both time- and dose-dependent manners. MTX-induced cell death was associated with increased mRNA and protein levels of WOX1 and activation of caspase-9 and -3 in MTX-susceptible SCC cells. Transient expression of an siRNA construct targeting WOX1 in SCC-15 cells suppressed MTX-mediated cell death. Conversely, overexpression of WOX1 in SCC-9 cells enhanced death induced by MTX. Together, MTX induces apoptotic cell death in SCC via upregulating WOX1 expression. The molecular mechanism underlying MTX-mediated SCC cell death via WOX1 is being underway.

Keywords: WOX1, chemotherapy

Presenting author: Yvonne Huebner

Early detection of apoptosis in human T leukaemia cells using dielectrophoresis

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In this study, we have investigated the ability of dielectrophoresis (DEP) to detect early apoptosis on the basis of changes in the biophysical properties of cells. DEP is the motion of polarizable particles in a non-uniform electric field. Cells can either be attracted to or repelled from the electrodes; this is termed positive and negative DEP, respectively. The direction of the movement is frequency dependent, and analysis of the cross-over frequency between negative to positive DEP reveals the biophysical properties of cells. DEP was used to investigate the biophysical properties of Jurkat human T-lymphocyte cells that were treated with anti-CD95 antibody (CH11) to induce death receptor-mediated apoptosis. A decrease in cytoplasmic conductivity and an increase in membrane permittivity were detected by DEP as early as 30 min after CD95 activation whereas phosphatidylserine exposure measured by Annexin V binding was evident only after 1h. Changes in the membrane permittivity can be a sign for membrane folding, while a drop in cytoplasmic conductivity means that ions are leaking out of the cell. Similarly when Jurkat cells were treated with staurosporine, a decrease in cytoplasmic conductivity and an increase in membrane permittivity were observed by DEP after 1 h, whereas phosphatidylserine exposure measured by Annexin V binding was evident only after 2h incubation time. This study demonstrates the potential use of DEP as a rapid and non invasive tool that can be adapted for high-throughput screening to detect early apoptosis by monitoring the biophysical properties of cells.

Keywords: phosphatidylserine, dielectrophoresis, permittivity, conductivity

Presenting author: Martina Hyzdalova

The interaction of butyrate with TNF-α during differentiation and apoptosis of colon epithelial cells: role of NF-κB activation

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We demonstrated that TNF-α suppressed differentiation and potentiated cell death induced by NaBt in both adenocarcinoma (HT-29 and CaCo-2 cell lines) and normal (FHC cell line) human colon cells. Since TNF-α is a typical activator of NF-κB pathway, we studied the role of activation of this transcription factor in cell differentiation and death during the TNF-α and NaBt co-treatment using HT-29 and FHC cells.

To study NF-κB activation, four different approaches were used – luciferase reporter construct pBIIX-LUC, electromobility shift assay (EMSA), and p65 nuclear translocation and IκB-α degradation using Western blot. To analyze cell differentiation we measured intracellular alkaline phosphatase activity (spectrophotometry). Cell death was expressed as number of cells with the characteristic apoptotic nuclear morphology (DAPI stainig).

TNF-α induced rapid NF-κB activation in both cancer HT-29 and normal FHC cell lines. However, this effect was modulated by NaBt differently in normal and cancer cells. In HT-29 cells, the NF-κB activity peaked after 4 hours of the TNF-α and NaBt co-treatment in comparison with the TNF-α alone. However, this initial increase of NF-κB activity was not observed in FHC cells. During additional time of TNF-α and NaBt co-treatment, NaBt decreased the TNF-α-mediated NF-κB activity in both cell types used. Moreover, we also detected a different response of HT-29 and FHC cells to the TNF-α and NaBt effects after the pre-treatment with the NF-κB activation inhibitor parthenolide.

We found significant differences in the mechanism of the NaBt and TNF-α co-effects between normal and cancer cells, suggesting that the NF-κB pathway is more effectively involved in these processes in cancer cells. Our findings imply the possible role of TNF-α in the development of adenoma or carcinoma during prolonged chronic inflammation of the colon via suppression of the differentiation and potentiation of apoptosis induced by butyrate in colon epithelial cells.

This work was supported by grant no. KJB500040508 of the Academy of Sciences of the Czech Republic, by grant no. 1QS500040507, and by grant no. 524/07/1178 from the Grant Agency of the Czech Republic.

Keywords: butyrate, TNF-alpha, differentiation, apoptosis, colon epithelium, NF-kappaB

Presenting author: Saska Ivanova

Cellular localization of MAGI-1 caspase cleavage products and their role in apoptosis

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MAGI-1 is a *m*embrane *a*ssociated *gu*anylate *k*inase (MAGUK) present at the adherent and tight junctions, where it acts as a structural and signalling scaffold. Recently, we have reported MAGI-1 as a caspase target during FasL-, STS- and UV-induced apoptosis in different cell models (Gregorc et al., 2007). Cleavage of MAGI-1 at Asp761 between the second and the third PDZ domains by caspases was found to be an important step in proper, fast and efficient cell detachment during apoptosis, since the dominant negative mutant of MAGI-1 delayed cell detachment, but not the other signs of apoptosis, i.e. nuclear fragmentation and PS exposure (Gregorc et al., 2007). Since Laura et al. (2002) showed that the C-terminal part of MAGI-1 starting with the fifth PDZ domain resides in the nucleus, whereas the whole region containing PDZ domains 1, 2, 3 and 5 was found to be necessary for targeting MAGI-1 to the tight junctions, we further investigated the cellular distribution and possible proapototic role of MAGI-1 caspase cleavage products. Full length MAGI-1 exhibited submembrane localization, while the N-terminal caspase cleavage product of MAGI-1 is translocated to the cytosol and the C-terminal caspase cleavage product accumulated in the nucleus. When overexpressed, both N- and C-terminal MAGI-1 caspase cleavage products in MDCK cells exhibited minor proapoptotic activity, although their role in apoptosis is probably more passive.

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Keywords: MAGUK, MAGI-1, apoptosis, caspase

Presenting author: Shawkat-Muhialdin Jangi

Pemetrexed induces DNA damage and Caspase dependent and independent Apoptosis in Human Melanoma cells.

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Pemetrexed (alimta, LY231514) is a novel multitargeted antifolate antimetabolite agent that inhibits folate-dependent enzymes: thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase, required for the *de novo* synthesis of nucleotides for DNA replication resulting in cell cycle arrest and apoptosis. Pemetrexed is currently used in the treatment of mesothelioma and it has shown clinical activity in non-small cell lung cancer, breast, colorectal, bladder, cervical, gastric and pancreatic cancer. However its effect in human melanoma has not been studied yet. Melanoma is a lethal skin cancer and its incidence is rising every year. Metastatic melanoma is still representing a challenge for oncologists since the current treatment options are limited and noncurative in the majority of cases. Therefore, the effort to find and/or develop novel compounds and new strategies in the treatment of metastatic melanoma is mandatory.

In the current work, we have found that pemetrexed induces dose and time dependent cytotoxic effect on two human melanoma cell lines, A375 and HT144. Through immunofluorescent and electron microscopic studies we have found typical changes of apoptosis in the form of cell compaction, chromatin condensation and nuclear fragmentation. Furthermore, we confirmed and quantified apoptosis by flow cytometry detecting the percentage of hypodiploid cells at subG0-G1. We found that pemetrexed increased, dose and time dependently, the percentage of hypodiloid cells. Furthermore, we observed that pemetrexed dissipates mitochondrial membrane potential, and releases cytochrome c and apoptosis inducing factor (AIF) from mitochondria to the cytosol, in addition to increasing the production of reactive oxygen species in the treated human melanoma cells. In addition, through colorimetric assay, we found that pemetrexed activates caspase-2,-3,-8,-9 and caspase-10. The activation of caspase-3 has been confirmed by western blotting and immunocytochemistry. Moreover, caspase inhibitors could reverse only about 40% of the cell death induced by pemetrexed, particularly caspase-3 and -9 inhibitors. This result, in combination with the finding of AIF release from mitochondria, indicates that apoptosis is caspase dependent and independent. Finally, through comet assay we found that pemetrexed induces DNA damage in the treated cells. These results may have a potential therapeutic application in the treatment of human melanoma.

Keywords: apoptosis, pemetrexed, caspase, melanoma

Presenting author: Irmela Jeremias

Cytotoxic drug-induced, p53-mediated upregulation of Caspase-8 overcomes resistance against TRAIL-induced apoptosis in tumor cells

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Apoptosis resistance is crucially involved in cancer development and progression, represents the leading cause for failure of anti-cancer therapy and is caused, e.g., by downregulation of pro-apoptotic intracellular signaling molecules such as Caspase-8.
We found that the cytotoxic drugs Methotrexat and 5- Fluorouracil were both able to sensitize resistant tumor cells for induction of apoptosis by p53-mediated upregulation of Caspase-8. Increase in Caspase-8 mRNA and protein expression disabled TRAIL-induced proliferation and restored sensitivity towards TRAIL-induced apoptosis which was inhibited by transfection of p53 decoy oligonucleotides, p53 shRNA and Caspase-8 shRNA. Upregulation of Caspase-8 and sensitization towards TRAIL-induced apoptosis was found both in a broad panel of tumor cell lines with downregulated Caspase-8 and in TRAIL-resistant primary tumor cells of children with acute leukemia.
Taken together, we have identified Caspase-8 as an important novel p53 target gene regulated by cytotoxic drugs. These findings highlight a new drug-induced modulation of physiological apoptosis pathways which may be involved in successful anticancer therapy using Methotrexat and 5- Fluorouracil in leukemia and solid tumors over decades.

Keywords: resistance, TRAIL

Presenting author: Siriporn Jitkaew

Programmed cell clearance: caspase-independent phosphatidylserine externalization in normal non-apoptotic neutrophils upon co- cultivation with PLB-985 macrophages

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Phosphatidylserine (PS) externalization on the surface of apoptotic cells serves as a crucial recognition signal for macrophages. Moreover, this process is essential for triggering of anti- inflammatory responses in macrophages resulting in resolution of inflammation. In neutrophils undergoing apoptosis, PS externalization is mediated through a caspase-dependent pathway. In addition, NADPH oxidase-generated reactive oxygen species are believed to contribute to an alternative mechanism of PS exposure (Fadeel *et al*., Blood, 1998). Recent studies have shown that macrophage-induced nitrosative stress contributes to cell clearance by inhibiting aminophospohlipid translocase function resulting in PS exposure in non-apoptotic target cells (Tyurina *et al*. J. Biol. Chem., 2007). To better determine the mechanisms of macrophage-induced PS exposure in non-apoptotic cells, we subjected normal human neutrophils to co-culture with PLB-985 macrophages and then examined PS exposure in neutrophils using conventional annexin V/PI staining protocols. Upon co-culture with PLB-985 macrophages, we found that PS exposure in normal neutrophils was increased. However, this PS exposure was not mediated by a caspase-dependent or NADPH oxidase-dependent pathway. Next, we tested whether macrophage-induced PS exposure in normal neutrophils plays a role in cell clearance. To this end, we added normal neutrophils to human monocyte-derived macrophages (HMDM). Of note, phagocytosis of normal neutrophils following co-culture with PLB-985 macrophages by HMDM was not efficient compared to neutrophils undergoing spontaneous apoptosis despite high levels of PS exposure in both types of target cells. However, the clearance of the non- apoptotic neutrophils could be restored by the addition of MFG-E8, a PS-binding molecule. These results suggest that although PS molecules may serve as an essential ”eat me” signal, efficient clearance of cells with exposed PS requires its interaction with bridging molecules. Taken together, our data suggest that macrophage- induced PS exposure in normal neutrophils might act as a feedback loop during inflammation by triggering PS-dependent anti-inflammatory responses in macrophages resulting in the resolution of inflammation.

Keywords: Phosphatidylserine externalization, programmed cell clearance

Presenting author: Gabor Juhasz

Autophagy gene 7 is required for neuronal health, stress tolerance, and longevity in Drosophila

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Autophagy (cellular self-digestion by lysosomes) is activated in response to starvation in all eukaryotic cells, and ensures survival by recycling dispensable cellular constituents for re-use in synthetic processes. In Drosophila, autophagy is also induced in most polyploid larval tissues during metamorphosis, and may potentially contribute to their destruction or reorganization.
We generated local deletions in Drosophila Atg7, a gene encoding an essential component of the core autophagic machinery. The mutants are fully viable and fertile, despite major defects in the induction of autophagy in response to starvation. Dying larval midgut cells also show a reduced level of autophagy and impaired DNA fragmentation at the beginning of metamorphosis, but the larval midgut shrinks and is shed normally into the lumen of the forming adult midgut.
Emerging mutant adult flies are hypersensitive to starvation and oxidative stress, and show a reduced lifespan when compared to controls. Also, polyubiquitinated protein aggregates accumulate in the neurons of Atg7 mutants, leading to neurodegeneration. These results suggest that the normal level of autophagy is not only required to better survive various stress conditions, but is also crucial for continuous cellular renewal.

Keywords: Atg7, autophagy, Drosophila, lifespan, neurodegeneration, stress

Presenting author: Minna Jääskeläinen

TRAIL and Its Receptors Regulate Apoptosis in Fetal Human Ovaries

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Introduction: Apoptosis is an essential component of ovarian development and function. Of the 7-8 million oocytes that are formed in the human ovary, most are depleted before birth. The remainder form a finite pool of resting follicles, which are also depleted until all follicles have undergone apoptosis and menopause ensues. TRAIL is a transmembrane protein that induces cell death by binding to its Death Receptors (DR4 and DR5). TRAIL induced apoptosis can be prevented by expressing decoy receptors (DcR1 and DcR2), which also bind trail. TRAIL is expressed in variety of human tissues but its role in human ovarian development is unknown.
Objective: To evaluate the role of TRAIL pathway in regulation of apoptosis during ovarian development.
Materials and methods: Ovarian tissues with normal karyotype (fetal age 11-41 weeks) were collected from aborted fetuses or neonates died shortly after birth. Expression of TRAIL pathway components was studied using immunohistochemistry and *in situ* hybridisation. Human granulosa cell line (KGN) was used to study the effects of TRAIL *in vitro*. Apoptosis was analyzed using caspase activation assay and in situ 3´-end labelling.
Results: TRAIL protein was present in fetal ovaries from 11th week onwards. Oocyte TRAIL expression was constantly higher than that of somatic cells and expression in oocytes increased towards birth. TRAIL mRNA was also detected throughout fetal life. However, unlike protein expression, mRNA levels remained relatively unchanged. DcR2 and DR5 proteins were expressed both in stromal cells and oocytes all fetal life. Their expression in oocytes, substantially increased towards birth. DcR1 and DR4 were absent in all studied samples. In cultured granulosa cells, TRAIL led to rapid and potent induction of apoptosis in a dose-dependent manner.
Conclusion: TRAIL participates in regulation of apoptosis in human fetal ovaries. TRAIL is also an effective inductor of granulosa cell apoptosis. The presence of both death- and decoy receptors in fetal ovaries suggest that TRAIL induced apoptosis is regulated at the cellular level through differential expression of these factors. The balance between the antagonist receptors might be an important factor in determining the survival of the oocyte.

Keywords: Human ovary, Apoptosis, TRAIL, DR5, DcR2

Presenting author: Magdalena Kalinowska-Herok

The mechanisms of activation of the major apoptotic nuclease DFF40/CAD by chromatin proteins histone H1 and HMGB1.

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The major apoptotic nuclease, DNA fragmentation factor 40kD (DFF40) also termed Caspase-activated DNase (CAD), is primarily responsible for internucleosomal DNA cleavage during the terminal stages of programmed cell death. Previously we demonstrated that several chromatin proteins, including HMGB1/2, histone H1 and topoisomerase II, greatly enhances naked DNA cleavage by DFF40/CAD *in vitro.* Here we investigated the mechanism of stimulation of DNA cleavage by histone H1 and HMGB1. Addition of histone H1 and HMGB1 either during or after caspase-3 treatment of DFF causes the same stimulatory effect on DNA cleavage, indicating that these proteins affect DFF40/CAD enzyme activity, but not caspase-3-dependent activation of the nuclease. We have found that each of the six somatic cell histone H1 isoforms, which differ in primary sequence, equally activate DFF40/CAD. Using a series of truncation mutants of recombinant mouse histone H1-0, we have demonstrated that the H1-0 C-terminal domain (CTD) is responsible for activation of DFF40/CAD. We show further that the intact histone H1-0 CTD and certain synthetic CTD fragments bind to DFF40/CAD. These interactions enhance the ability of DFF40/CAD to bind to DNA. We have concluded that the interactions between the histone H1 CTD and DFF40/CAD may target and activate linker DNA cleavage during the terminal stages of apoptosis. In contrast to histone H1, HMGB1 neither binds to DFF40/CAD nor enhances its ability for stable binding to DNA. Comparison of the stimulatory activities of different forms of HMG-box containing proteins, indicates that the HMG-box alone is responsible for such stimulation. We postulate that changes induced in DNA conformation upon HMG-boxes binding makes the substrate prone to cleavage by DFF40/CAD, and that such phenomenon may also contribute to preferential cleavage of linker DNA.

Keywords: nuclease DFF40/CAD, apoptosis, histone H1, HMGB1 proteins, DNA fragmentation

Presenting author: Marcin Kaminski

Mitochondria - oxidative signalling organelles in activation-induced death of T cells.

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Reactive oxygen species (ROS) generated upon T cell activation are crucial for induction of CD95L expression and, consequently, for Activation Induced T cell Death (AICD). The molecular source and the signalling pathway mediating activation-induced ROS production are still unclear.

Our experimental results show that the proximal T cell receptor (TCR) signalosome, consisting of ZAP70, LAT, SLP76, PLCγ1 and PKCθ, is crucial for ROS generation. Upon activation PKCθ translocates to the mitochondria. Application of cells depleted in mitochondrial DNA (mtDNA) led to identification of mitochondria as source of activation-induced ROS. Pharmacological inhibition of the mitochondrial respiratory chain complex I (NADH-quinone oxidoreductase) or siRNA-mediated knockdown of the complex I assembly factor chaperone NDUFAF1 resulted in blocking of ROS production. Mitochondria derived ROS signal is essential for CD95L expression, since inhibition of complex I assembly by NDUFAF1-specific siRNA prevents AICD. Furthermore, application of metformin, an antidiabetic drug and mild, non-toxic complex I inhibitor, also decreased activation-induced ROS production, CD95L expression and AICD.

Thus, PKC θ-dependent ROS release by mitochondrial complex I constitutes a crucial signalling event leading to AICD of T cells.

Keywords: ROS, TCR , apoptosis, complex I, CD95L (FasL), metformin

Presenting author: Chahrazade Kantari

The pro-inflammatory role of apoptosis-induced proteinase 3 is dependent on its association with phospholipide scramblase 1.

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Background: Neutrophils are essential actors in the defense against pathogens. Because neutrophils contain highly toxic microbicidal mediators, their apoptosis, is crucial for the resolution of inflammation. Proteinase 3 (PR3) is an antibiotic protein mainly stored in the neutrophil azurophilic granules. In the laboratory, it has previously been described that PR3 can be expressed at the plasma membrane of resting neutrophils. Recently, we have described a new pro-inflammatory role of PR3 showing that 1) it was expressed at the neutrophil plasma membrane during apoptosis and 2) that this expression interfere with the resolution of the inflammation by decreasing the phagocytosis by macrophages. We have hypothesized that apoptosis-induced PR3 membrane expression was mediated by a membrane partner, the phospholipid scramblase 1 (PLSCR1), a protein involved in the redistribution of phospholipids at the plasma membrane during apoptosis.
Results: By confocal microscopy, we have shown that PR3 co-localises with PLSCR1 at the neutrophil plasma membrane under resting conditions and that this colocalization increased during apoptosis. Next, we have shown by co-immunoprecipitation experiments, a physical interaction between these two proteins. In our cellular model of RBL (rat basophilic cells) stably transfected with PR3 (RBL/PR3) or its inactive mutant (RBL/PR3S203A), we have shown that PLSCR1 was associated but not cleaved by PR3, and that, like PR3, PLSCR1 was externalized at the plasma membrane during apoptosis. Finally by siRNA experiments, we have shown that the decreased expression of PLSCR1 induced an important decreased apoptosis-induced PR3 membrane expression.
Conclusions: To conclude, our results clearly demonstrate that the molecular mechanisms of PR3 membrane expression during apoptosis are dependent on its functional association with PLSCR1.

Keywords: PR3 , phospholipid scramblase 1, apoptosis, neutrophils, inflammation

Presenting author: Sarvenaz Karimpour

Investigation into ATP and GTP binding abilities of Nucleotide-Binding-Oligomerization domain of Human Neuronal Apoptosis Inhibitory Protein

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Human Apoptosis Inhibitory Protein (NAIP) is a 160 kDa protein shown to inhibit programmed cell death. The protein consists of three distinct domains of BIR (baculoviral IAP repeat) domain, NOD (nucleotide binding oligomerization domain) and LRR (Leucine rich repeat) domain. It has been suggested that the BIR domain inhibits apoptosis through the inhibition of caspases. The structure and the function of the NOD and the LRR domains, on the other hand, remain obscure. Thus we have decided to produce the NOD domain of NAIP in E. coli and purify the recombinant protein. To do so, the cDNA coding amino acids 423-762 was PCR amplified and subcloned into pET32a plasmid. Then, BL21-DE3-Trxb E. coli transformed with pET32a-NOD construct was induced to produce the protein by 0.5 mM IPTG. High amount of protein was produced in the form of inclusion bodies. Consequently, numerous refolding strategies were adopted in order to obtain a soluble protein. Six molar concentration of Guanidin hydrochloride solubilized the protein completely. However, refolding proved to be difficult due to aggregation of the protein during dialysis. However, eight molar concentrations of urea or 0.4% of sarkosyl could successfully be used for protein solubilization and refolding. Thus, the solubized protein was bound to Ni-NTA resin through the histidine tag at the N-terminus of the protein and refolded. The soluble portion of the refolded protein was eluted from the column using 300 mM immidazole under native conditions. Circular dichroism spectroscopy showed that the protein mostly consists of α-Helical structure, which in this respect is similar to the NOD domain of APAF-1. The effect of AMP, ATP, and GTP on the secondary structure was also investigated. Micromolar concentrations of AMP did not have a noticeable effect on the CD spectra of the protein, while the addition of ATP and GTP resulted in an elipticity decrease at 222 nm and an elipticity increase at 208 nm. These results suggest that both ATP and GTP but not AMP interact with the NOD domain of NAIP.

Keywords: apoptosis, naip, nod, nucleotide-binding

Presenting author: Smitha Karunakaran

Cell-specific activation of redox – apoptotic signaling in MPTP mouse model of Parkinson's disease

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Parkinson's disease (PD), a neurodegenerative disorder is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) in the ventral midbrain and their terminals in the striatum. Mitochondrial dysfunction seen as complex I loss and oxidative stress are major contributors to the pathogenesis and progression of PD. However, the precise mechanisms involved in the region and cell type specific vulnerability of neurons are yet to be understood. Loss of the glutathione (GSH) is seen as an early event in animal models of PD and in substantia nigra of PD patients obtained at autopsy. Exposure to 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) results in loss of dopaminergic neurons in substantia nigra and is considered a good animal model of PD. MPTP exposure results in loss of GSH and increased formation of protein glutathione mixed disulfides (PrSSG) indicating the oxidative modification of cysteine residues in key proteins. The redox status of proteins plays an important role in cell signaling pathways that govern both cell survival and death. We observed that exposure to MPTP activates a redox sensitive death signaling cascade initiated by apoptosis signal regulating kinase 1 (ASK1) at early time periods after a single dose of MPTP in ventral midbrain. This is followed by the activation of stress activated protein kinases (SAPK/MAPK) leading to increase in p53 levels and its translocation to the nucleus. p53 mediated transcriptional activation was evident as observed by the upregulation of Bax in the ventral midbrain after MPTP administration. These events culminated in the activation of caspase 3 selectively in midbrain but not in striatum. Further, we observed a decrease in the levels of DJ1, a redox sensitive protein in the ventral midbrain not in striatum and translocation of the death associated protein, Daxx from nucleus to cytoplasm only in the SNpc neurons not in the neighbouring reticulata neurons of the substantia nigra. Pretreatment with thiol antioxidant α-lipoic acid abolished DJ1 downregulation, translocation of Daxx and activation of ASK1. Our studies demonstrate that exposure to MPTP activates the Daxx/ASK1/SAPK pathway in ventral midbrain, which further activates p53 mediated transcription of Bax thus initiating the apoptotic pathway. Thiol anti-oxidants and/or SAPK/MAPK inhibitors are neuroprotective and attenuate the loss of dopaminergic neurons caused by MPTP.

Keywords: MAPK signaling, MPTP , ASK1, oxidative stress, cell death

Presenting author: Daniela Kassahn

Degranulation of preformed Fas (CD95) ligand in activated T cells

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Fas ligand (FasL, CD95L) is a member of the Tumor Necrosis Factor family and a potent inducer of apoptosis. It is mainly expressed on activated T cells and NK cells, where it can mediate target cell killing by engaging its receptor Fas. The expression of FasL is tightly regulated by both, transcriptional events as well as post-transcriptional mechanisms. In particular, activated T cells store preformed FasL in secretory vesicles and release it to the cytoplasmic membrane upon restimulation. The aim of this study is to investigate the biochemical processes leading to subcellular redistribution of FasL upon T cell activation.
Restimulation of murine T cell blasts with immobilized anti-CD3 antibody or PMA/Ionomycin results in functional FasL cell surface expression by de novo protein synthesis and the release of preformed FasL from secretory vesicles (degranulation). In contrast, stimulation of T cell blasts with the phorbol ester PMA alone results in FasL cell surface expression, which is solely dependent on FasL degranulation. Stimulation with the calcium ionophore Ionomycin alone was not sufficient to induce FasL degranulation. PMA induced degranulation is protein kinase dependent and mediated by actin filament dependent intracellular transportation system. Surprisingly, an involvement of PMA activated protein kinase C and protein kinase A isoforms could be excluded. Although FasL partially colocalizes with the secretory lysosomal marker CD63, PMA only induced cell surface expression of FasL but not CD63. This suggests that the degranulation of different granular proteins is regulated by distinct biochemical pathways. Future studies aim at investigating the differential subcellular transportation of FasL versus other granular proteins, i.e. perforin, granzyme B, lamp1 and CD63 in different T cell subsets. The understanding of the degranulation mechanisms of cytotoxic T cell effector molecules may give important insight into the differential use of perforin versus death ligand mediated killing.

Keywords: FasL, secretory lysosomes, degranulation

Presenting author: Anna Kawiak

Pro-apoptotic activity of a plant-derived naphthoquinone, ramentaceone.

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Naphthoquinones represent a group of compounds, which exhibit various biological activities including anticancer properties. Ramentaceone (7-methyljuglone) is a naturally occurring naphthoquinone, which has been reported to possess cytotoxic activity *in vitro*. The mechanisms underlying the induction of cell death by this compound have not previously been investigated.
Ramentaceone was isolated from *in vitro* cultured *Drosera* plants and its pro-apoptotic activity was determined in HL-60 cells. Apoptosis was confirmed by examining the morphological features of cells, the occurrence of chromatin condensation and DNA fragmentation. The treatment of cells with ramentaceone induced a dose and concentration-dependant increase in the hypodiploid cell number, caspase-3 activation and loss in the mitochondrial membrane potential.
Apoptosis was found to be mediated through a redox-dependant mechanism. The level of intracellular reactive oxygen species (ROS) increased 3-fold in ramentaceone-treated cells. The pretreatment of cells with N-acetylcysteine (NAC) inhibited ROS generation and suppressed apoptotic cell death induced by ramentaceone. Diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, inhibited the generation of ROS by ramentaceone as well as the pro-apoptotic activity of this compound. Thus, our observations suggest that the induction of apoptosis by ramentaceone is mediated via NADPH oxidase-dependant generation of ROS.

Keywords: apoptosis, NADPH oxidase, naphthoquinone, ramentaceone, reactive oxygen species

Presenting author: Fatma Kaya Dagistanli

Effects of nicotinamide on pancreatic beta cell regeneration and survivin expression in STZ treated neonatal rats

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Streptozotocin causes DNA breaks in rat pancreatic beta (β) cells and stimulates nuclear poly ADP-ribose synthetase (PARP). Nicotinamide (NA) may also protect β cells by its action in inhibiting PARP. The aims of this study is to determine the insulin gene expression with protein and mRNA levels, to show possible β cell proliferation and the extra islet source of β cells with pdx-1, to evaluate the relation of NA treatment and survivin expression on β cell regeneration in the early stages of the neonatal streptozotocin (n-S TZ) diabetic model. On the second day after birth 100 mg/kg STZ was given i.p to two groups of the newborn rats. First group was STZ treated newborn group. To the second group, starting from third day, 500mg/kg/day NA was given for 5 days. Third group was control group. At 7th day the pancreas tissue were fixed in neutral formalin and embedded in paraffin. The serial tissue sections were carried out in situ hibridization with insulin probe and immunostaining with insulin, survivin and pdx-1 antibodies. Islet sizes of STZ treated groups were smaller than control groups. Insulin immunopositive and islet beta cells contain insulin mRNA signals were lower at STZ treated groups than control group. In addition in NA treated groups the number of islet pdx-1 immun positive cell significant higher than untreated STZ group whereas lower than control group. In NA treated group little islets which contain 5-15 cells at exocrine tissue. Insulin and pdx-1 immunopositive cells in extra islet cells and duct epithelium and insulin mRNA positive cells. In control group survivin immun positive cells localizated most area which similar insuline disperse in treated and untreated STZ groups.Survivin immuno positive cells found in exocrine tissue and duct epithelium same as insulin and pdx-1 immunopositivity in NA treated group. Our results show that the NA treatment involved an induction of neogenesis of new beta cells in extra islet pancreas and survivin may have a role in regulation of beta cells regeneration.

Keywords: Survivin, nicotinamide, n-STZ, Beta cell

Presenting author: Nadine Keller

NMR Structure of the Initiator Procaspase-8 - New Insights in its Activation Mechanism.

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Caspases are key mediators of apoptosis, a fundamental process in development, tissue homeostasis, and inflammation and related to various neurodegenerative diseases and cancer. Two different types of caspases have been distinguished in apoptotic signaling pathways. Apical initiator caspase are recruited to activation complexes, are activated and cleave the executioner caspases, which are thus activated and process downstream targets. In the absence of an apoptotic stimulus, caspases exist as monomeric (initiator) or dimeric (executioner) zymogens containing a prodomain and the catalytic domain comprising a small and a large subunit.
Caspase-8 is the most apical caspases in the so called extrinsic apoptotic pathway. Upon an apoptotic stimulus, such as Fas ligand binding to the Fas receptor, monomeric procaspase-8 is recruited to the DISC (death-inducing signaling complex) comprising Fas, FADD and caspase-8. After autoprocessing dimeric active caspases-8 is released from the DISC.
The structure of active dimeric caspase-8 was solved by X-ray analysis. However, structure determination of monomeric procaspase-8 by X-ray was not possible so far. Here we present an NMR structure of the unprocessed inactive procaspase-8. The monomer shows a six-stranded β-sheet, surrounded by five alpha-helices, which is a common feature for all caspases. Although this alpha/beta fold is highly conserved there are substantial differences in the loops surrounding the activation site of procaspase-8 when compared to active caspases-8. Furthermore, this is the first structure of an initiator caspases, which shows the location of the intersubunit linker, which is removed during proteolytic cleavage during the activation process. Therefore, we gain new insights into activation mechanism of caspases-8.

Keywords: procaspase-8, structure, activation

Presenting author: Darren Kidd

A time course investigation into the effects of colchicine on cell cycle parameters and apoptosis induction.

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Compounds that disrupt the cell cycle attract much interest as potential anti-cancer treatments. An important subset of these are the aneugens, which affect spindle microtubules during cell division. To further understand their wider effects on cell cycle, we have performed experiments using colchicine, and a p53 mutant clone of L5178Y cells. Key parameters measured were viability, cell cycle distribution and apoptosis induction.
Cultured cells were treated for 24 hours with a dose range from 5 to 50 ng/mL, washed and allowed to recover in fresh media for up to 72 hours. Cell samples were taken at end of treatment, 24-, 48- and 72-hours into recovery and cell viability measured by cell counts expressed relative to the solvent control. A dose-related decrease in cell number was seen at each sample time. The highest dose tested (50 ng/mL) reduced viability to 18% at end of treatment but cell viability had recovered to 36% 72 hours later.
Cell cycle analysis of DNA content by flow cytometry was performed using ethanol-fixed cells stained with propidium iodide. Increases in the G2 peaks and the presence of polyploid cells were observed at the higher doses (20-30 ng/mL). The severity of both anomalies decreased with time as the cultures progressed towards a normal cell cycle distribution**.**
Induction of apoptosis was assessed using two methods. First, phosphatidylserine lipid flipping was measured by immunofluorescence labeling (annexin V) and flow cytometry. A dose-related increase in apoptosis occurred at higher doses (25-40 ng/mL), giving a >10 fold increase in annexin V compared to the negative control. Second, a luminescent detection method was used to detect the presence of caspase 3 and 7. As with the annexin V analysis, doses of 25 ng/mL and above showed consistently increased apoptosis at all time points.
It is well known that colchicine arrests cell division and that this can lead to abnormal complements of the haploid chromosome number. Our data have shown increased levels of annexin V, caspase 3/7 activity and polyploidy in the presence of colchicine. We suggest that colchicine's effect on mitotic segregation of chromosomes triggers apoptosis.

Keywords: colchicine; flow cytometry; annexin V; caspase

Presenting author: Ana Kitanovic

Bioenergetic aspects of MMS-induced apoptosis in *S.cerevisiae*

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A very important aspect of DNA damage response is the modulation of major energy metabolism pathways. This may be closely linked to the oxidative burst in cells exposed to stress. Reactive oxygen species (ROS) are byproducts of energy metabolism, with 0.2% to 2.0% of oxygen consumption resulting in ROS formation. ROS can damage DNA, proteins and lipids (St-Pierre *et al.*, 2002). Given that many pathways and enzymes involved in transcription, DNA replication and DNA repair are ATP-dependent, it is also likely that alterations in energy metabolism and changes in the ATP-level may affect these processes. In addition, an increase in ATP level and mitochondrial membrane potential ($\Delta\Psi$) is considered necessary for certain steps of apoptosis (reviewed in Skulachev, 2006).

Here we demonstrate that the low doses of the alkylating agent methyl methanesulfonate (MMS) trigger the activation of environmental stress response (ESR), increase ROS production and mitochondrial membrane potential ($\Delta\Psi$), and induce mitochondrial biogenesis. This is accompanied by a strong increase in cellular ATP-level and disintegration of the tubular mitochondrial network and of mitochondrial filaments into small roundish mitochondrial clusters. However, higher doses of MMS (0.1%) have a different effect leading to decreased mitochondrial biogenesis, lower $\Delta\Psi$ and reduced ATP production, and a collapse of the tubular mitochondrial network. Cultivation of cells in acetate/low glucose medium, that was proven to induce apoptosis (Knorre *et al.,* 2005), or minimal medium, could efficiently decreases sensitivity to MMS and allow more cells to survive the treatment with this toxic agent. The effect seems to be connected with increased NADPH resulting in an elevated NADPH/NADP+ ratio, enhanced ROS production, elevated ATP-level and an early activation of general stress response.

In conclusion, we believe that in *S.cerevisiae* treatment with relatively low concentrations of MMS leads to important alterations in cellular bioenergetic conditions, which serve to induce repair mechanisms, but at the same time, remove seriously damaged cells by apoptosis. We propose that the intensified energy metabolism and increase in ATP could serve as a monitoring parameter of cellular damage and by this ensure a proper balance between apoptotic and anti-apoptotic signals.

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Keywords: DNA-damage, MMS, ATP, NADP, NADPH

Presenting author: Konrad Kleszczynski

Lipophilicity of fluorochemicals-dependent cytotoxicity and mitochondrial disfunction

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Perfluorinated carboxylic acids (PFAs) represent derivatives of naturally occurring compounds and have been widely used in various industrial fields for decades. They are known to be environmentally persistent. Thus far numerous reports have been focused on reproductive toxicity of PFAs in animals but few studies have been carried out on toxicity towards human cells.
Preliminary studies focused on estimation of EC_{50} values by viability tests performed at varying time-exposures on CF_6-CF_{18} PFAs with human colon carcinoma (HCT116) cells. Cytotoxicity increased with the elongation of fluorocarbon chain length (PFHxA>PFHpA>PFOA>PFNA>PFDA>PFDoA>PFTeDA) while higher lipophilicity (CF_{16} and CF_{18}) did not deepen the effect but even partially reversed it. The effect was intensified after longer exposure (72 h); at relatively low 40 μM PFTeDA, the viability decreased to ~50%.
Comparative mechanistic analysis with 200 μM perfluorodecanic acid [PFDA] and its natural analogue as a reference – decanoic acid [DA] were carried out in time-dependent manner by GC-MS and flow cytometry, respectively. PFDA after 4 h caused visible loss of cell transmembrane potential up to 42% comparing with 12% for DA. Following exposures (24 - 72h), particularly with PFDA, revealed significant disorders in downstream events as dissipation of mitochondrial transmembrane potential ($\Delta\Psi_m$), H_2O_2 and $^{\bullet}O_2^-$ generation, cytochrome c release and activation of caspase 9 and -3. In order to evaluate the importance of functional mitochondria in PFDA-induced apoptosis, cells were co-treated with PFDA and inhibitor of mitochondrial transition pore (MPT) - Cyclosporin A (CsA). Presence of CsA reduced $\Delta\Psi_m$ dissipation, cytochrome c release and apoptotic fragmentation of nuclear DNA, indicating a role for PFDA-induced apoptosis dependent on the mitochondrial intrinsic pathway.
We claim that more pronounced and longer lasting PFAs effects compared with natural acids on cultured cells may have similar mechanism of action.

Keywords: perfluorinated carboxylic acids (PFAs), mitochondrial transmembrane potential, reactive oxygen species, apoptosis

Presenting author: Max Koeppel

IRF2BP2 is a new target gene of p53 identified by ChIP-on-chip

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Using global ChIP-on-chip, we have identified several hundreds of new binding-sites for the p53-family. Expression analysis of genes linked to p53 binding sites shows that IRF2BP2, a repressor of Interferon-regulatory factor 2 which has oncogenic functions, is upregulated after genotoxic insults mediated through Act.D.
We have investigated this upregulation further and found that it is specific to p53. Although p73 is able to bind to the binding-site upstream of the IRF2BP2 gene, the expression levels of IRF2BP2 do not change significantly upon p73 induction. We wanted to analyze the role of this transcriptional co-repressor during the progression of cell cycle and its function in stress response pathways. We can show that IRF2BP2 can differentially regulate p53 target genes, dependent as well as independent of p53. Very interestingly, overexpression of IRF2BP2 prevents U2OS from apoptosis after chemoherapeutic treatment, but drives them into G1-arrest as shown by FACS-analysis. Furthermore knock-down of this factor combined with chemotherapeutic treatment shows an accumulation of cells in G2-phase, before they show increased induction of apoptosis.
We identified IRF2BP2, a gene which was known to be involved in the immune response, as a direct target of p53. This gene is involved in a specific cell cycle arrest pathway and prevents cells from undergoing apoptosis upon chemotherapeutic treatment. Very interestingly, the response to this treatment can be re-established upon knock-down of IRF2BP2. Thus, we have identified and characterized a new target gene of p53 that might play an important role in the growth arrest versus apoptosis decision of p53.

Keywords: p53, IRF2BP2, CHIP-chip

Presenting author: Spela Konjar

Cathepsins in the life cycle of perforin in NK cells

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Perforin is stored in lytic granules that contain proteoglycans and lysosomal enzymes. In lytic granules perforin is cleaved. It was reported that this cleavage is inhibited by E-64, suggesting that cysteine cathepsins could be involved in this process (1). In addition, surface cathepsin B was reported to provide self-protection for degranulating CTLs (2), although recent studies showed that cathepsin B is not essential for the protection of CTLs from perforin (3).

We investigated role of cysteine cathepsins in the life cycle of perforin. Initial in vitro studies showed that perforin is cleaved by cathepsins B, K, L and S. It was examined how cathepsins B, K, L and S affect the lytic activity of perforin in in vitro system. Co-localizations of cathepsins with perforin were studied in YT NK cells and NK-92 cells. We measured activity of cathepsins during stimulation of YT NK cells with IL-2. We did cytotoxic assay for NK-92 treated with E64-d.

From our in vitro studies we concluded that, perforin is cleaved and deactivated by cathepsins B, K, L and S. During IL-2 activation of YT NK cells the activity of cathepsins is up-regulated. We found partial co-localization of perforin and cathepsin L. We also found out that cytotoxicity of NK-92 is decreased during treatment with E-64d.

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Keywords: cathepsins, perforin, NK cells

Presenting author: Natasa Kopitar-Jerala

Overexpression of stefin B in the nucleus of T98G astrocytoma cells delays caspase 3 activation in staurosporine-induced apoptosis

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Stefin B (also called cystatin B) belongs to the cystatin family of endogenous cysteine protease inhibitors. Mutations in the gene encoding stefin B are responsible for the primary defect in Unverricht-Lundborg disease (EPM1). The removal of cathepsin B from stefin B-deficient mice greatly reduced neuronal apoptosis, but did not rescue the ataxia and seizure phenotypes, indicating that there are other factors involved. In our previous work we observed increased sensibility to PKC inhibitor staurosporine-induced apoptosis in the thymocytes of stefin B-deficient mice when compared to the wild-type thymocytes. (1).
In T98G astrocytoma cell line stefin B was over-expressed in the nucleus and in cytoplasm. DEVDase activities were measured 5 and 9 hours after induction of apoptosis with staurosporine, respectively. Only in the cells where stefin B was over expressed into the nucleus, we have observed delay in caspase 3 activation at a 5 hour time point post apoptosis induction. As a conclusion, the functions of stefin B in the nucleus could be important for the progression of apoptosis induced by staurosporine.

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Keywords: apoptosis , cystatin, nucleus, staurosporin

Presenting author: Dagmar Kulms

Proapoptotic action of NF-kappaB is promoted by UVB-induced inhibition of I-kappaB resynthesis

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Activation of the transcription factor NFκB by IL-1 is generally associated with the induction of antiapoptotic pathways. Accordingly, NFκB inhibits death ligand-induced apoptosis (CD95L, TRAIL) via upregulation of antiapoptotic IAPs and FLIP. However, apoptosis induced by UVB is enhanced upon costimulation with IL-1 in a NFκB-dependent manner, indicating that NFκB can act also in a proapoptotic fashion. We could show that NFκB activation in the presence of UVB represses the genes of antiapoptotic proteins (IAP, FLIP). Simultaneously, extensive amounts of TNF are released which activate the TNFR -1 in an autocrine fashion. Triggering of TNF-R1 can transduce either pro- or antiapoptotic signals depending on whether the proapoptotic adapter protein FADD or the antiapoptotic adapter proteins TRAF-1 and -2 are recruited to TRADD. We have demonstrated that NFκB activation in the presence of UVB represses the TRAF genes. Therefore, the balance at the TNF-R1 is shifted towards promotion of the proapoptotic pathway which in concert with downregulation of IAPs and FLIP finally results in enhancement of UVB-induced apoptosis.
IL-1-dependent NFκB activation is facilitated by IKK2 mediated phosphorylation of its cellular inhibitor IκB. Ubiquitination followed by proteasomal degradation allows NFκB to translocate into the nucleus and bind to responsive promoter elements. Once activated NFκB induces transcription of its inhibitor resulting in reappearance of IκB in the cytoplasmic fraction of the cell within 2h, consequently terminating NFκB dependent transcription.
In the case of costimulation of cells with IL-1 + UVB, we noted that reappearance of IκB was completely inhibited over hours, whereas IκB transcription remained unaffected. Instead, lack of IκB recurrence was shown to be due to immediate proteasomal degradation of newly synthesized IκB within the cytoplasm. The underlying mechanism involves constitutive Ser-phosphorylation of IKK2. Lack of IκB resynthesis leads in turn to prolonged nuclear persistence of NFκB without alteration in NFκB subunit composition. As a consequence NFκB maintains a shifted transcriptional activity, evident from enhanced TNFα production paralleled by enduring repression of anti-apoptotic genes. As a result both effects cooperate to mediate enhancement of UVB-induced apoptosis.

Keywords: Apoptosis, UVB, IL-1, TNF

Presenting author: Donat Kögel

Cancer type-specific effects of proteasome inhibitors: Implications for reactivation of apoptosis in brain tumors

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The proteasome has emerged as a novel target for antineoplastic treatment of hematological malignancies and solid tumors, including those of the CNS. To identify cell death pathways activated by PIs, we treated human SH-SY5Y neuroblastoma cells and a panel of malignant glioma cell lines with the proteasome inhibitors (PIs) MG132 and epoxomicin. PIs were capable to induce release of cytochrome c from the mitochondria and activation of caspases in SH-SY5Y cells. Analysis of global gene expression revealed that PIs triggered transcriptional activation of the BH3-only gene PUMA. Further characterization of transcriptional responses and apoptosis demonstrated that the p53/PUMA pathway plays an important role in the sensitivity of neuroblastoma and colon cancer cells to cell death induced by PIs. In contrast to neuroblastoma and colon cancer cells, glioma cells did not reveal PI-dependent induction of PUMA and apoptosis, indicating that combined therapeutic approaches with other apoptosis inducers such as death ligands are required to efficiently induce cell death in this type of cancer. Analysis of six glioma cell lines revealed that only two of them were sensitive to TRAIL. However, apoptosis could be efficiently potentiated in TRAIL-resistant glioma cells with the BH3 mimetics BH3-I2´ and HA14-1, and even more potently with PIs. Reactivation of TRAIL-induced apoptosis by PIs did not depend on functional p53, but depended on JNK/c-Jun-dependent activation and enhanced surface expression of death receptor DR5. Our data suggest that PIs (re)activate apoptosis at multiple levels of cell death signalling in a cancer type-specific manner. Novel therapeutic approaches using TRAIL or agonistic TRAIL receptor antibodies in combination with PIs may represent a promising approach to reactivate apoptosis in therapy-resistant cancers such as high grade gliomas.

Keywords: astrocytoma, proteasome, apoptosis, death receptor

Presenting author: Violeta Lamarca

Two isoforms of the mitochondrial outer membrane protein PSAP/Mtch1 share two independent proapoptotic domains.

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Presenilin 1-associated protein, also known as Mitochondrial Carrier Homolog 1, is a mitochondrial protein that induces apoptosis when overexpressed in cultured cells (JBC, 277:48913-48922, 2002). There are two ubiquitously expressed isoforms of PSAP/Mtch1, generated by alternative splicing, that differ in the length of a hydrophilic loop located between two predicted transmembrane domains. Although PSAP/Mtch1 contains a mitochondrial carrier domain conserved in several inner membrane carriers, our results indicate that it is an outer membrane protein. Import of outer membrane proteins is not fully understood yet. In this work we show that PSAP/Mtch1 contains multiple mitochondrial targeting motifs dispersed along the protein, but a transmembrane domain in the correct position and orientation is needed for correct membrane insertion. Two independent regions in the amino terminal side of the protein are responsible for its proapoptotic activity.

Keywords: PSAP/Mtch1, mitochondria, apoptosis, presenilin

Presenting author: Leila Laredj

PML nuclear domains are transducers of DNA injury stimuli

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Promyelocytic leukaemia nuclear domains (PML-NDs) are intra-nuclear macromolecular protein complexes that appear to regulate multiple cellular processes. Most mammalian nuclei contain 10 -20 PML-NDs, each around 0.5 microns in diameter, comprising a shell of sumoylated, polymerised PML protein and several species of cargo protein. Their relevance in carcinogenesis is attested by two key observations. First, promyelocytic leukaemia cells have lost the ability to form PML-NDs, but if this is restored experimentally, there is reversion to normal phenotype. Second, PML knock-out animals, although live-born, are cancer-prone. Cells from such knock-out animals show diminished apoptosis following exposure to many inducers of cellular injury, including ionising radiation (IR).

Here we show that PML-NDs vary in size, number and position in response to IR. Close contact is established between PML-NDs and the radiation-induced repair foci (identified by their gamma-H2AX content) some 4 - 8h after injury. Untransformed human fibroblasts (eg WI38) and colorectal cancer epithelial cells (HCT116) differ in the reversibility of these changes, but in both cell types the quantitative changes in PML-NDs correlate closely with ultimate cell fate. The kinetics of the PML-ND response to IR are sensitive to the presence of the DNA damage signal transducers ATM and CHK2. New methods will also be described for analysis of the changes in PML cargo protein following DNA damage.

PML-NDs may audit residual DNA damage and so participate in the still poorly understood decision between apoptosis and survival following IR.

Keywords: PML, DNA damage, cell fate, CHK2

Presenting author: Giagkos Lavranos

The normal apoptotic wave in the developing rat prostate is suppressed in castrated male rats

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Aim: Estimation of the role and extend of apoptosis in the fetal and neonatal rat prostate gland. Observation of the effect of post-castration androgen depletion in the regulation of prostate development.

Materials and Methods: 200 male Wistar rats from the Athens Pasteur Institute colony were separated in 2 equal groups. The first half was subjected to bilateral surgical castration at the 6th day after birth, while the remaining animals served as the control group. Afterwards, the prostate was explanted and weighted in both groups at the age of 7, 14, 21, 28, 42 and 56 days after birth. The dissected tissue was histologically processed and sections from both the control and castrated group were stained with hematoxylin/eosin. Immunohistochemical detection of PCNA, a marker of cell proliferation and, Bax, an apoptotic marker, was performed in the selected age groups. The results were compared with those calculated via RT-PCR and Western blot.

Results: Mean prostate weight was higher in the normal group, compared to the castrated animals, for all age groups examined. This difference was statistically significant with a p value < 0.05 for animals aging 21 days or more. Using the PCNA antibody, a higher labeling index was observed for normal subjects (66.67, 34.69, 56.6, 9.84, 6.43, 9.84 versus 28.7, 20.32, 18.42, 8.5, 5.5, 1.54 %) in all age groups. From a statistical point of view, this difference was very significant ($p<0.001$). Finally, Bax labeling was 3.4-3.5 % for normal rats contrary to 1.5 and 0.8% for castrated animals, at 28 and 56 days of age, respectively.

Discussion: Androgen depletion results in rapid prostate degeneration, a result observed both macroscopically and microscopically. The study of cell proliferation stresses the subsequent decrease in cell survival in the castrated group. However, it appears that this reaction also deregulates a normal process of cell selection, which is represented by the apoptotic wave observed in normal subjects at 28 days onwards. On the other hand, such an adaptive mechanism is minimized in the castrated population.

Support: The project was funded by the European Social Fund and National Resources-(EPEAEK II) PYTHAGORAS (grant "Pythagoras" 70/3/7361)

Keywords: prostate, apoptosis, castration, androgens

Presenting author: Christophe Le Clorennec

Overexpression of Epstein-Barr virus latent membrane protein 1 (LMP1) in EBV Latency III B-cells induces ligand independent autoactivation of CD95 and caspase 8 mediated apoptosis.

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EBV oncoprotein LMP1 has been described as the major transforming protein in various cell types, by rerouting the TNF-receptor family signalling pathway. Despite this undoubtedly role in EBV-associated transformation of cells, toxicity of LMP1 is a well known experimental feature, may be because it contradicts the dogma of its transformation power. This phenomenon may be important to understand in the view of natural immune control of the EBV burden in the immunocompetent host (Le Clorennec, 2006). To understand the toxic effect of LMP1 in LCLs, we cloned wild type LMP1 into a novel double inducible episomal vector pRT-1. We found that over-expression of LMP1 induce over-expression of CD95, CD95 aggregation, activation of caspase 8, 9 and 3, followed by late stages of apoptosis (PARP cleavage, sub-G1 peak). Immunoprecipitation of CD95 and caspase 8 suggested that LMP1 induction was associated with formation of CD95-dependant DISC. Induction oxygen species (OS) has been shown to induce aggregation and autoactivation of CD95. We found that inhibition of the formation of OS by catalase resulted in a decrease of LMP1-dependant apoptosis. We also show that induction of latency III is associated with induction of OS. Therefore, our results are in agreement with a model according which (i) EBV latency III increases the pool of OS of the infected B-cell, rendering the cell permissive to CD95 auto-activation, (ii) increasing levels of LMP1 induces both expression of the pro-apoptotic CD95 and survival of the cell through NF-kB activation, and (iii) increasing CD95-autoactivation due to inappropriate over-expression of LMP1 finally overwhelms the antiapoptotic effect of NF-kB. From this model, it is expected that at the single cell level, LMP1 expression results from an equilibrium between NF-kB dependant survival of cells and CD95-dependant apoptosis, explaining the heterogeneity of LMP1 expression from cell to cell and pointing a new mechanism of regulation of LMP1.

Keywords: LMP1, APOPTOSIS, CD95, CASPASE

Presenting author: Christophe Lemaire

Chemosensitization by Knockdown of Adenine Nucleotide Translocase-2

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Mitochondrial membrane permeabilization (MMP) is a rate-limiting step of apoptosis, including in anticancer chemotherapy. Adenine nucleotide translocase (ANT) mediates the exchange of ADP and ATP on the inner mitochondrial membrane in healthy cells. In addition, ANT can cooperate with Bax to form a lethal pore during apoptosis. Humans possess four distinct ANT isoforms, encoded by four genes, whose transcription depends on the cell type, developmental stage, cell proliferation, and hormone status. We previously showed that the *ANT2* gene is up-regulated in several hormone-dependent cancers. Here, we demonstrated that knockdown of ANT2 by RNA interference (i) induced no major changes in the aspect of the mitochondrial network or cell cycle but provoked minor increase in mitochondrial transmembrane potential and reactive oxygen species level and (ii) reduced intracellular ATP concentration without affecting glycolysis. At expression and functional levels, ANT2 depletion was not compensated by other ANT isoforms. Most importantly, ANT2, but not ANT1, silencing facilitated MMP induction by lonidamine, a mitochondrion-targeted antitumor compound already used in clinical studies for breast, ovarian, glioma, and lung cancer as well as prostate adenoma. The combination of ANT2 knockdown with lonidamine induced apoptosis irrespective of the Bcl-2 status. These data identify ANT2 as an endogenous inhibitor of MMP and suggest that its selective inhibition could constitute a promising strategy of chemosensitization.

Keywords: Apoptosis, mitochondria, chemotherapy, ANT

Presenting author: Anthony Lemarie

specific inhibition of mitochondrial respiratory chain complex II for apoptosis induction.

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Through a genetic high-throughput screen, our group isolated various proapoptotic genes, notably SDHC (Succinate Dehydrogenase subunit C) and SDHD, two membrane-anchoring components of mitochondrial respiratory chain complex II (succinate:ubiquinone oxidoreductase or SQR). This complex is also a component of the tricarboxylic acid cycle (TCA) that catalyzes the oxidation of succinate to fumarate by the SDHA subunit (SDH activity). Our previous study[1] demonstrated that SDHC, a tumour suppressor protein in humans, is part of a major apoptosis sensor, since numerous anti-cancer drugs inhibit complex II for apoptosis induction. In the present study, we have addressed the biochemical nature of complex II inhibition leading to apoptosis.
We measured in parallel the SDH activity and the total SQR activity through ubiquinone reduction in Hela cells treated with different anti-cancer drugs and showed that drug-triggered apoptosis is preceded by a inhibition of the SQR activity without alteration of the SDH activity. We also assessed the effects of two direct inhibitors of complex II: 3-nitropropionate (3NP), which inhibits SDH activity at the SDHA subunit, and 2-thenoyltrifluoroacetone (TTFA), which blocks the ubiquinone-binding site in the SDHC/SDHD subunits. We observed that 3NP induced an equal inhibition of both SDH and SQR. In contrast, TTFA led to an inhibition of SQR but did not impair SDH confirming that this inhibitor acts downstream of SDHA and only at the ubiquinone-binding site. Importantly, as stated above, anti-cancer drugs had the same differential effect on SDH and SQR activity. In line with that TTFA, but not 3NP, was able to induce apoptosis in a time- and dose-dependent manner. This cell death was accompanied by superoxide production, prior to apoptosis. Both events can significantly be inhibited by pre-treatment with various antioxidants. Moreover, pre-treatment with specific SDH inhibitors or TCA inhibitors can significantly reduce TTFA-induced superoxides and apoptosis.
So far, our results suggest that TTFA or numerous anti-cancer drugs lead to specific inhibition of complex II for ubiquinone reduction in the absence of SDH inhibition, allowing an electron leak to molecular oxygen between these two enzymatic sites, which leads to superoxide production and apoptosis.

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Keywords: apoptosis, anticancer drugs, mitochondrial respiratory chain, complex II

Presenting author: Yee-Shin Lin

Pathologic role of streptococcal pyrogenic exotoxin B antibodies in the poststreptococcal rheumatic heart disease

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An immune-complex pathogenesis is thought to be involved in the rheumatic heart disease following group A streptococcal infections. Molecular mimicry between streptococcal and host antigens is one of the mechanisms for post-infectious autoimmune disease. Our previous studies showed that antibodies against streptococcal pyrogenic exotoxin B (SPE B) play a role in the pathogenesis of glomerulonephritis in a mouse model. In this study, we investigated whether anti-SPE B antibodies are also involved in rheumatic heart disease pathogenesis. We generated a panel of monoclonal anti-SPE B antibodies and found that one of these clones, 10G, showed cross-reactivity with endothelial cells, mouse heart tissue, and human valve. 10G monoclonal antibodies induced endothelial cells to undergo apoptosis. We further found a dominant epitope recognized by 10G at SPE B amino acid 296-310 residues. The binding activity of 10G to endothelial cells was inhibited by preabsorption with this epitope. The titers of anti-SPE B antibodies are significantly higher in the rheumatic heart disease patient sera as compared with those of the normal control sera. The anti-SPE B antibody titers in the rheumatic heart disease patient sera are positively correlated with the endothelial cell-binding activity. Therefore, anti-SPE B antibodies play a role in group A streptococcus-induced rheumatic heart disease. These antibodies could bind to endothelial cells and induce apoptotic cell death, and the dominant epitope at SPE B was mapped.
(This work is supported by grant NHRI-EX96-9429SP from the National Health Research Institutes.)

Keywords: streptococcal pyrogenic exotoxin B, rheumatic heart disease, endothelial cell apoptosis

Presenting author: Saskia Lippens

Caspase-14 protects against epidermal UVB photo-damage and water loss

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Caspase-14 belongs to a conserved family of aspartate-specific proteinases. It is a short prodomain caspase whose expression is almost exclusively restricted to the suprabasal layers of the epidermis and the hair follicles. Moreover, proteolytic activation of caspase-14 is associated with stratum corneum formation. In order to determine the role of caspase-14, we generated caspase-14 deficient mice. The skin of these was shiny and lichenified, indicating an altered stratum corneum composition. However, no major histomorphological or immunohistochemical differences were observed. Electron microscopic analysis revealed a significantly higher number of alveolar keratohyalin F-granules, the profilaggrin stores, in the caspase-14 deficient epidermis. Accordingly, caspase-14 deficient epidermis is characterised by an altered profilaggrin-processing pattern and by reduced skin hydration levels and increased water loss. We show that recombinant caspase-14 can directly cleave profilaggrin *in vitro*. Importantly, the skin of caspase-14 deficient mice was highly sensitive to the formation of cyclobutane pyrimidine dimers upon UVB irradiation, leading to increased levels of UVB-induced apoptosis. Our data indicate that the UVB filtering capacity of the stratum corneum is reduced in the absence of caspase-14. Taken together, these results demonstrate that caspase-14 deficient mice can serve as a model to better understand the pathways that lead to proper development of skin barrier functions preventing water loss and protecting against UVB light.

Keywords: caspase-14 skin

Presenting author: Andrew Logan

The role of c-FLIP in colorectal cancer chemoresistance

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Death receptors, such as Fas and TRAIL, trigger death signals when bound by their natural ligands, principally through the activation of caspase 8. Subsequent to ligand binding, an adaptor protein FADD is recruited to the intracellular death domain (DD) of the death receptor via homotypic interactions. FADD also contains a second protein-protein interaction domain at its N-terminus known as the death effector domain (DED). This domain permits interaction with other DED containing molecules such as caspase 8 leading to formation of the death inducing signalling complex (DISC). Binding of caspase 8 molecules to the DISC results in dimer formation and catalytic cleavage via a two-step mechanism. Through this mechanism caspase 8 is activated at the DISC. A key regulator of death receptor-mediated apoptosis is c-FLIP, which prevents the activation of caspase 8 at the DISC. Silencing of c-FLIP using siRNA has been shown to sensitize a panel of colorectal cancer cell lines to chemotherapy-induced apoptosis. Conversely the overexpression of c-FLIP has been shown to abrogate chemotherapy-induced apoptosis. c-FLIP has therefore been implicated as a potential therapeutic target in the treatment of colorectal cancer. Two splice variants of this protein, c-$FLIP_L$ and c-$FLIP_S$, have been shown to inhibit death receptor-mediated apoptosis by forming a proteolytically inactive heterodimer with caspase 8. c-$FLIP_L$ and c-$FLIP_S$ both have two DEDs, which include a conserved motif RXDL which is thought to be important in interacting with FADD and caspase 8. The aim of the project is to generate c-FLIP motif mutants to map interaction with caspase-8 and FADD. Disruption of these interactions would be expected to prevent c-FLIP recruitment to the DISC. c-$FLIP_L$ also contains a C-terminus caspase-like domain, which has a tyrosine residue in the place of the caspase cysteine residue rendering it proteolytically inactive. c-FLIP's catalytic inactivity presents a challenge in targeting it as a therapeutic target. The second aim of the project is to investigate c-FLIP binding to novel candidate proteins identified in a Y-2-H screen with the goal of identifying novel regulators of c-FLIP function. The significance of candidate proteins RACK1 and Ku70 are being examined in relation to c-FLIP activity and function. The targeting of c-FLIP may be a promising therapeutic approach for the treatment of colorectal cancer.

Keywords: c-FLIP, caspase 8, apoptosis, DISC

Presenting author: Monica Lopez-Guerra

A selective Ikappa B kinase inhibitor induces apoptosis and enhances the antitumoral effect of different chemotherapeutic drugs in chronic lymphocytic leukemia cells

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Chronic lymphocytic leukemia (CLL) is a clinical heterogenous entity derived from the monoclonal expansion of long-life B-lymphocytes with low proliferative potential. Constitutive activation of the NF-kappaB pathway has been implicated with the pathogenesis of this malignancy. Thus, inhibition of NF-kappaB might be useful to antagonize different target genes, involved in the adverse biology of CLL. BMS-345541 (4(2'-aminoethyl)amino-1,8-dimethylimidazo(1,2-a)quinoxaline) is a highly selective inhibitor of IKK that inhibits NF-kappaB-dependent transcription of pro-inflammatory cytokines both in vitro and in vivo. The compound appears to bind to an unidentified allosteric binding site of the IKK catalytic subunits.
Our results demonstrated that this specific Ikappa B kinase inhibitor induced apoptosis in primary CLL cells, at doses ranging from 2 to 5 microM, independently of p53 status or ZAP70 expression. Indeed, following IκB and p65 dephosphorylation, BMS-345541 led to induction of apoptosis, characterized by a time-dependent Bax and Bak conformational activation, mitochondrial depolarization, phosphatidylserine exposure and caspases activation. However, pretreatment with the pancaspase inhibitor z-VAD.fmk didn't abrogate completely the lethal phenotype, suggesting that the BMS-345541 apoptotic signaling activates both caspase-dependent and caspase-independent factors. Furthermore, apoptosis induced by this inhibitor was accompanied by downregulation of NF-kappaB dependent antiapoptotic genes, being the most clearly target affected cFLIP. Interestingly, BMS-345541 sensitized CLL primary cells to TRAIL and enhanced the antitumoral effect of different chemotherapeutic drugs, such as mitoxantrone or dexamethasone.
Altogether, these findings indicate that NF-kappaB inhibition represents a promising field for the determination of further potent antitumoral strategies in the treatment of CLL patients.

Keywords: NFkappaB , CLL , apoptosis

Presenting author: Nuria López-Royuela

Rapamycin potentiates glucocorticoid-induced apoptosis in human myeloma cells by increasing Bim and Puma levels and enhancing caspase activation

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Glucocorticoids are widely used in anti-myeloma therapy and their apoptotic action can be potentiated by rapamycin, a mTOR inhibitor. However, the molecular mechanisms underlying glucocorticoid-induced apoptosis, alone or in combination with rapamycin remain poor characterized. We show here that dexamethasone (DEX) induce apoptosis in human MM cell lines characterized by Bax and Bak conformational changes, $\Delta\Psi_m$ loss, cytochrome c and AIF release, caspase activation and PS exposure. Co-treatment with the pan-caspase inhibitor Z-VAD-fmk prevented PS exposure and most of apoptotic morphology development, but only partly reduced cell death. RT-MLPA and Western blot analysis showed up-regulation of Bim at mRNA and protein levels. Treatment with siRNA for Bim fully prevented DEX-induced apoptosis, indicating Bim was the key mediator of DEX-induced apoptosis. Treatment with rapamycin alone caused growth arrest in G_1/S, increase in Puma and Bcl-2 mRNA and protein levels and decrease in those of surviving. Rapamycin strongly potentiated DEX-induced caspase activation and apoptosis, which occurred with increasing levels of Bim, Puma and Bcl-2. Gene silencing of Bim reduced DEX-induced apoptosis, more strongly in case of combined treatment with Rapamycin. Combined gene silencing of Bim and Puma with the corresponding siRNAs abrogated toxicity. These results show up to Bim and Puma as the key mediators of DEX-induced apoptosis in myeloma cells and provide a molecular explanation for the observed potentiating effect of rapamycin.

Keywords: Dexamethasone, Rapamycin, Multiple Myeloma

Presenting author: Erica Lorenzon

The extracellular matrix glycoprotein EMILIN2 triggers apoptosis via the extrinsic apoptotic pathway

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EMILINs are a family of extracellular matrix (ECM) glycoproteins characterized by the presence of an EMI-domain at the N-terminus and a gC1q-like domain at the C-terminus. EMILIN1, the archetype molecule of the family, is involved in elastogenesis and hypertension etiology whereas EMILIN2 function has so far not been uncovered. Here we provide evidence that EMILIN2 is a pro-apoptotic molecule and significantly reduces the viability of different tumor cell lines. The mechanism of action involves direct binding of EMILIN2 to the death receptor DR4 and partially to DR5. Binding to death receptors triggers receptor clustering, co-localization with lipd rafts, DISC assembly and caspase activation. Thus EMILIN2 bears the unique feature for an ECM molecule to mimic the activity of TRAIL, the natural ligand for these receptors. The use of different deletion constructs allowed the identification of the region responsible for the activation of the extrinsic apoptotic pathway. Moreover different deletion constructs of the DR4 receptor identify the portion responsible for the binding to EMILIN2.
Moreover EMILIN2 is a negative regulator of tumor growth *in vivo*: fibrosarcoma cell lines over-expressing EMILIN2 develop smaller tumors in nude mice compared to mock-transduced cells. Taken together these data demonstrate an unexpected direct and functional interaction of an ECM constituent with death receptors and implies further unknown regulative mechanisms of the tumor microenvironment.

Keywords: EMILIN2, apoptosis, death receptors

Presenting author: Sofie Lust

Xanthohumol: a new potential proteasome inhibitor that activates the pro-apoptotic arm of the unfolded protein response in B-CLL cells

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B-Chronic Lymphocytic Leukemia (B-CLL) is incurable by current methods because of increasing resistance to chemotherapy. Therefore, new options are needed for the treatment of CLL. Proteasome inhibitors represent a potential therapeutic strategy. One such agent, bortezomib, was described to promote apoptosis in CLL cells. Moreover, bortezomib has been shown to induce ER stress and unfolded protein response (UPR) in refractory multiple myeloma. The UPR is mainly a self-protective mechanism activated when protein folding is disrupted and misfolded proteins are accumulating in the ER. However, sustained ER stress eventually leads to cell death. The three major ER sensors are ATF6, PERK and IRE1, normally bound to the chaperone BiP. Upon ER stress, BiP is released from the luminal domain of the three ER stress transducers. Pro-survival signals are delivered via IRE1 (XBP1) and ATF6 while pro-apoptotic signals are generated via PERK (CHOP).

We have previously demonstrated that the hop-derived flavonoid Xanthohumol (X) was capable of killing B-CLL cells in a dose- and time-dependent way as evidenced by PARP cleavage and Annexin V staining (Lust et al., 2005). However, the mechanism behind this observation was unknown. In this study we demonstrate that X is a new potential proteasome inhibitor which induces apoptosis of B-CLL cells at least partially via activation of ER stress and the UPR. Upon treatment of freshly isolated B-CLL cells with X, the expression levels of BiP and Hsp70 were upregulated, indicative for ER stress. Apart from a prolonged phosphorylation of eIF2α, suggestive for PERK activation, X stimulated the splicing of XBP1 mRNA while ATF6 seemed not to be involved. Activation of PERK was associated with a pro-apoptotic outcome as demonstrated by upregulation of CHOP, generation of ROS, downregulation of the anti-apoptotic proteins Mcl-1, Bcl-x_L, Bcl-2, cleavage of PARP, and processing of caspase-3. Moreover, we demonstrate that X was able to inhibit the 20S proteasome activity which may explain the induction of ER stress and the activation of the UPR in B-CLL cells.

Our results further suggest that tackling organelles like the proteasome and the ER is a valuable strategy in treatment of B-CLL.

Keywords: CLL, UPR, ER stress, proteasome inhibitor

Presenting author: Agnieszka Maciag-Gudowska

S.aureus cytoprotective effect on monocyte-derived macrophage cell death

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Macrophages, as well as neutrophiles, are known to be essential to innate host defense against invading microorganisms. It has been previously reported that infection of macrophages by different bacterial pathogens often induce programmed cell death (PCD) of long-lived leukocytes which leads to pathogen killing and clearance of infection or elimination of macrophages from the side of inflammation.

Staphylococcus aureus considered to be an extracellular pathogen is able to invade a variety of mammalian non-professional phagocytes but can also survive engulfment by professional phagocytes such as neutrophils and monocytes. In both of these cell types *S. aureus* promptly escapes from the endosomes/phagosomes and proliferates within the cytoplasm, which quickly leads to host programmed cell death - apoptosis. Based on this information we focused on *S. aureus* (Newman strain) induced programmed cell death analysis in hMdM. Our findings indicate that macrophages infected by *S. aureus* do not undergo apoptosis. At numerous time points several tests were applied to detect apoptosis including PARP cleavage, chromatin condensation (DAPI staining), DNA fragmentation (TUNEL and DNA laddering) and microscopic examination for host cell plasma membrane blebbing, each of which were negative, despite initial transient phosphatidyl serine exposure on the cell surface (annexin V binding) and procaspase 3 activation.

In addition, *Staphylococcus aureus* seems to modulate the sensitivity of host macrophages to undergo staurosporine-induced apoptosis. Following stimulation with staurosporine and other potential inductors of apoptosis (butyric acid, anti-Fas antibodies) high activity of executive caspase 3 was detected but surprisingly this effect was significantly reduced in macrophages after previous *S.aureus* phagocytosis.

Taken together these data indicate that *S. aureus* may be able to manipulate macrophages in different manner compared to the other cell types in which it triggers apoptosis. Our findings suggest that *S.aureus* cytoprotective effect on monocyte-derived macrophages can prevent host cell elimination, allows intracellular bacterial survival and due to this contribute to dissemination of the infection and may be detrimental to the host.

Keywords: S.aureus, hMdM, apoptosis

Presenting author: Ashley Mackey

Redox survival signalling in retina-derived 661W cells

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Reactive oxygen species have long been implicated in processes involving cellular damage and subsequent cell death, especially in organs such as the eye that are constantly exposed to excitatory signals. However, recent studies have shown that oxidant species can also act as intracellular signalling molecules promoting cell survival, but very little is known about this in the retina. The present work demonstrates for the first time that hydrogen peroxide is generated rapidly and acts as a pro-survival signal following several apoptotic stimuli in retina-derived 661W cells and in the retina ganglion cell line RGC-5. Concentrating on 661Ws and serum deprivation, we systematically investigated pro-survival and pro-death pathways and discovered that the rapid and transient burst of hydrogen peroxide activates survival pathways, such as AKT. Subsequently, activation of the apoptotic machinery only takes place following a decline of hydrogen peroxide to basal levels. To substantiate this pro-survival role, we inhibited the oxidant burst, which exacerbated cell death. Furthermore, maintaining the oxidant signal using exogenous hydrogen peroxide enhanced cell survival. Finally, we demonstrate that the oxidant burst is not an event exclusive to cell systems but that is also present in retinal explant cultures, implying a greater physiological relevance. Overall, the results presented in this study provide an exciting insight into the role that rapid hydrogen peroxide production plays in the response of retinal cells to apoptotic stimuli.

Keywords: Apoptosis, Hydrogen Peroxide,ROS, Survival

Presenting author: Louisa Mahaira

Anavex 7-1037, a novel small synthetic sigma ligand, induces apoptosis in human HCT116 colon cancer cells in vitro and suppression of HCT116 tumor growth in SCID mice

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Sigma receptors are unique drug-binding proteins that are present in the central nervous system as well as in various peripheral tissues. It has been shown that sigma receptor subtypes are highly expressed in tumor cell lines from various tissues. Their high density in various tumor cell types and particularly in proliferating cells, makes sigma receptors potential target for diagnostic imaging as well as therapeutic agents. Anavex 7-1037 [chlorohydrate of 5-(Tricyclo[3,3,1,1^{3,7}]dec-1-yl)-dihydro-3-(dimethylamino-methyl)-5-phenylfuran-2(3H)-one is a synthetic compound with low molecular weight exhibiting high affinity for sigma-1 (nanomolar) and moderate (micromolar) affinity for sigma-2 and sodium channels. The compound was tested initially *in vitro*, against HCT116 human colon cancer cells and found to exhibit a significant anticancer activity by inducing *anoikis* in a dose and time dependent manner. In a short term (2-days 5-dose, SRB) assay the compound exhibited activity at low micromolar range, while in a long term (14-days 3-dose, clonogenic) assay activity was found to be below 1μM. Anavex 7-1037 induced initially detachment of cells as early as 6 hours after the addition of 20μM to the culture and subsequent apoptosis that was accompanied by activation of both caspases -8 and -9 and subsequent activation of caspase 3 as this was estimated by FACS and western blot analysis. BrdUdr incorporation assays revealed that the compound blocked DNA synthesis and suggested that apoptotic cells may originate from the S-phase of the cell cycle. Anavex 7-1037 was further administered i.p. to SCID mice bearing HCT116 tumors (xenografts) at a concentration of 100mg/kg for 5 days/week, for a 3 week period and found to cause growth delay with a %ΔT/ΔC lower than 40%, suggesting a significant *in vivo* anticancer activity against HCT116 xenografts, according to NCI criteria (%ΔT/ΔC<42%). These results were further confirmed by detecting the presence of the cell proliferation-associated nuclear protein, Ki67, in thin tumor sections, by immunohistochemistry. In a nutshell, the present work suggests that Anavex 7-1037 may possess chemotherapeutic potential for the treatment of colorectal and probably of other types of human cancer.

Keywords: sigma ligand, colon cancer, xenografts

Presenting author: Hayat Mahmud

Targeted induction of tumor cell death by an immunotoxin-like antibody fusion protein carrying human apoptosis inducing factor (AIF)

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Recombinant immunotoxins consisting of a tumor-specific antibody domain and a bacterial toxin have demonstrated potent antitumoral activity in clinical studies. However, prolonged therapy can be complicated by the development of neutralizing antibodies against the toxin domain. Therefore attempts are being made to design less immunogenic molecules by employing cell-death inducing proteins of human origin, such as the human serine protease granzyme B of cytotoxic lymphocytes [1,2]. Apoptosis inducing factor (AIF) may represent another pro-apoptotic effector protein suitable for the generation of immuno toxin-like molecules. AIF is a mitochondrial flavoprotein with NADH oxidase activity that has a vital function in healthy cells. In addition, in many models of apoptosis AIF translocates from the mitochondria to the cytosol, from where it can enter the nucleus and induce chromatin condensation and DNA fragmentation.

Here we have generated a truncated AIFΔ1-100 derivative which lacks the mitochondrial import signal. Bacterially expressed AIFΔ1-100 protein was functionally active and induced apoptotic cell death upon microinjection into Vero cells. For specific targeting to tumor cells, AIFΔ1-100 was genetically fused to an ErbB2-specific scFv(FRP5) antibody fragment and the non-toxic translocation domain of *Pseudomonas* exotoxin A as an endosome escape activity. ErbB2 (HER2) is a receptor tyrosine kinase that transmits important growth and survival signals, and is overexpressed by many human tumors of epithelial origin. In FACS analysis, the resulting scFv(FRP5)-E-AIFΔ1-100 protein displayed enhanced binding to ErbB2-overexpressing breast cancer cells, but to a lower degree also to ErbB2-negative controls. Untargeted AIFΔ1-100 displayed similar binding to ErbB2-negative cells, most likely due to electrostatic interactions of positively charged AIF with negatively charged molecules on the cell surface. In microscopic assays, scFv(FRP5)-E-AIFΔ1-100-treated ErbB2-expressing renal carcinoma cells (Renca-lacZ/ErbB2) displayed apoptotic morphology very similar to staurosporine-treated controls, whereas isogenic ErbB2-negative Renca-lacZ cells remained unaffected. Concentration-dependend cell killing activity of scFv(FRP5)-E-AIFΔ1-100 could be confirmed in MTT cell viability assays. In this case at high protein concentrations a less pronounced antiproliferative effect was also observed towards ErbB2-negative targets.

Our preliminary results demonstrate that recombinant AIFΔ1-100 retains its apoptosis-inducing activity and can be targeted to ErbB2-expressing tumor cells via fusion to a scFv antibody domain. Ongoing experiments aim at a more detailed analysis of the cell binding and apoptosis-inducing activities of scFv(FRP5)-E-AIFΔ1-100 and strategies to reduce antigen-independent, AIF-mediated cell binding.

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Keywords: AIF, cancer therapy

Presenting author: Fayaz Malik

Withanolide induces apoptosis in HL-60 through mitochondrial dysfunction, caspase activation and NFkB suppression

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Withania somnifera has found several therapeutic uses in Ayurveda. It contains several withanolides of which Withaferin-A (WA) predominantly found in the leaves is reported for its anti-angiogenesis and cancer cells cytotoxicity. We report here the molecular mechanisms involved in cell cytotoxicity by withaferin-A. The withanolide stimulates early mitochondrial potential loss in human leukaemia HL-60 cells, which is responsible for induction of apoptosis measured by several end-points of apoptosis, viz. increased sub-Go DNA fraction, DNA fragmentation by Tunnel assay, phosphatidylserine externalisation, altered nuclear morphology and PARP cleavage. The decision to trigger cell death appeared to reside in mitochondrial because the withanolide brings about early generation of ROS and loss of mitochondrial potential with subsequent translocation of Bax to mitochondria, release of cytochrome c into the cytosol, a point of no return for cell death. Withanolide time dependently induce caspases 3, and –9 activation in HL60 cells. Withanolide inhibited the DNA binding of the transcription factor NF-ĸB was measured by EMSA and cause nuclear cleavage of p65/Rel. withanolides also activate the extrinsic pathway by enhancing the expression of TNF-R1 and caspase 8. The studies demonstrate that withaferin-A induces apoptosis by activation of mitochondrial -independent and -dependent pathways.

Keywords: Apoptosis, Mitochondrial Membrane potential, Caspase, NFkB

Presenting author: Eleonora Marrazzo

ΔNp73β is involved in mitotic catastrophe

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The p73 locus gene has a complex structure encoding a plethora of isoforms. The different ΔN truncated isoforms of p73 may exert different activities depending on the cellular context. The β isoform of ΔNp73 seems to have a particular pattern of action even if its role in cell cycle and mitosis is still under investigation. To gain further knowledge of ΔNp73β's function, we investigated the effects of its over-expression in tumor cellular models, using the tetracycline-inducible expression system. In the human lung carcinoma cell line H1299, ΔNp73β over-expression resulted in suppression of cell growth and in cell death. Surprisingly stable over-expression of ΔNp73β impaired the genomic stability of tumor cells, leading to the formation of tetraploid cells. The cells become enlarged and multinucleate, with incorrect mitotic figures, and died by apoptotic-independent pathways. Our data suggest that ΔNp73β-induced aberrant mitosis evades the control of the mitotic spindle assay checkpoint, leading to tetraploidy and cell death through mitotic catastrophe rather than apoptosis. The various C-terminal regions of ΔNp73 may influence the final cellular phenotype and we assume that the β one in particular could be an important in both cell growth control and regulation of mitosis.

Keywords: mitotic catastrophe, tetraploidy, genomic instability, mitosis

Presenting author: Wim Martinet

Nitric oxide selectively induces macrophage apoptosis in atherosclerotic plaques via stimulation of endoplasmic reticulum stress

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Rupture-prone atherosclerotic plaques are characterized by a thin fibrous cap containing numerous macrophage-derived foam cells and few smooth muscle cells (SMCs). Decreasing the ratio between macrophages and SMCs might favor plaque stabilization. It has previously been demonstrated that macrophages expressing inducible nitric oxide synthase (iNOS) become hypersensitive to killing by exogenous nitric oxide (NO) donors. In this study, administration of the NO donor molsidomine to cholesterol-fed rabbits (20 weeks 0.3% cholesterol) containing atherosclerotic plaques with iNOS expressing macrophages preferentially eliminated plaque macrophages, but did not affect plaque size. Macrophages and SMCs treated in vitro with the NO donors spermine NONOate or S-nitroso-N-acetylpenicillamine (SNAP) showed several signs of endoplasmic reticulum (ER) stress, including upregulation of C/EBP homologous protein (CHOP), hyperphosphorylation of eukaryotic initiation factor 2α (eIF2α), inhibition of de novo protein synthesis and splicing of X-box binding protein 1 (XBP1) mRNA. These effects were similar in macrophages and SMCs, yet only macrophages underwent apoptosis. Plaques from molsidomine-treated atherosclerotic rabbits showed a 2.7-fold increase in CHOP expression as compared to placebo. Beside NO, selective induction of macrophage death could be initiated with the well-known ER stress inducers thapsigargin and tunicamycin, suggesting that induction of ER stress during treatment with NO donors initiates selective macrophage death. Previous work in our laboratory showed that local administration of the protein synthesis inhibitor cycloheximide can induce macrophage apoptosis in plaques from cholesterol-fed rabbits without influencing the viability and reactivity of SMCs or the endothelium. We therefore assume that not only differential expression of proapoptotic proteins such as CHOP, but also inhibition of translation triggered by phosphorylation of eIF2α may be responsible for NO-induced macrophage death. In conclusion, ER stress after NO treatment leads to selective depletion of macrophages in atherosclerotic plaques, probably via upregulation of proapoptotic proteins and/or inhibition of protein synthesis. NO donors are widely used by patients with coronary artery disease to relief the symptoms of ischemia evoked by atherosclerosis and can be administered safely for many years. Accordingly, NO donors, if necessary in combination with other therapies, would offer new opportunities for a long-term macrophage depletory effect in atherosclerotic plaques, thereby promoting plaque stability.

Keywords: nitric oxide, ER stress, atherosclerosis, macrophage

Presenting author: Olga Martins de Brito

Mitofusin-2 Regulates Juxtaposition of Endoplasmic Reticulum to Mitochondria.

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Juxtaposition between endoplasmic reticulum (ER) and mitochondria is a common structural feature, providing the physical basis for intercommunication during Ca2+ signalling; yet, molecular mechanisms controlling this interaction are unknown. Mitofusin-2 (MFN2), a mitochondrial dynamin-related protein mutated in Charcot-Marie-Tooth type IIa (CMTIIa) is found enriched at the ER-mitochondria interface. Mouse embryonic fibroblasts lacking Mfn2 displayed disrupted ER and loose ER-mitochondria interactions. Mitochondrial Ca2+ uptake in response to stimuli that generate inositol 1,4,5-trisphosphate was reduced, providing evidence that its efficiency is affected by MFN2-dependent tethering to the ER. Selective targeting of MFN2 to the ER complemented morphology of the organelle and its interaction with mitochondria. MFN2 on the ER bridged the two organelles by engaging in homo- and heterotypic complexes with MFN 1 or 2 on the surface of mitochondria. Thus, MFN2 regulates ER-mitochondria juxtaposition, which is required for efficient mitochondrial Ca2+ uptake.

Keywords: ER-mitochondria interaction, Mfn2, Ca2+, CMTIIA

Presenting author: Eva Matalova

Dental apoptosis: caspases behind

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Tooth germs develop as epithelio-mesenchymal organs during mammalian embryogenesis and apoptosis belongs to important morphogenetic events accompanying this process. Based on temporospatial data, many roles of apoptosis have been suggested in odontogenesis, however, the exact mechanisms of dental apoptosis remain unclear. Primary enamel knot – an essential signalling centre of developing teeth – undergoes gradual apoptotic elimination at the bell stage (E15.5 in the mouse) and represents a great model to study dental cell death.

Impact of individual caspase absence on dental apoptosis and tooth shape morphology was investigated in knock-out mice and inhibitor treated mandibular explant cultures. Caspase-8 (extrinsic pathway), caspase-9 (intrinsic pathway), and caspase-3 (central step in both pathways) were in focus. E15.5 knock-out mice were employed to study primary enamel knot (PEK) apoptosis in case of gene deficiency. Explant cultures treated with pharmaceutical inhibitors started at E12.5 and E13.5, respectively, up to 72 h. Apoptosis was evaluated by appearance of apoptotic bodies and TUNEL test, proliferation by PCNA staining in serial histological sections.

In caspase 3 knock-out mice/129X1/SvJ, the location of the first molar tooth germs was shifted posteriorly in the upper jaw. In contrast, in the caspase-3 -/-/B57BL/6, altered morphology of the first molar tooth germs was found in both jaws. In particular, the concave region corresponding to former PEK appeared to be strongly disorganised. No apoptotic bodies were found in the mutant molars and TUNEL labelling showed negative or very weak staining. In caspase-9 deficient mice, the tooth shape corresponded to the wild type, however, no apoptosis was found in PEK, navel, stalk or surrounding tissues. All mutant mice showed the same PCNA staining pattern as the wild type mice.

General inhibition of caspases in tooth germ cultures led to lack of apoptotic bodies and specific DNA fragmentation in the PEK, and confirmed dental apoptosis as caspase dependent. Caspase-3 inhibition caused significant decrease of apoptotic cells within the PEK area after 24 h inhibition. Caspase-9 inhibition blocked caspase-3 activation as showed by immunohistochemistry and a decrease in apoptotic cell numbers was identified. PCNA staining did not detect any differences between experimental and control groups.

Interestingly, despite knock-out and inhibitor mediated block of apoptosis in the PEK, the cells seem to disappear. Compensatory caspase mechanisms and other forms of cell death are therefore aims for the further research.

Thanks to KA Roth and RA Flavell labs for the knock-out mice and COST Action B23, IRP IAPG No. AVOZ 50450515, GAAV (B500450503) for financial support.

Keywords: tooth development, cell death, intracellular pathways

Presenting author: Rada Mateva

The effects of myosmine on the process of apoptosis

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Recently it was shown that nicotine inhibits apoptosis induced by the drugs gemcitabine, cisplatin, and taxol and other drugs. It means that nicotine may function as tumor promotor.
Myosmine, 3-(1-pyrroline-2-yl)pyridine, is a minor alkaloid found in tobacco plants. It was also detected in various edibles and staple foods. Independent from smoking status, myosmine is present in human plasma and saliva. Whereas other tobacco alkaloids such as nicotine and nornicotine have been widely discussed, the mutagenic impact of myosmine has not been investigated in detail. Moreover, there is no data about it's apoptogenic action. Some researchers connect the rapidly increasing incidents of an adenocarcenome of the oesophagus in Europe with the possible cancerogenic activity of the myosmine. The purpose of this study was to determine the effects of myosmine on the process of apoptosis. The type of cell death caused by myosmine was studied on murine leukemia cells. The modulation of apoptosis induced by cisplatin, etoposide, 5-fluorouracil was evaluated.

Keywords: myosmine apoptosis

Presenting author: Laurence Michel

Downregulation of NF-κB canonical pathway by proteasome or IKK2 inhibition induces apoptosis of Cutaneous T Cell lymphoma.

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Severe forms of cutaneous T cell lymphoma (CTCL) - mostly mycosis fungoides in its advanced stages and Sézary syndrome - are associated with a poor response to death-inducing chemotherapeutic agents, including mitochondrial drugs [Michel *et al*, 2003]. The NF-κB signalling pathway has been involved in the physiological regulation of apoptosis in many cell types, and in the resistance of several malignancies to cell death, thus raising this complex as an appealing candidate target in cancer therapy. We previously demonstrated that NF-κB is constitutively activated in CTCL cells and plays a key role in their survival and chemoresistance [Sors *et al*, Blood 2006]. Indeed, selective inhibition of NF-κB nuclear translocation was obtained by overexpression of a non phosphorylable form of IκBα, leading to subsequent apoptosis in CTCL cells. Similarly, the proteasome inhibitor PS-341 (Velcade™) completely reverses the constitutive nuclear translocation of NF-κB and leads to apoptosis induction in both CTCL cell lines and tumor cells from patients with Sézary syndrome. Bortezomib-induced apoptosis of CTCL cells was associated with an up regulation of the pro-apoptotic Bax member of the bcl-2 family, while expression of anti-apoptotic molecules Bcl-x(L) or Bcl-2 was not altered. We further aimed to down regulate more specifically the NFκB canonical pathway by investigating *in vitro* the effects of a pharmacologic inhibition of the IκB kinase (IKK) subunit 2 (IKK2) on CTCL survival and chemoresistance by using the anilinopyrimidine derivative AS602868. Our data showed that the inhibition of IKK2 by AS602868 downregulates constitutive NF-κB activation in tumor cells from SS patients and in CTCL cell lines (HuT-78, SeAx, MyLa), and induces a potent apoptotic cell response. AS602868 presents additive effects with conventional antineoplastic agents such as etoposide for CTCL death induction. Of interest, AS602868 does not significantly alter viability of PBMCs from healthy donors. Proapoptotic effects of AS602868 on CTCL cells are associated with an up regulation of Bax dimers, and a decrease in survivin expression.
Altogether, our results demonstrate that the NF-κB pathway is constitutively activated in CTCL, plays a key role in the resistance of these malignant cells to apoptosis and provide attractive targets for new chemotherapeutic approaches.

Keywords: NF-kB / Cutaneous T lymphoma / Resistance to apoptosis

Presenting author: Jaromi Mikes

p53 status does not affect overall efficiency of photodynamic therapy however influences cell death signaling in colon adenocarcinoma in vitro model HCT-116

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Photodynamic therapy (PDT) represents a new rapidly-developing anticancer approach based on administration of a non- or weakly-toxic photosensitizer and its activation with light of appropriate wavelength. Hypericin, one of the promising photosensitizers, is known to induce apoptosis with high efficiency in various cell line models. It is non-toxic in dark however produces singlet oxygen and superoxide anions with high efficiency when irradiated with light of wavelength around 600 nm.

As reported previously by us alteration of p53 function might play a role in cell death signalling in human colon adenocarcinoma HT-29 cells treated by PDT under variable conditions of hypericin concentration and light dose. To evaluate an impact of p53 status on effectivity of PDT with hypericin, experiments with human colon cancer cells HCT116 p53 wild-type ($p53^{+/+}$) and p53 null ($p53^{-/-}$) had been performed.

Under conditions of single light dose and variable hypericin concentrations insignificant differences in metabolic activity or cell proliferation had been observed. Even a cell cycle distribution in $p53^{+/+}$ as well as in $p53^{-/-}$ cells showed similar trend to arrest cells in G_2/M-phase 24 hrs and arrest in S-phase 48 hrs after PDT. However analysis of nuclear morphology revealed a higher apoptosis incidence in $p53^{+/+}$ cells with no effect to overall toxicity. Consequent analysis of phosphatidylserine externalisation by Annexin V confirmed differences in apoptotic index. Caspase-3 activity analysis and Western blot analysis of pro- and anti-apoptotic members of Bcl-2 family exposed differences in cell death signalling control.

Considering our results we can conclude that even if p53 status alters cell death incidence it does not affect overall toxicity of photodynamic therapy with hypericin and therefore its application might be extended on wider range of malignancies.

Acknowledgements

This study was supported by the Science and Technology Assistance Agency under contract No. APVT-20-003704 and by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic No. VEGA-1/2329/05.

Keywords: photodynamic therapy, PDT, hypericin, p53, apoptosis

Presenting author: Hanna Mild

Cathepsin D deficiency reduces the susceptibility to apoptosis

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During the last decade it has become increasingly clear that other proteases than caspases are important during the apoptotic process. Apoptosis has in many models been shown to be associated with an early lysosomal membrane permeabilisation resulting in release of lysosomal proteases, cathepsins, to the cytosol. The exact proapoptotic effect of cathepsins is not clear, although cleavage of the proapoptotic Bcl-2 family member Bid has been demonstrated. Cathepsin D is one of the cathepsins that have been shown to participate in the apoptotic signalling. In order to investigate the function of cathepsin D we use mouse embryonic fibroblasts of cathepsin D knock out origin. The cells are transfected with the wild type cathepsin D gene or variants with missense mutations causing either inactivation or missorting of the protease. The aim of our project is to elucidate the importance of cathepsin D´s proteolytic activity and cellular location for the apoptotic process using the transfectants. We have started to investigate their susceptibility to apoptosis after exposure to the protein kinase inhibitor staurosporine, the lysosomotropic detergent MSDH or UVB radiation. For all inducers, Wt cells were more susceptible to apoptosis induction than cat $D^{-/-}$ cells. After UVB radiation the caspase-3 activity was significantly lower in cat $D^{-/-}$ cells compared to Wt cells. Our results this far show that cell with cathepsin D deficiency show higher apoptotic resistance. Our forthcoming studies using the different mutated variants will reveal if cathepsin D´s proteolytic activity and normal cellular location is necessary for its proapoptotic function.

Keywords: cathepsin D, apoptosis, lysosomes

Presenting author: Ana Milojkovic

p14^{ARF} regulates cell division cycle checkpoint control and induces cell death via autophagy in the absence of caspase activation

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The tumor suppressor p14^{ARF} is encoded by the human INK4A gene locus and has been shown to play a unique role in regulation of cell cycle arrest and apoptosis following oncogenic stress. To dissect cell death signaling induced by p14^{ARF}, we employed p53 wild type MCF-7 breast carcinoma cells which have lost the key executioner caspase-3 and stably re-transfected these cells with procaspase-3. Expression of p14^{ARF} induced dominant G1 arrest in both caspase-3 deficient mock and caspase-3 re-expressing MCF-7 transfectans. Checkpoint abrogation by the kinase inhibitors caffeine or roscovitine was accompanied by loss of cell viability as determined by the number of PI positive cells in both caspase-3 and mock transfectants. In caspase-3 proficient MCF-7 cells, p14^{ARF} induced apoptosis (type I cell death). This was executed via the mitochondrial pathway as evidenced by breakdown of the mitochondrial membrane potential, cytochrome c release and caspase activation. In contrast, caspase-3 deficient MCF-7 cells died through a non-apoptotic mechanism that was characterized to be autophagic. This type II cell death was visualized by the use of a GFP-tagged light-chain 3 (LC3, ATG6) protein which is concentrated in autophagosomes. Fluorescence microscopic studies revealed a punctuated fluorescence pattern of the GFP-LC3 which was not the case in caspase-3 proficient cells. Inhibition of the DNA damage response by caffeine/roscovitine impaired expression of the CDK inhibitor p21$^{CIP/WAF-1}$ and this is linked to induction of cell death by p14^{ARF}. Thus, loss of p21 facilitates p14^{ARF}-induced apoptosis in caspase-3 dependent manner or autophagy in the absence of efficient executioner caspase activation. These data indicate that cell cycle arrest programs interfere with p14^{ARF} induced apoptosis that strictly depends on caspase-3 and that loss of the executioner caspase-3 facilitates induction of autophagic cell death.

Keywords: apoptosis, caspases, autophagy,cell cycle

Presenting author: Banafsheh Mirnikjoo

Regulation of PS externalization during apoptosis by Ca2+ dependent vesicle fusion

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One of the hallmarks of apoptotic cells is the appearance of phosphatidylserine (PS) at the cell surface which occurs as a result of its redistribution from the cells inner-to-outer membrane leaflet. This provides a recognition signal for recruitment of phagocytes that engulfs the dying cell. Our studies address the mechanisms by which PS translocates across the membrane bilayer. Several papers have shown the importance of Ca^{2+} during apoptosis and have raised the possibility that alterations in Ca^{2+} homeostasis might be related to PS externalization. Our results indicated that chemically-induced increases in cytosolic Ca^{2+} levels trigger concomitant transbilayer PS movements in eukaryotic cells. Using a series of fluorescent lipid probes, we have identified a novel apoptosis- and caspase-dependent vesicle trafficking pathway that provides a significant increase in cytosolic Ca^{2+} levels that result in fusion of endocytoic vesicles with the plasma membrane. This process is inhibited by cytochalasin B, suggesting that microfilaments play a critical role in trafficking of the vesicles to the plasma membrane. Taken together our studies indicate that Ca^{2+} released form intracellular stores during apoptosis triggers specific trafficking to- and fusion of intracellular membranes with the plasma membrane that triggers the reorientation of PS from the cells inner to outer membrane leaflet.

Keywords: Apoptosis,PS, calcium and vesicle

Presenting author: Takashi Momoi

PERK/eIF2alpha-MEDIATED CONSTITUTIVE AUTOPHAGY/LYSOSOME REGURATES ER STRESS-CELL DEATH BY DEGRADING ABNORMAL PROTEIN AGGREGATES AS ERAD-II

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Dysferlin is a type-II transmembrane protein and the causative gene of limb girdle muscular dystrophy type 2B and Miyoshi myopathy (LGMD2B/MM), in which specific loss of dysferlin labeling has been frequently observed. Recently, a novel mutant (L1341P) dysferlin has been shown to aggregate in the muscle of the patient. Little is known about the relationship between degradation of dysferlin and pathogenesis of LGMD2B/MM. Here, we examined the degradation of normal and mutant (L1341P) dysferlin. Wild-type (wt) dysferlin mainly localized to the ER/Golgi, associated with retrotranslocon, Sec61, and VCP(p97), and was degraded by endoplasmic reticulum (ER)-associated degradation system (ERAD) composed of ubiquitin/proteasome. In contrast, mutant dysferlin spontaneously aggregated in the ER and induced eukaryotic translation initiation factor 2 (eIF2) phosphorylation and LC3 conversion, a key step for autophagosome formation, and finally, ER stress cell death. Unlike proteasome inhibitor, E64d/pepstatin A, inhibitors of lysosomal proteases did not stimulate the accumulation of the wt-dysferlin, but stimulated aggregation of mutant dysferlin in the ER. Furthermore, deficiency of *Atg5* and dephosphorylation of eIF2, key molecules for LC3 conversion, also stimulated the mutant dysferlin aggregation in the ER. Rapamycin, which induces eIF2 phosphorylation-mediated LC3 conversion, inhibited mutant dysferlin aggregation in the ER. ER stress (PERK/eIF2alpha phosphorylation) upregulates Atg12 mRNA, constitutively stimulating the LC3 conversion, and stimulates the autophaosome formation, which degrades the abnormal protein aggregates. Mutant dysferlin aggregates in the ER-stimulated autophagosome formation to engulf them via activation of ER stress-eIF2 phosphorylation pathway. We propose two ERAD models for dysferlin degradation, ubiquitin/proteasome ERAD(I) and autophagy/lysosome ERAD(II). Mutant dysferlin aggregates on the ER are degraded by the autophagy/lysosome ERAD(II), as an alternative to ERAD(I), when retrotranslocon/ERAD(I) system is impaired by these mutant aggregates.

Keywords: Constitutive autophagy, ER stress, cell death, ERAD

Presenting author: Laura Mondragón Martínez

Compounds inhibitors of cellular apoptosis: Novel APAF- modulators

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Through reconstitution of a functional apoptosome *in vitro* different chemical libraries were screened in order to find lead compounds that inhibit the apoptosome-mediated activation of procaspase-9.[1,2] A N-alkylglycine Apaf-1 specific inhibitor (peptoid 1) was identified.[3] This molecule showed poor solubility and low membrane permeability which forced to design new derivatives for further analyses *in vivo*. Three different approaches were followed in order to improve peptoid 1 cell performance: fusion to cell penetrating peptides, synthesis of conformationally restricted analogues (cyclopeptoids) and conjugation to a polymeric carrier (poly-(L-glutamic acid)-peptoid conjugate).[4] In order to evaluate the activity of this third generation of peptoid 1 analogues, a systematic study using different techniques and cellular models was carried out. Promising results with some of the derivatives were found being the cyclopeptoid (QM31) and the poly-(L-glutamic acid)-peptoid conjugate (QM56) the most active modulators.[5] The next step in a potential therapeutic application of these compounds has been focused on the optimization of QM31 and QM56 structure. New conformationally restricted derivatives of QM31 have been synthesized. At the same time, a new generation of polymeric conjugates has been designed employing different linkers to join the active moiety to the polymeric carrier.
Finally, in order to move towards a clinical application, the ability of whether our apoptosome inhibitors could inhibit hypoxia-induced apoptosis in cardiomyocytes as an ex- vivo model of myocardial infarction. The caspase-activity experiments together with the MTT assays demonstrated a reduction of apoptosis up to a 90 %. apoptosis in cardiomyocytes, which clearly opens the possibility for clinical applications in cardiovascular diseases.[5]

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Keywords: apoptosome, inhibitors, Apaf-1, caspase-9

Presenting author: Rodrigo Mora

Microglial factors induce caspase-independent programmed cell death of glioma cells

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The role of microglia, the brain resident macrophages, in glioma biology is still ill defined. Despite their cytotoxic potential, these cells that significantly infiltrate the tumour mass seem to support tumour growth and development rather than tumour eradication. A better characterisation of microglia anti-tumour activities would definitely help designing appropriate immunotherapies against brain tumours. We report herewith the first detailed characterisation in the mouse system of (LPS+IFN-gamma)-induced cytotoxic activity of primary microglia towards glioma cell lines. Upon stimulation, microglia from various strains secreted toxic factors into the culture supernatant that specifically killed the glioma cells lines, MT539MG and SMA560, but not primary cultures of astrocytes or neurons. The toxic factor(s) was/were of proteic nature as indicated by heat-sensitivity, and distinct from TNF-alpha that was released by microglia upon stimulation and towards which the glioma cells were highly resistant. The time-dependent increase of cell death was concomitant to the accumulation of acidic vesicles, phosphatidylserine exposure, appearance of double-membrane cytoplasmic vesicles, extensive zeiosis and a very late loss of DNA in cells that had lost membrane integrity. This later loss of DNA could be reduced partially in SMA560 but not in MT539MG cells by addition of the pan-caspase inhibitor Z-VAD-fmk to the supernatant of stimulated microglia. The inhibitors of autophagy: 3-MA, Asparagine and Wortmannin; protected only slightly from cytotoxicity. Pepstatin A, the inhibitor of cathepsin D showed to be strongly protective as well as N-Acetyl-Cysteine, an inhibitor of sphingomyelin breakdown. Altogether, these results indicate that microglia can be induced to release factors that efficiently and specifically trigger in glioma cells a mode of death involving ceramide production, autophagy, cathepsin D and caspase activity. The identity of the(se) factor(s) is under investigation.

Keywords: Microglia, Ceramide, Autophagy, Glioma

Presenting author: Maryline Moulin

Potential roles of membrane fluidity and ceramide in hyperthermia and alcohol stimulation of TRAIL apoptosis

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We recently reported that a mild heat shock induces a long lasting stimulation of TRAIL-induced apoptosis of leukemic T-lymphocytes and myeloid cell lines, but not normal T-lymphocytes which correlates with an enhanced ability of TRAIL to recognize its receptors. As shown here, this phenomenon could be inhibited by the xanthogenate agent D609, a sphingomyelin/ceramide pathway inhibitor. A caspase-dependent and D609-sensitive two-fold increase in ceramide level was elicited by heat shock plus TRAIL combined treatment. One day after heat shock, a similar increase in ceramide was induced by TRAIL. Sphingolipids/ceramides are known to regulate membrane integrity, and heat shock increases membrane fluidity. In this regard, the heat shock plus TRAIL combined treatment resulted in a D609-sensitive membrane fluidization which was far more intense than that induced by heat shock only. We also report that membrane fluidizers, that mimic the effect of heat shock, such benzyl alcohol and ethanol, potently stimulated TRAIL-induced apoptosis. As heat shock, these alcohols increased, in a D609-sensitive manner, membrane fluidity in the presence of TRAIL, the recognition of TRAIL death receptors, and ceramide levels. These results suggest that stress agents that trigger ceramide production and an overall increase in membrane fluidity are stimulators of TRAIL apoptosis.

Keywords: TRAIL, DR5, Apoptosis, Membrane, Heat Shock

Presenting author: Mirna Mourtada-Maarabouni

Protein Phosphatase 4: A Novel Modulator of Apoptosis

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Many intracellular signalling pathways initiating a wide range of responses include reversible phosphorylation of one or more of their components. At the molecular level, apoptosis also is often regulated by the levels of phosphorylation of particular proteins, i.e. by the balance of between opposing kinase and phosphatase activities on that protein. Protein phosphatase 4 is a novel PP2A-related serine/threonine phosphatase. PP2A is already implicated in the control of cell proliferation, cell cycle and tumorigenesis. Down-regulation studies using siRNA has indicated an antiapoptotic role of PP2A, suggesting that a different protein phosphatase may mediate the proapoptotic effects. We have previously identified PP4 as an important gene influencing the regulation of both apoptosis and cell proliferation in human leukaemic cell lines. We have further investigated the effects of the modulation of PP4 on these cell lines. Our results implicate PP4 in regulation of PEA-15 and BAD activities by affecting their phosphorylation state. PP4 also appears to modulate Histone Deacytelases (HDACs) activity. Our data also show that modulation of PP4 levels has significant effects on the mutation frequency at the HPRT locus after UV exposure. Since the behaviour of cell lines often differs from that of the corresponding normal cells, we manipulated the endogenous level of PP4 expression in normal human lymphocytes stimulated with the mitogen phytohaemagglutinin (PHA). Our results strongly suggest that PP4 mediates the anti-survival effects in these cells. Overall, our data suggest that PP4 plays an important proapoptotic role in T lymphocytes.

Keywords: PP4, apoptosis, human lymphocytes

Presenting author: Annika Mueer

Regulation of mitochondrial apoptosis signaling by p14^{ARF} critically depends on Puma/Bax in p53-proficient cells

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The p14^{ARF} tumor suppressor protein plays a central role in mediating apoptosis, cell cycle arrest, and senescence in response to various cellular stress signals. We previously reported that p14^{ARF} is capable of triggering p53-independent cell cycle arrest and apoptosis programmes, respectively. Whereas the p53-independent induction of mitochondrial apoptosis by p14^{ARF} is fully independent from Bax, the activation of mitochondria in the p53-proficient cells is mediated either by Bax or Bak. However, the upstream signaling requirements for the activation of Bax by p14^{ARF} remain unclear. We show here that the induction of mitochondrial apoptosis upon adenovirus-mediated expression of p14^{ARF} critically depends on the presence of Puma in p53-proficient cells. In contrast to Puma-proficient HCT116 wild-type cells, isogeneic HCT116 cells homozygously deleted for the puma gene are almost completely resistant towards p14^{ARF}-induced apoptosis as evidenced by a lack of nuclear DNA fragmentation and activation of caspases upon expression of p14^{ARF}. Notably, apoptosis induction by p14^{ARF} in p53-proficient cells is mediated exclusively by pro-apoptotic Bax, but not Bak. A N-terminal conformational change of the Bax protein is followed by activation of mitochondria, i.e. a breakdown of the mitochondrial membrane potential, and induction of caspase-9 (LEHDase)-like and caspase-3/7 (DEVDase)-like activities. Whereas the concomitant loss of p21 strongly enhanced p14^{ARF}-induced apoptosis in Puma-proficient cells, the deletion of p21 in Puma-deficient cells did not sensitize for apoptosis induced by p14^{ARF}. Finally, we show that reconstitution of Puma fully restores sensitivity towards p14^{ARF}-induced apoptosis. Taken together, these data demonstrate that p14^{ARF}-induced mitochondrial apoptosis critically depends on the BH3-only protein Puma and its target Bax in p53-proficient cells and is independently modulated by the cell cycle regulator p21.

Keywords: p14ARF, PUMA, apoptosis

Presenting author: Mathis Mueller-Ohldach

The poly(ADP-ribose)polymerase of the fungal ageing model *Podospora anserina* is a putative target of apoptotic processes

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Podospora anserina is a filamentous ascomycete which is characterized by a defined lifespan. Wild-type strain s displays a lifespan of about 25 days. After this period of linear growth morphological and physiological changes occur leading to the development of a senescence syndrome and eventually to the death of the fungus. These changes are largely due to the accumulation of deficient mitochondria during aging. The key role of mitochondria in this degenerative process raises the question of whether or not an apoptotic machinery is involved in lifespan control. *In-silico* analysis of the genome sequence led to the identification of several putative apoptosis factors. One of those factors is a putative poly(ADP-ribose)polymerase (PARP). PARPs belong to a protein family linked to DNA repair, apoptosis and ageing. For instance, in mammals PARP degradation is a hallmark of caspase dependent apoptosis. PaPARP has a high similarity to the human form of PARP-2. A C-terminal GFP fusion of PaPARP is, as expected, located in the nucleus. Numerous attempts to knock-out the gene by homologous recombination did not succeed, leading to the conclusion that the deletion of the gene might be lethal. Verification of this assumption is under way by replacing *PaParp* in a transgenic strain carrying an ectopic *PaParp* integration. A future aim is to demonstrate the role of PaPARP in the apoptotic machinery of *P. anserina* by biochemically assessing its catalytic properties. There are already several mutants created overexpressing *PaParp* constitutionally. The lifespan as well as the resistance of these strains against genotoxic stresses is under investigation. Using bovine PARP as a model for the cleavage of PARP during apoptosis, we used an immunochemical assay to asses whether there is a PARP degrading activity in *P anserina* whole cell extracts present.

Acknowledgement: Financial support is provided by the European Commission (LSHL-CT-2004-512020, Acronym: MiMage) and by grants of the Deutsche Forschungsgemeinschaft.

Keywords: PARP, fungi, Podospora anserina, metacaspases

Presenting author: Georg Müller

Dexamethasone pretreatment enhances necrosis-like neuronal death in ischemic rat hippocampus

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Transient forebrain ischemia (TFI) leads to selective CA1 pyramidal cell death in hippocampus during which apoptotic and other forms of programmed cell death (PCD) are activated and which is exacerbated by glucocorticoids (GC). However, it remains unknown whether glucocorticoids affect neuronal apoptosis and necrosis in cerebral ischemia. Therefore we investigated the relative contribution of apoptosis-like and necrosis-like CA1 neuronal death in rats subjected to 10 min of TFI (survival 1, 2, 4, 5, 7 days) pretreated with dexamethasone (DEXA). We found that DEXA increased ischemic CA1 cell death from 67.7±19% to 95.7±1% (survival 7 days). When we counted all neurons that showed signs of irreversible damage – either apoptosis-like or necrosis-like- between survival days 1-7, we found that DEXA decreased the proportion of apoptosis-like neurons from 4.2% to 1.3%; conversely it increased the proportion of dying CA1 neurons showing necrosis-like nuclear changes from 95.8% to 98.7%. Irrespective of treatment, all apoptosis-like neurons showed immunoreactivity (IR) for activated caspase-3 (casp-3), whereas only in ischemic animals without DEXA casp-3 IR could be found in 4.2% of ischemic neurons, showing necrosis-like nuclear changes. In addition, we found that cell death associated induction of the microglia/macrophage phagocytosis marker ED1 was suppressed by DEXA in ischemic rats. We conclude that dexamethasone enhances ischemic cell death by necrosis-like mechanisms. The concurrent suppression of phagocytosis may have consequences for tissue repair and regeneration.

Keywords: cerebral ischemia, glucocorticoids, apoptosis, necrosis

Presenting author: Olivia Ndozangue-Touriguine

Intracellular mechanisms associated with resistance to TRAIL-mediated apoptosis in a human metastatic colon cancer cell line.

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Agonistic antibodies against TRAIL receptor or soluble recombinant TRAIL are tested in clinical phase I and II studies in a variety of cancers including colorectal tumors. The increased sensitivity of tumor cells compared to their normal counterpart to TRAIL-induced apoptosis has prompted these studies. However resistance to TRAIL can occur in cancer cells and would impair the efficiency of this new therapeutic regimen. We aim at identifying the molecular mechanisms that can mediate this resistance. Our cellular model consists of a cell line derived from the primary tumor of a colon cancer (SW480) and a cell line derived from a lymphnode metastasis of the same tumor (SW620). Whereas SW480 cell readily die when treated with recombinant TRAIL, SW620 cells are totally resistant. We checked that this difference could not be explained by a variation in the density of TRAIL-R1, TRAIL-R2, TRAIL-R3 or TRAIL-R4. Expression levels of the anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-xL, Mcl-1) and the pro-apoptotic molecules Bax, Bid and Bim are identical in both cell lines. Immunoprecipitation of the DISC (Death Inducing Signaling Complex) at early times (from 15mn to one hour) after TRAIL ligation shows that caspase-8 is recruited and activated in both cell lines and that the amount of FLIP recruited to the DISC is comparable. However, we noticed a sustained recruitment of RIP selectively in SW620 cells. This pointed to a possible involvement of the NF-κB pathway. We found that NF-κB is indeed activated in SW620 exposed to TRAIL. The use of an NF-κB inhibitor, Bay 11-7082, allowed us to show that this pathway is involved in the resistance of SW620 cells. A proteasome inhibitor, bortezomib, which can also contribute to NF-κB inhibition was also very efficient in sensitizing SW620 cells to TRAIL-induced apoptosis. Bortezomib was more potent in allowing SW620 cell death than Bay11-7082 indicating that additional protective mechanisms were activated in addition to the NF-κB pathway. SW480 and SW620 are type II cells which necessitate the activation of the mitochondrial loop during TRAIL-mediated apoptosis. We found that SW620 cells are perfectly able to relocalize Bid to the mitochondria and have even generated stable clones overexpressing truncated Bid (tBid-GFP). Despite a clear mitochondrial localization of tBid-GFP the cells do not die indicating a strong resistance to the effect of BH3-only proteins. We are currently trying to identify the proteasome target that can confer this resistance to SW620 cells.

Keywords: TRAIL, resistance, colon cancer cell line, NF-kB, proteasome inhibition

Presenting author: Francesca Nicolau-Galmés

ATM activation in response to terfenadine-induced DNA damage and apoptosis on melanoma cells.

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Previously we have found that terfenadine, an H1 Histamine receptor antagonist, induced apoptosis in melanoma cells independently of H1 histamine receptor. The apoptosis induced by terfenadine is caspase-2 dependent and involves the mitochondrial pathway. Moreover, we have found that terfenadine, through calcium modulation, induces DNA damage tested by comet assay. Since ATM recognizes double-strand breaks and it is activated in response to DNA damage, we have studied the activation of ATM on A375 melanoma cells treated with terfenadine. We detected a time dependent increase of ATM phosphorylated at Ser^{1981} on terfenadine-treated melanoma cells. Moreover, we found a very significant increase of ATM activation after 30 min of terfenadine treatment by flow cytometry and, using confocal microscopy, we found that activated ATM was localized in the nucleus between the marginal and compacted chromatin in the apoptotic cells. In order to elucidate the molecular signalling triggered by ATM, we have studied p53 and p73 pathways. We have found that terfenadine arrests the cell cycle and increases the phosphorylation of p53 at Ser^{15}. Furthermore, terfenadine treatment increases the expression of p73 and PUMA. These results suggest that p53 induces cell cycle arrest and DNA repair rather than apoptosis and that p73 could activate p53-independent apoptosis by transcripcional upregulation of PUMA.

Keywords: terfenadine, apoptosis, ATM

Presenting author: Magali Nicolier

Cervical carcinoma cells display apoptosis under staurosporine exposure through caspase-dependent and caspase-independent pathways

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Previous data from our laboratory have demonstrated that staurosporine (ST), a broad spectrum protein kinase inhibitor, induces apoptosis of cervix cancer-derived HeLa (HPV18+, p53wt) and CaSki (HPV16+, p53wt) cells, as well as C33A cells (HPV-, p53mt). ST causes p53 (either wild type or mutated) and Bax translocation to mitochondria and dissipation of mitochondrial membrane potential, a decisive event of the intrinsic apoptotic pathway. To further characterize pathways involved in the ST-induced apoptosis, mitochondrial pro-apoptotic proteins, cytochrome c and Apoptosis-Inducing Factor (AIF), were studied by confocal microscopy and western blots on purified nuclear, mitochondrial and cytosolic fractions. In the 3 cell lines studied, staurosporine caused an early release (30min) of mitochondrial cytochrome c, followed by cleavage of procaspase-9, -3 and of nuclear poly(ADP-ribose) polymerase (PARP) and ultimately cell death. The addition of z-VAD-fmk, a pan caspase inhibitor, did not change the kinetics of cytochrome c release but led to an attenuated apoptosis. Moreover, in the carcinoma cells, ST caused a late release (4h) of the caspase-independent death effector AIF from mitochondria into the cytosol. Such factor was thereafter partly translocated into the nucleus. Interestingly, the AIF release was concomitant with the cleavage of mitochondrial PARP. The addition of 3-aminobenzamide, a PARP inhibitor, led to a decreased AIF release. Thus, staurosporine orchestrate apoptotic cell death very likely via two partially overlapping molecular mechanisms: a caspase-dependent apoptosis and a PARP-mediated cell death, which opens new fields from therapeutic intervention of cervical cancer.

Keywords: cervical carcinoma, apoptosis, mitochondria, caspase-dependent pathway, caspase-independent pathway

Presenting author: Cathrine Nilsson

Correlation between lysosomal function and cisplatin sensitivity in normal oral keratinocytes and head and neck squamous carcinoma cells

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In cancer cells several functional alterations in the endo-lysosomal system has been correlated to increased resistance to apoptotic cell death pathways. Changes in lysosomal pH and in the expression and trafficking of lysosomal proteases are common. In this study the lysosomal compartment of normal oral keratinocytes (NOKs) and five head and neck squamous carcinoma cell lines, grown under identical serum free conditions, were compared in relation to sensitivity to cisplatin. The two most cisplatin resistant cancer cell lines, as determined by MTT reduction assay, were found to have the highest lysosomal pH (4.9 and 4.7 compared to 4.3 in NOKs) while the most sensitive cell line had a pH of 4.4. The amounts of the cathepsins were altered in the tumor cell lines compared to NOKs, showing both overexpression and repression. As compared to NOKs, all tumor cell lines secreted high amounts of the cathepsin proforms into the medium. Exposure to cisplatin induced caspase-3 dependent apoptosis, which could be reduced by inhibition of either cathepsin D or cysteine cathepsins. Increasing the lysosomal pH, using the lysosomal proton pump inhibitor Bafilomycin A, also had protective effect. The study indicates that a correlation between sensitivity to cisplatin and lysosomal pH exists. In addition, we show that cisplatin induces apoptosis that is, at least partly, dependent on lysosomal cathepsins.

Keywords: cancer, cathepsin, cisplatin, lysosome, pH

Presenting author: Carolin Oberle

Cathepsins and lysosomal membrane permeabilisation in apoptosis induction

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Lysosomes are membranous bags for hydrolytic enzymes used for a controlled bulk digestion of intracellular macromolecules. They require an acid environment for optimal activity in order to protect the rest of the cell from general degradation. Recently these organelles have gained more attention due to their possible role during apoptotic signalling. In particular, it was shown that certain apoptotic stimuli, such as TNFα can trigger increased lysosomal membrane permeability leading to the release of lysosomal proteases such as cathepsin B, L and/or D. Once in the cytosol, these enzymes were proposed to trigger caspase-dependent and –independent death signalling pathways. An unresolved issue is whether this cathepsin mediated process is an early triggering or a downstream amplifying event for apoptosis. Here we use factor dependent monocytes (FDMs) deprived of IL-3 to investigate this aspect. While these cells are undergoing apoptosis, they demonstrate a loss of lysosomal membrane integrity (LMP) and the release of cathepsin B, L and D into the cytosol. Interestingly, all features of LMP and cathepsin release were entirely blocked in Bax/Bak double knock-out cells (DKO), pointing to a possible mitochondrial dependency of these lysosomal processes. To investigate whether the Bax/Bak dependency is a unique feature of IL-3 deprivation, we examined additional apoptotic stimuli like UV-irradiation and etoposide treatment. Again, a clear Bax/Bak requirement of LMP and cathepsin release was detectable. Our data indicate that in response to several different apoptotic stimuli, LMP occurs downstream of mitochondrial outer membrane permeation (MOMP), thus most likely serving the role of an amplification loop rather than an initiating event of apoptosis signalling. Further studies will show by what molecular mechanism Bax/Bak and MOMP contribute to LMP.

Keywords: cathepsin, apoptosis, lysosomes

Presenting author: Eftychia Oikonomou

TRAIL Induced Apoptosis in Freshly Isolated Colon Cancer Cells: An *In Vitro* and *In Vivo* Sensitivity via Death Receptor Upregulation

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Most data on the therapeutic potential and expression of TRAIL as well as resistance to FASL death in colorectal cancer has come from *in vitro* studies using tumour cell lines. To gain a clearer understanding about the susceptibility of patient tumours to TRAIL and FASL, we derived primary human cancer epithelial cells from patients with colon cancer at different stages. Characterisation of the primary cultures PAP60 and MIH55 determined their highly proliferating advantage, transforming capability and tumourigenicity *in vitro* and *in vivo*. Although FASL treatment appeared to cause little apoptosis only in the PAP60 primary culture, increased apoptosis independent of p53 was observed in both primary PAP60 and MIH55 and control cell lines Caco-2, HT29 and DLD-1 after treatment with SuperKiller TRAIL. An increase in PARP cleavage and activation of caspase-3 was also observed. Expression analysis of death receptors (DR) in the original parental tumours, the primary cultures before and after engraftment as well as the mouse xenografts, revealed a significant upregulation of both DR4 and DR5, with the latter being the most differentially expressed. In addition, DR expression pattern was correlated to differences in sensitivity of the cells to TRAIL and FASL induced apoptosis by means of RT-PCR and FACS analysis. Treating patient tumour xenograft/ SCID mouse models with Killer TRAIL *in vivo* for 5 consecutive days suppressed tumour growth and upregulation of DR4 and DR5 that directly correlated to its antitumour activity. This is the first demonstration of TRAIL induced apoptosis in characterised tumourigenic primary human cultures (*in vitro*) followed by the antitumour activity in xenograft models (*in vivo*).

Keywords: Colon Cancer Cells, TRAIL, FASL, Epithelial Isolation, Invasion, FACS, Xenografts

Presenting author: Deepu Ooommen

Hsp27 delays endoplasmic reticulum stress mediated apoptosis induced by Thapsigargin in PC12 cells

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Induction of heat shock proteins (Hsps) has been implicated in inhibition or attenuation of apoptosis associated with cardiovascular and neurodegenerative diseases where endoplasmic reticulum (ER) stress plays a significant role. Although inhibition of conventional apoptotic pathways by Hsps has been extensively studied, information on Hsp regulation on ER stress-induced apoptosis is limited. Hsp27 belongs to the family of small Hsps and is highly induced upon stress. In addition to its role as chaperone, Hsp27 is cytoprotective and anti-apoptotic. Hsp27 is also known to inhibit mitochondrial mediated apoptosis and pro-apoptotic kinases. We report here that overexpression of Hsp27 delayed ER stress-induced apoptosis in rat pheochromocytoma cell line, PC12. There was reduced processing of caspase-9 and caspase-3 in Hsp27 transfected cells as well as significant inhibition of caspase-3 activity. Loss of mitochondrial membrane potential was significantly attenuated in Hsp27 transfected cells. However overexpression of Hsp27 did not potentiate the protective arm of unfolded protein response (UPR) since the expression of Grp78 and appearance of spliced XBP-1 was similar to vector controls. These results indicate that Hsp27 exerts its anti-apoptotic activity by maintaining mitochondrial integrity during the activation of ER stress.

Keywords: Hsp27, ER stress, apoptosis

Presenting author: Daniela Opel

Sensitization of glioblastoma cells for death receptor- or anticancer drug-induced apoptosis by PI3K inhibition

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Glioblastoma is the most common and most aggressive primary brain tumour. Activation of the PI3K/Akt/mTOR pathway, e.g. because of mutations of the *PTEN* tumour suppressor gene, has been reported to correlate with increasing tumour grade, decreased apoptosis and adverse clinical outcome in human malignant glioma. However, the therapeutic potential of targeting the PI3K/Akt/mTOR cascade for apoptosis sensitization of glioblastoma has not yet been investigated in detail. Here, we report that inhibition of PI3K by LY294002 strongly sensitized glioblastoma cell lines for death-inducing ligands (TRAIL, agonistic anti-CD95 antibodies) as well as for various anticancer drugs (Doxorubicin, Taxol, Vincristin or Etoposide). Strikingly, only combined treatment with LY294002 and TRAIL, but neither agent alone, markedly reduced clonogenic growth of glioblastoma cells, demonstrating that the combination treatment synergized to suppress long-term survival of glioblastoma cells. In contrast to PI3K inhibition, blockade of mTOR-mediated signaling either by chemical inhibitors, e.g. RAD001 (everolimus) and rapamycin, or by genetical approach via siRNA did not significantly alter sensitivity of glioblastoma for TRAIL- or Doxorubicin-induced apoptosis. Analysis of apoptosis pathways revealed that inhibition of PI3K cooperated with TRAIL or Doxorubicin to trigger loss of mitochondrial membrane potential, release of cytochrome c from mitochondria and full activation of the caspase cascade. Inhibition of caspases by the broad range caspase inhibitor zVAD.fmk completely abolished apoptosis in response to combined treatment with LY294002 and TRAIL or Doxorubicin, indicating that apoptosis occurred in a caspase-dependent manner. Importantly, LY294002 also sensitized patients' derived primary glioblastoma cells for TRAIL- or Doxorubicin-induced apoptosis. By demonstrating that inhibition of PI3K significantly enhanced both death receptor- and anticancer drug-induced cell death in glioblastoma cell lines and primary tumour samples, our findings have important implications for the development of novel strategies in glioblastoma therapy. Thus, PI3K inhibitors represent a promising approach to enhance the anti-tumour activity of TRAIL or chemotherapy in glioblastoma.

Keywords: apoptosis, PI3K, Akt, glioblastoma, TRAIL, chemotherapy

Presenting author: Mar Orzáez

Apoptosis induction in cancer cells through inhibition of cyclin A/cdk2

Mar Orzáez Nuria Canela, , Raquel Fucho, Francesca Mateo, Ricardo Gutierrez, Antonio Pineda-Lucena, Oriol Bachs, and Enrique Pérez-Paya

The protein-protein complexes formed between different cyclins and cyclin-dependent kinases (CDKs) are central to cell cycle regulation. As cell cycle deregulation represents a hallmark of cancer, development of modulators for this type of protein-protein complexes is a relevant strategy for the development of anticancer agents

Here we describe the identification of an all D-amino acid hexapeptide, termed NBI1, that inhibits the kinase activity of the cyclin-dependent kinase 2 (cdk2)-cyclin A complex through selective binding to cyclin A. The inhibition mechanisms of this peptide is non competitive for substrates and non competitive for ATP. Moreover docking experiments show that NBI1 binds to a concave pocket on the surface of cyclin A that could represent a new allosteric site for the development of drugs.

Furthermore, fusion of NBI1 to cell penetrating peptide carriers allows cellular internalisation, inhibits cell proliferation and induces apoptosis in a panel of different tumour cell lines. Mechanism of NBI apoptosis induction compared with other pro-apoptotic drugs will be also discussed..

Keywords: cyclin A, Apoptosis, cancer

Presenting author: Astrid Ottosson

Velcade/Bortezomib induces G2/M-phase cell cycle arrest and caspase-dependent apoptosis in chemoresistant B lymphoma cells

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Our group has shown that the prototypic Raji B lymphoma cell line fails to undergo intrinsic (mitochondria-dependent) apoptosis in response to the anti-cancer agent etoposide due to lipid raft sequestration of apoptotic protease-activating factor-1 (Apaf-1) (Sun et al., Blood, 2005). Recently, we also showed that synthetic Smac (second mitochondrial activator of caspases) can potentiate the effects of lactacystin (proteasome inhibition)-induced apoptosis in chemoresistant B lymphoma cells in an Apaf-1-dependent manner (Sun et al., Leukemia, 2007). We are now investigating the effects of the novel proteasome inhibitor, Velcade/Bortezomib, a drug that was recently approved for the treatment of advanced or relapsing multiple myeloma. Results from our ongoing studies suggest that Velcade/Bortezomib induces G2/M-phase cell cycle arrest in B lymphoma cells harboring a wild-type (WT) p53 gene (DG75) and in cells with mutant (mt) p53 (Raji). Furthermore, Velcade/Bortezomib can induce the activation of caspase-3 and apoptosis, as assessed by the quantification of cells with hypodiploid DNA content, in the etoposide-resistant DG75 cell line. This cell death could be abrogated by adding the pan-caspase inhibitor zVAD-fmk. The effect of Velcade/Bortezomib on caspase-3 activity was more pronounced in B lymphoma cells with WTp53 (DG75) when compared to cells with p53mt (Raji), and we are currently testing whether p53-dependent signaling is required for the apoptosis induction in our model system. Based on these findings, it is possible that Velcade/Bortezomib could be used for the treatment of B lymphomas that are resistant to conventional chemotherapeutic agents.

Keywords: Chemoresistance, B lymphoma, Velcade/Bortezomib, Cell cycle arrest

Presenting author: Tim Overkamp

Cooperative effect of p21$^{CIP/WAF-1}$ and 14-3-3σ on cell cycle arrest and apoptosis induction by p14^{ARF}

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By linking the p53 and Rb signaling pathways, p14^{ARF} (p19^{ARF} in the mouse) plays a central role in the regulation of cellular proliferation. Whereas its ability to induce a G1 cell cycle arrest entirely depends on a functional p53/p21 signaling axis, G2-arrest triggered by p14^{ARF} is p53/p21-independent and occurs via inhibition of p34^{cdc2} (Cdk1). In contrast to DNA damage, which induces mitotic catastrophe in 14-3-3σ-deficient cells, expression of p14^{ARF} triggered apoptotic cell death irrespective of the presence or absence of 14-3-3σ. Notably, p14^{ARF}-induced apoptosis was substantially augmented in cells nullizygous for both 14-3-3σ and p21$^{CIP/WAF-1}$ as compared to wild-type cells or cells lacking either gene alone. Thus, p14^{ARF} targets the distal DNA damage signaling pathway but prevents mitotic entry by down-regulating cdc2 and induces apoptotic cell death out of the G2-phase of the cell cycle. Furthermore, these data delineate that disruption of G2/M cell cycle checkpoint control critically determines the cellular sensitivity towards p14^{ARF}-induced mitochondrial apoptosis but not mitotic catastrophe.

Keywords: p14ARF, 14-3-3sigma, cell cycle

Survivin expression in STZ treated newborn rat endocrine pancreas

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Survivin is a member of apoptosis inhibitor protein family which play role regulation of the cell cycle and apoptosis inhibition. In this study we aimed to investigate between survivin and regeneration of the beta cell in STZ treated neonatal rats (nSTZ). In this study four groups which contain seven wistar albino newborn rats were used. On the second day after birth 100mg/kg STZ was given two groups. On the five and ten days after the birth all groups were decapitated. Pancreas tissue were fixed in %10 formaline and embedded paraffin. Digoxigenin labeled insulin was used for in situ hybridization. Insulin, survivin and pdx-1 antibodies were used for immunohistochemistry on serial parafine sections. Islet sizes of STZ treated groups were smaller than control groups. The number of insulin positive beta cells were lower at five days goups than control group ,on the other hand there were no difference between control group and the ten days group. In the tissue sections of n-STZ groups ,the area of the cells containing insulin m-RNA signals were lower than insulin immunopositive cells. In the islets of the five days n-STZ group the number of pdx-1 immunopositive cells was lower than control groups. In STZ treated five days groups survivin positive reaction were seen in a few islet cells. In STZ treated ten days groups islet cells had survivin positive reaction as same control groups. Survivin ,pdx-1 and insulin expression showed almost similar distribution on the serial sections of the pancreas tissue all of the groups. These findings suggest that beta cells protect themselves from STZ with increase survivin expression. Survivin may play a role islet beta cell regeneration after STZ administration

Keywords: survivin, rat, pancreas, n-STZ

Presenting author: Vincenza Pace

Caspase activation in extrinsic apoptosis: comparison between *in silico* and *in vitro* results

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Apoptosis is modulated by extrinsic and intrinsic signals that activate complex intra-cellular signalling networks in which caspases play a pivotal role. Caspases are a family of conserved cysteine proteases that cleave target proteins at specific aspartate residues. The activation of these proteins starts up a complex network of processes whose outcome is the cell death. The complexity of the apoptotic process pushed researchers to assemble mathematical models that could help to identifying the crucial steps of apoptosis. We built a mathematical model that considers the dynamical interactions between pro-caspases C8 and C3, active caspases C8* and C3*, and the inhibitor proteins BAR and IAPs (1,2). We simulated the time evolution of the protein concentrations by solving the system differential equations for several sets of parameters, thus trying to mimic the quantitative cell response to external stimuli (3). The next step in our work was to check the biological reliability of our model. First, we used recombinant Apo2L/TRAIL to induce extrinsic apoptosis in 143B.TK- synchronized cells. Second, we analyzed by flow-cytometry the time evolution of both C8* and C3* concentrations. Third, we compared *in silico* results (simulations) with the results obtained in the experimental system. We found a consistent agreement between *in silico* and *in vitro* results thus proving that the model could be a reliable tool in exploring the apoptosis pathway.

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Keywords: Mathematical modelling, caspase activation

Presenting author: Helena Paidassi

Seeking additional C1q ligands on the apoptotic cell surface

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Efficient clearance of apoptotic cells is critical for development, tissue remodelling and maintenance of homeostasis, and is crucial to control the immune responses mediated by macrophages and dendritic cells. However, little is known about the molecules that contribute “eat-me” signals on the apoptotic cell surface. C1q, the recognition unit of the C1 complex of complement, also senses altered structures from self and is a major actor of immune tolerance.
We have recently demonstrated that the polar head of phosphatidylserine (PS) is one of the Clq ligand on apoptotic cells. The C1q-PS interaction takes place at early stages of apoptosis, likely in newly organized membrane patches, and is mediated by the globular region of C1q. Nevertheless, there is increasing evidence that apoptotic cell recognition involves sensing of multiple “eat-me” signals. Given its multimeric structure, and the known versatility of its recognition properties, C1q may be expected to sense different ligands which collectively would provide strong “eat-me” signals, hence allowing efficient removal of apoptotic cells. We are currently investigating the involvement of other candidate ligands. First, recent studies have demonstrated the involvement of calreticulin (CRT) in apoptotic cell clearance. Using surface plasmon resonance (SPR), we demonstrate that CRT binds to both the collagen tail and the globular region of C1q. We are now using cellular techniques to investigate whether CRT behaves as a C1q ligand on the apoptotic cell surface. Second, it has been reported that the cell surface glycosylation pattern undergoes modifications during apoptosis. We have corroborated this observation in our own cellular model using biotinylated lectins. In addition, using SPR, we provide evidence that C1q has the ability to bind certain neutral carbohydrates. Further experiments are being performed to assess whether CRT and carbohydrates act as C1q ligands on the apoptotic cell surface. These data will be presented at the meeting.

Keywords: apoptotic cell clearance, C1q, immune tolerance, innate immunity, anticancer immunotherapy

Presenting author: Beata Pajak

Sodium butyrate-dependent sensitization of human colon adenocarcinoma COLO 205 cells to TNF-α-induced apoptosis

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COLO 205 colon adenocarcinoma cells are highly resistant to extrinsic apoptosis induced by immunomodulatory cytokines. One of the antiapoptotic mechanisms is the expression of cFLIP protein, which inhibits TNF-α-induced cell death. The use of metabolic inhibitors, such as sodium butyrate (NaBt), the potent repressor of histone deacetylase, sensitizes tumor cells to TNF-α-mediated apoptosis. The Western-blot analysis revealed that in COLO 205 cells the susceptibility to apoptogenic stimuli results from time-dependent reduction in $cFLIP_L$ protein assembled with DISC complex. At the same time, the level of transmembrane TNF-α receptor 1 (TNF-R1) was elevated which is consistent with the exaggerated rate of cell death. Since preincubation of COLO 205 cells with *N*-acetyl-L-cysteine (NAC), or sodium ascorbate (ASC) did not protect cells from combined NaBt- and TNF-α-induced apoptosis, we concluded that deletion of cancer cells is not evoked by oxidative stress. Our results suggest that the combination of TNF-α with NaBt targets antiapoptotic protein(s) and may provide more efficient and less toxic treatment in colon cancer therapy.

Keywords: TNF-alpha, colon cancer, apoptosis resistance, cFLIP, sodium butyrate

Presenting author: Nicole Paland

Prevention of p53-dependent apoptosis inhibition in smooth muscle cells by Cancer Associated Fibroblasts (CAFs)

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Prostate cancer is the most common cancer in men in developed countries. To date, no cure is available for patients in advanced disease stages and little is known about the initiation of prostate carcinomas. The prostate is composed of the epithelium and the stroma which consists of smooth muscle cells and fibroblast. The stroma is very important during embryonal development of diverse structures in the prostate or mammary gland. In adult tissues stromal cells are responsible for the maintenance of a homeostatic equilibrium and in controlling cell size and cell functions of the epithelium they surround. Stromal cells are also important the wound healing process and other pathological conditions through modification of the extracellular matrix. In the case of disturbed homeostasis regulation stromal cells can contribute to the initiation and progression of cancer. During the progression of cancer the stroma undergoes several changes: altered fibroblast in the proximity of the tumor, so called “cancer associated fibroblasts” (CAFs) appear and the amount of smooth muscle cells decreases. Many cancer cells harbour mutations in tumour-suppressor genes in the stromal compartment of human carcinomas, among them is p53. p53 levels are rapidly elevated as a response to stress signals including oncogene activation, DNA damage or physiological stress leading either to apoptosis or growth arrest. Alterations and loss of p53 was shown in stromal tissues of carcinomas of the prostate. In our laboratory, normal human smooth muscle cells were immortalized and diverse mutations such as a p53 knock down were introduced. These cells were exposed to DNA damage under normal conditions and in conditioned media from cancer associated fibroblast (CAFs). Smooth muscle cells without knocked down p53 were resistant to cisplatin induced apoptosis under normal conditions, but when cisplatin was administered in conditioned media from CAFs, apoptosis was observed. Furthermore, in immortalized smooth muscle cells and in those with the p53 mutation R175H an increase in apoptosis was observed when it was induced in conditioned media from CAFs. This observation implies that CAFs secrete a factor that either interferes with p53-dependent apoptosis regulation.
Here we show for the first time a possible cause for the loss of smooth muscle cells during the progression of prostate carcinomas.

Keywords: p53, prostate cancer, smooth muscle cells, cancer associated fibroblast, apoptosis inhibition

Presenting author: Julian Pardo

Granzyme B induced cell death exerted by *ex vivo* CTL: discriminating requirements for cell death and some of its signs

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Granzyme B (gzmB) of cytotoxic lymphocytes is essential for recovery from intracellular pathogens, but the molecular basis is still unresolved. Here, we analyzed gzmB-mediated death pathways under more physiological conditions using *ex vivo* virus-immune CTL that express perf and gzmB, but not gzmA ($gzmB^+$CTL). We show that $gzmB^+$CTL kill targets independent of caspases and mitochondrial signaling. In addition the data reveal that $gzmB^+$CTL independently induce pro-apoptotic processes either via caspases 3/-7, leading to plasma membrane perturbance and ROS production, or via Bid/Bak/Bax, resulting in cytochrome c release and that both pathways elicit $\Delta\Psi_m$ suppression. Our data provide evidence for a pleiotropic pro-apoptotic function of gzmB suitable to counteract evasion strategies of pathogens and to control tumors.

Keywords: CTL, granzyme B, apoptosis, immune evasion

Presenting author: Laura Pattacini

Anti-rheumatic biologic agents induce Fibroblast-like Synoviocytes Anoikis through a PTEN-FAK mediated pathway.

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by hyperplasia of fibroblast-like synoviocytes (FLS) and inflammatory cell infiltration. Synovial macrophages secrete tumor necrosis factor α (TNF-α), which induces the production of pro-inflammatory cytokines, such as IL-6. Treatment with TNF-α antagonists, infliximab, adalimumab or etanercept, or with the anti-IL-6 receptor tocilizumab has been shown to improve disease symptoms. Nevertheless, the precise mechanism of action of these drugs has not been fully understood. In our study, we have evaluated the effects of the different drugs on FLS anoikis. FLS were obtained from synovial fluids of 5 RA and 5 osteoarthritis (OA) patients and were exposed to the different drugs. Thereafter, mononuclear cells from peripheral blood of the same patients were added. Seven days afterward, apoptosis induction was determined by light microscopy, by nucleosome ELISA and by TUNEL. We observed that the four different drugs induced apoptosis of RA FLS to a different extent. In fact, one sample resulted only sensitive to Etanercept, one responded to the two anti-TNF-α antibodies and three samples showed a comparable response for all tested drugs. We failed to observe any apoptosis induction in samples from OA patients. By a binding assay, we showed that all the four drugs bind to FLS surface. We then investigated the intracellular activated pathways. We found that the activating phosphorylation of PTEN and the total protein levels were increased. FAK phosphorylation of the serine 397 residue was decreased, whereas the protein level did not change. MAPK expression did not change after cell treatment of the different drugs. On the contrary, p53 levels increased as well as the levels of the pro-apoptotic factor bax, at almost the same extent. The expression of the anti-apoptotic protein Bcl-2 was reduced in only in cells treated with either etanercept or tocilizumab. Caspase 3 resulted to be activated by the different treatments. In our study, we have shown that the four biologic agents either in use or in clinical trial for RA treatment can exert their effects at least in part through their capacity to induce FLS apoptosis.

Keywords: Rheumatoid Arthritis, Fibroblast-like synoviocytes, Anoikis, FAK

Presenting author: Michela Pellegrini

Late-onset systemic autoimmunity in mice lacking expression of the proapoptotic p66Shc protein

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The p66 kDa isoform of ShcA acts as a promoter of apoptosis, an activity which in fibroblasts has been ascribed to its capacity to enhance intracellular ROS production. We have previously demonstrated that p66Shc is expressed in T-cells, where it is upregulated in response to apoptogenic stimulation. p66Shc expression in T-cells correlates with enhanced susceptibility to apoptosis and blunted responses to mitogenic stimuli. The defect in T-cell apoptosis found in p66Shc deficient T-cells, as well as their enhanced proliferative response to TCR agonists, suggest a potential impact of p66Shc deficiency in selection of the immune repertorie and immune homeostasis. In the present work we show that p66Shc deficient mice are susceptible to lupus-like autoimmune disease on the nonautoimmune-prone 129sv genetic background. Old p66Shc deficient mice (over 10 months) show splenomegaly characterized by white pulp hyperplasia and the presence of florid germinal centers, as well as accumulation of large numbers of spontaneously activated T and B cells both in the spleen and lymph nodes. p66Shc deficiency also results in production of circulating anti-ds DNA autoantibodies, hypergammaglobulinemia that preferentially affects IgG1 and IgG2a subclasses, and increased frequencies of peritoneal B-1 cells, which are know to produce low affinity polyclonal autoantibodies. Microscopic analysis revealed glomerular deposition of immune complexes in the kidney that correlated with histological and ultrastructural glomerular alterations and proteinuria. Moreover, p66Shc deficient mice showed a trend to develop alopecia, with a prevalence in females, due to destruction of hair follicles by lymphoid infiltrates and deposition of immune complexes in the skin. The pathological features found in p66Shc deficient mice are consistent with lupus-like autoimmunity. These findings underscore the role of p66Shc as a critical regulator of lymphocyte survival whose absence promotes the development of systemic autoimmunity.

Keywords: p66Shc, autoimmunity

Presenting author: Clara Pereira

Loss of ADP/ATP carrier leads to aggregation of mitochondria and delayed degradation during apoptotic cell death in *Saccharomyces cerevisiae*

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Acetic acid has been shown to activate a form of programmed cell death in *Saccharomyces cerevisiae* that exhibits some apoptotic characteristics, including cytochrome *c* release (Ludovico *et al.*, 2001; Ludovico et al., 2002). Recently, we observed ADP/ATP carrier (AAC) is required for mitochondrial outer membrane permeabilization (MOMP) and cytochrome *c* release in yeast apoptosis. Acetic acid treatment was also reported to cause disruption of the mitochondrial network and subsequent degradation of the mitochondria (Fannjiang *et al*., 2004). In this work we proposed to study the process of fragmentation/degradation of mitochondria in acetic acid-induced apoptosis and the role of AAC/MOMP in these processes. We show that non-selective autophagy (macro- or micro- autophagy) is not activated by acetic acid, and that degradation of mitochondria following the apoptotic induction is probably the result of a selective *UTH1*-dependent process. Additionally, using mitochondria-addressed GFP, we could observe that the fragmentation of mitochondria in wild-type cells which originates spots distributed throughout the cell, is altered in AAC-deleted cells. This morphological alteration consisted on a few number of big spots which resulted from the aggregation of fragmented mitochondria. The disturbance of the mitochondrial dynamics was associated to a slower degradation of this organelle as assessed by monitoring protein degradation by western-blot. The results show that AAC proteins play a role in the morphological remodelling of mitochondria and in the ensuing degradation, giving a new insight to the contribution of AAC proteins to the intrinsic apoptotic pathway.

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C.P. has a fellowship from POCTI (Fundação para a Ciência e Tecnologia, Portugal).

Keywords: yeast, mitochondria, autophagy, ADP/ATP carrier

Presenting author: Ana Petelin

Cysteine cathepsins trigger caspase-dependent cell death through cleavage of Bid and antiapoptotic Bcl-2 homologues

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As a model for defining the role of lysosomal cathepsins in apoptosis, we characterized the action of the lysosomotropic agent L-Leucyl-L-Leucine methyl ester (LeuLeuOMe) using distinct cellular models. LeuLeuOMe induces lysosomal membrane permeabilization, resulting in release of lysosomal cathepsins that cleave the proapoptotic Bcl-2 family member Bid and degrade the antiapoptotic members Bcl-2 or Bcl-xL. The papain-like cysteine protease inhibitor E-64d largely prevented apoptosis, Bid cleavage and Bcl-2/Bcl-xL degradation. The pancaspase inhibitor z-VAD-fmk failed to prevent Bid cleavage and degradation of Bcl-2, Bcl-xL and Mcl-1 homologues, but substantially decreased cell death suggesting that cathepsin-mediated apoptosis in these cellular models mostly follows a caspase-dependent pathway. Moreover, *in vitro* experiments showed that one or more of the cysteine cathepsins B, L, S, K, and H could cleave Bcl-2, Bcl-xL, Mcl-1, Bak and Bim EL, whereas no Bax cleavage was observed. On the basis of inhibitor studies we demonstrate that lysosomal disruption triggered by LeuLeuOMe ocurrs prior to mitochondrial damage. We propose that degradation of anti-apoptotic Bcl-2 family members by lysosomal cathepsins synergizes with cathepsin-mediated activation of Bid to trigger a mitochondrial pathway to apoptosis.

Keywords: cysteine cathepsins, Bcl-2 homologues, apoptosis

Investigation of DNA fragmentation during apoptosis in fission yeast

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Programmed cell death or apoptosis is a highly regulated cellular process. Recently, apoptosis-like processes have been also found in the yeasts *Saccharomyces cerevisiae* and *Schizoccharomyces pombe*. Therefore, they are successfully used as model organisms for investigating the regulation of apoptosis. The Bcl-2 family of proteins known to be major regulators of apoptosis is present in higher eukaryotes but not in yeasts. Two members on Bcl-2 family are anti-apoptotic Bcl-X_L and pro-apoptotic Bax proteins. We compared both nucleotide sequences of human and mouse Bcl-X_L and Bax genes. The sequence alignment showed approximately 90 % homology between these genes.
We applied Comet assay on yeast cells developing the modification called *Yeast Comet Assay (YCA)*. The *YCA* facilitates detection of single and double-stranded DNA breaks. As genomic DNA fragmentation is one of the hallmarks of the process the YCA method could enable perceptive investigation of DNA fragmentation during apoptosis.
Here, we are presenting our results on DNA degradation during apoptosis in *S. pombe* cells transformed with either Bax or Bcl-X_l gene.

Keywords: apoptosis, Yeast Comet Assay, S. pombe, Bax, Bcl-Xl

Presenting author: Mark Pickard

Fau regulates apoptosis in human cell lines – an essential role for Bcl-G

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The Fau gene encodes a ubiquitin-like protein, termed Fubi, fused to ribosomal protein S30. Several lines of evidence indicate a tumour suppressor role for Fau, but the mechanism of this activity is unclear. However recent studies have shown that i) Fau induces apoptosis in mouse W7.2c cells and ii) Fubi covalently modifies murine Bcl-G, a pro-apoptotic member of the Bcl-2 family. The aims of the present study were to examine if Fau regulates apoptosis in human cells and, if so, to determine if Bcl-G is essential for this regulation.

We employed the following cell lines: Jurkat and CEM-C7 (T-lymphoblastic leukemia cells), 293T/17 (embryonic kidney-derived) and T-47D (breast tumour-derived). These were transiently transfected with i) pcDNA3-Fau construct or pcDNA3, ii) siRNA to Fau or negative control siRNA or iii) siRNA to Bcl-G or negative control siRNA, followed by pcDNA3-Fau construct or pcDNA3. Cells were then either irradiated with UV light (254 nm) to induce apoptosis or unexposed (basal apoptosis), and apoptosis (microscopic examination of Caspatag and/or acridine orange staining) and cell viability were subsequently determined.

Fau overexpression was associated with increased basal apoptosis and decreased short-term cell viability in the absence of UV-irradiation in Jurkat, CEM-C7 and 293T/17 cells. The colony-forming ability of these cell lines was also reduced. In contrast, there was no consistent effect of ectopic Fau expression on UV-induced apoptosis. siRNA-mediated silencing of Fau expression had negligible effect on basal apoptosis and cell viability, but attenuated UV-induced apoptosis and loss of cell viability in all cell lines. Further experiments in 293T/17 cells revealed that prior knockdown of Bcl-G expression diminished Fau-induced changes in basal apoptosis and cell survival.

In conclusion, these findings are consistent with a pro-apoptotic regulatory role for Fau in human cells, in agreement with previous findings in murine cells. Furthermore our findings indicate that Bcl-G mediates this pro-apoptotic activity of Fau. This novel apoptotic regulatory role of Fau is an attractive mechanism to explain the putative tumour suppressor activity of the gene.

This work was supported by a grant from the BBSRC.

Keywords: Fau, Bcl-G, ubiquitin-like protein, tumour suppressor,apoptosis

Presenting author: Vsevolod Pinelis

THE NEUROPROTECTIVE EFFECTS OF HEPTAPEPTIDE SEMAX AND THREEPEPTIDE PRO-GLY-PRO IN CULTURED NEURONS TREATED WITH GLUTAMATE.

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N-terminal fragments of adrenocorticotropic hormone (ACTH) are well known for their potent neuroregenerative and cognitive activities (Strand et al. 1993). The heptapeptide Semax (Met-Glu-His-Phe-Pro-Gly-Pro) is an analogue of the ACTH(4–10) fragment, which is completely devoid of any hormonal activity associated with the full-length ACTH molecule (Ashmarin et al, 1997). Semax is currently used for treatment of brain hypoxia and ischaemia but despite clinical benefits, the cellular and molecular mechanisms underlying the action of Semax in the brain are mainly known. In present work we studied effects of Semax and its analog Pro-Gl-Pro (PGP) on glutamate-induced neuronal death, intracellular Ca^{2+} homeostasis deterioration and gene expression of BDNF and Trk B in cultured neurons. To explore Glu-induced $[Ca^{2+}]_i$ changes cultured neurons were loaded with fluorescent Ca^{2+} probe Fura-2FF. Cell viability was estimated using MTT-test or vital fluorescent dyes (propidium iodide or Hoechst 33258). In control cultures glutamate (100 μM, 0 Mg^{2+}, 10 μM glycine) induced the development of delayed Ca^{2+}deregulation (DCD) and 35% neuronal death (24 hours following glutamate challenge). Pretreatment of neurons with Semax or PGP (20, 50 and 100 μM, respectively) resulted in decrease number of neurons that revealed DCD, 2-3-fold increase of exon III BDNF and TrkB mRNA levels and two-fold diminish of the neuronal death. Mechanisms of these peptides action are discussed. Supported with the RFBR grant.

Keywords: neurons, peptides, glutamate, neuronal death

Presenting author: Olga Pletjushkina

Oxidative stress triggers the changes of mitochondrial reticullum morphology in HeLa cells

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We studied the changes in mitochondrial morphology induced by oxidative stress in HeLa cells. Addition of hydrogen peroxide caused the fission of mitochondria, and respiratory chain inhibitors piericidin and myxothiazol (affectors of Complex I or Complex III, respectively) accelerated this process. Preincubation of cells with mitochondria-targeted antioxidant, 10-(6'-ubiquinolyl)decyltriphenilphosphonium (MitoQ) or it's analogue SkQ, prevented fragmentation induced either by one agent or by combination of them. The comparative study of antioxidant properties of this two quinone derivatives was performed, and it was found that working concentrations of both antioxidants are nanomolar. We suggest that the process of mitochondrial fission depends on ROS, produced by mitochondria themselves.

To study the further steps of changes in mitochondrial morphology we used the uncoupler, FCCP. In these experiments we observed the clustering of mitochondria taking place after mitochondrial fission. In the presence of tubulin polymerization inhibitor nocodazole the gathering of mitochondria around the nucleus and formation of clusters happens much faster. Cytochalazin D, inhibitor of actin polymerization, does not affect the process.

We observed that mitochondrial clustering is the next step in changes of mitochondrial morphology after fragmentation induced by oxidative stress. We suppose that this process plays an important role in protecting cell from reactive oxygen species burst and subsequent cell death.

Keywords: mitochondria, oxidative stress, antioxidants

Presenting author: Marie-Laure Plissonnier

Can p53 status modulate PPARγ agonists effect on promoting apoptosis or cell growth inhibition in cervical cancer cells

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Introduction: Persistent infection by high-risk Human Papillomavirus (HPV) such as HPV16 and 18 are etiologically associated with the development of cervical cancer. The expression of E6 and E7 viral oncoproteins short-circuits apoptosis by disrupting the function of p53 and pRb respectively. These alterations confer a survival benefit to the cells and a resistance to anticancer treatment. Recent evidence indicates that a new class of drugs, the thiazolidinediones (TZD) such as ciglitazone, troglitazone, rosiglitazone and pioglitazone, which activate the peroxisome proliferator-activated receptor γ (PPARγ), exhibit antiproliferative, differentiation and apoptotic activities on different cancer cells in addition to their antidiabetic properties.
Methods: Our study aims to evaluate by flow cytometry and western-blotting the effect of TZD on cell proliferation and apoptosis in cervical cancer-derived cell lines: HeLa (HPV18+, p53wt), CaSki (HPV16+, p53wt) and C33-A (HPV-, mutated p53).
Results: Here, we report that in the three cancer cell lines, only ciglitazone and troglitazone induce apoptosis characterised by a cleavage of caspase-3,-8 and -9. Rosiglitazone induces a G0/G1 cell cycle arrest in C33-A cells. Contrary to the other ligands, pioglitazone has no effect.
Discussion: Our results suggest that TZD can induce apoptosis or inhibit cell proliferation of cervical cancer-derived cell lines according to the p53 status. These antineoplastic effects of PPARγ agonists may provide an exciting novel therapeutic approach for the treatment of cervical cancer.

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Keywords: HPV, cervical cancer, thiazolidinediones, p53, apoptosis

Presenting author: Ekaterina Popova

Mitochondrial reactive oxygen species regulate the architecture of cytoskeleton and cell motility.

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We have investigated the role of mitochondrial reactive oxygen species (ROS) in intracellular signaling using mitochondria-targeted antioxidant, 10-(6'-ubiquinolyl) decyltriphenilphosphonium (MitoQ). A specific targeting of this antioxidant to mitochondria was confirmed using fluorescent rhodamine-labeled MitoQ. The several cell cultures (normal or Ras-transfected) were treated with MitoQ. We revealed the significant changes in cytoskeleton, morphology and increase of cell motility. Analysis of fibroblast culture revealed a fraction of very large well-attached and symmetrical cells, enriched in α-smooth muscle actin (α-SMA) that is a selective marker of myofibroblasts. The changes observed in epithelial cells were typical for the beginning of epithelial-mesenchimal transition (EMT). We suppose that reducing of mitochondrial ROS by MitoQ can activate Rac-dependent signalling. It was found that short-term (2-3 h) treatment of fibroblasts with MitoQ followed by prolonged (7-60 days) culturing of the cells without added MitoQ caused the same effects. Also we tested the effects of mediums, collected from epithelial cells, pretreated with MitoQ the same way, on the same cell culture and found that they also cause the increase of cell motility. This may indicate that MitoQ gives rise to some autocrine mechanisms of supporting the changes, caused by this antioxidant.

Keywords: ROS, mitochondria, cell signalling

Presenting author: Faiy Psahoulia

Quercetin mediates preferential degradation of oncogenic Ras and causes autophagy in Ha-*RAS*-transformed human colon cells.

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In vitro experiments supported by animal investigations as well as epidemiological studies of human colorectal cancer suggest that the development of this disease can be modulated by dietary factors. In this study, we have investigated whether the flavonoid quercetin can modulate cell proliferation and survival by targeting key molecules and/or biological processes responsible for tumor cell properties. The effect of quercetin on the expression of Ras oncoproteins was specifically studied using systems of either constitutive or conditional expression of oncogenic *RAS* in human epithelial cells. Our findings suggest that quercetin inhibits cell viability as well as cancer cell properties like anchorage independent growth. These findings were further supported at the molecular level, since quercetin treatment resulted in a preferential reduction of Ras protein levels in cell lines expressing oncogenic Ras proteins. Notably, in cells that only express wild-type Ras or in those where the oncogenic Ras allele was knocked out, quercetin had no evident effects upon Ras levels. We have shown for the first time that quercetin drastically reduces half-life of oncogenic Ras but has no effect when the cells are treated with a proteasome inhibitor. This could account for its ability of quercetin to inhibit cell growth and tumor properties. Moreover, we propose that oncogenic Ha-*RAS* sensitizes the cells to autophagy, a type of cell death distinct from apoptosis that is caspase independent. This process was further promoted by quercetin and this accounts for the reduced cell viability of Ha-*RAS*-transformed cells after quercetin treatment. Since quercetin downregulates the levels of oncogenic Ras in cancer cells, we propose that this flavonoid could act as a chemopreventive agent for cancers with frequent mutations of *RAS* genes [(1)].

To further support our conclusions concerning the antitumor properties of quercetin, we have examined the potent sensitization of colon cancer cells by quercetin, so that anticancer drugs can enhance and/or accelerate apoptosis processes. Thus, our first results provide a challenging approach to enhance the efficiency of therapies based on apoptotic agents by overcoming resistance of tumor cells towards anticancer therapies and a framework for future experiments [(2)].

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Keywords: quercetin, colon cancer, ras, proteasome, autophagy, apotosis

Presenting author: Dmitry Pshezhetskiy

Oxidative stress–dependent sphingosine kinase-1 inhibition mediates monoamine oxidase A–associated cardiac cell apoptosis

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The mitochondrial enzyme monoamine oxidase (MAO) and its isoform MAO-A, play a major role in reactive oxygen species–dependent cardiomyocyte apoptosis and postischemic cardiac damage. In the current study, we investigated whether sphingolipid metabolism can account for mediating MAO-A– and reactive oxygen species–dependent cardiomyocyte apoptosis. In H9c2 cardiomyoblasts, MAO-A–dependent reactive oxygen species generation led to mitochondria-mediated apoptosis, along with sphingosine kinase-1 (SK1) inhibition. These phenomena were associated with generation of proapoptotic ceramide and decrease in prosurvival sphingosine 1-phosphate. These events were mimicked by inhibition of SK1 with either pharmacological inhibitor or small interfering RNA, as well as by extracellular addition of C2-ceramide or H2O2. In contrast, enforced expression of SK1 protected H9c2 cells from serotonin- or H2O2-induced apoptosis. Analysis of cardiac tissues from wild-type mice subjected to ischemia/reperfusion revealed significant upregulation of ceramide and inhibition of SK1. It is noteworthy that SK1 inhibition, ceramide accumulation, and concomitantly infarct size and cardiomyocyte apoptosis were significantly decreased in MAO-A–deficient animals. In conclusion, we show for the first time that the upregulation of ceramide/sphingosine 1-phosphate ratio is a critical event in MAO-A–mediated cardiac cell apoptosis. In addition, we provide the first evidence linking generation of reactive oxygen species with SK1 inhibition. Finally, we propose sphingolipid metabolites as key mediators of postischemic/reperfusion cardiac injury.

Keywords: sphingosine kinase-1, monoamine oxidases, ischemia/reperfusion, ROS, cardiac apoptosis

Presenting author: Anja Pucer

Possible role for cathepsins B and L in programmed cell death of glioblastoma cells induced by chemotherapeutic arsenic trioxide

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Glioblastoma multiforme (GBM) is the most common and malignant type of the primary brain tumors. Arsenic (As_2O_3), the component of the chemotherapeutic Trisenox™, is a promising drug in treating GBMs, inducing either autophagy and/or apoptosis of tumour cells. Lysosomal proteases - cathepsins play important roles in the regulation and/or execution of both types of programmed cell death (PCD). Cysteine cathepsins L and B are upregulated in GBM and therefore, we are interested whether they may affect the PCD response after arsenic treatment.
We used an established GBM line U87 to study the effects of arsenic on cell proliferation and cell death. Arsenic inhibited viability of U87 cells in a dose and time dependent manner, as shown by the MTT test. Cell proliferation, indicated by Ki-67 immunostaining, was inhibited by 25% after treatment with 2 μM arsenic for 24h. Under these conditions no caspase activation was detected, but 7% of cells showed autophagic activity, as indicated by the redistribution of the autophagy marker L-C3, with the percentage increasing at higher arsenic concentrations. Arsenic inhibited biosynthesis of both cathepsins: RNA levels were reduced to 55 % for cathepsin L and 77% for cathepsin B and activity was reduced to 42% and 69% for cathepsin L and B, respectively. In a reverse type of experiment, lowering the levels of cathepsin L, which would increase the apoptotic response to other drugs in this cell line, did not affect the arsenic induced PCD. In contrast, silencing of cathepsin B significantly inhibited authophagy, suggesting its role in arsenic induced PCD. Further work will reveal the selective roles of both cathepsins in response to arsenic based therapeutic drug in this GBM cell line.

Keywords: cathepsins, autophagy, glioblastoma, arsenic trioxide

Presenting author: Sharon Rashi-Elkeles

Using functional genomics to dissect the p53-mediated transcriptional response to DNA damage

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** Equal contribution*

The cellular response to DNA damage is vital for maintaining genomic stability and preventing cell death or cancer formation. The DNA damage response mobilized by double strand breaks (DSBs) rapidly activates an extensive signaling network that affects numerous cellular systems, leading to cell survival or programmed cell death. A key question is the mechanism by which the decision is made between cellular survival or death, and the choice of type of cell death. The complex transcriptional response to DNA damage is mediated by many transcription factors, among which p53 is a key player. We are combining microarray analysis and computational tools developed in our labs to dissect this response. Recently we showed that the p53-mediated response following DSB induction in mouse lymphoid tissues is primarily directed to genes that mediate apoptosis. Here, we recorded expression profiles in five cancerous and non-cancerous human cell lines after exposure to 5 Gy of ionizing radiation (IR). Interestingly, the predominant response was cell line-specific, with some common themes. Computational analysis identified significant enrichment for binding sites of p53 and the transcription factor NF-Y among promoters of up-regulated and down-regulated genes, respectively. The prominent functional categories among genes whose expression was modulated by the treatment were associated with cell cycle regulation. Thirteen genes were induced by IR in all the examined cell lines, the majority of them involved in negative regulation of cellular growth. Remarkably, ten out of these genes are regulated by p53. To further dissect the transcriptional networks induced by DNA damage we applied meta-analysis to 10 publicly available datasets on the transcriptional responses to IR and to our data. Computational promoter analysis demonstrated that a significant number (76) of the 297 genes that were predominantly regulated in these datasets carried p53 binding site signatures. Importantly, p53 regulation had previously been documented in only 28 of them; the rest constitute a rich source of novel potential p53 targets. Further validation of these new p53 targets is being carried out using real-time PCR and chromatin immunoprecipitation. Initial results attest to the power of our combined computational and experimental approach to identify novel damage response genes. In parallel, we are characterizing the type of cell death that follows IR damage in these cell lines.

Keywords: DSB; IR; Transcriptional response; p53; Meta-analysis

Presenting author: Jan Trige Rasmussen

Structure and function of lactadherin in relation to its ability to recognize cellular exposed PS

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Lactadherin is a widely distributed glycoprotein (~ 50 kDa) that was originally characterized by its association with milk fat/lipid globule membranes. The protein is also known as PAS-6/7, bovine-associated mucoprotein, BA-46, P47, and MFG-E8. Lactadherin comprise two epidermal growth factor (EGF) homology domains (with an RGD peptide motif in the second EGF domain), and two C domains homologous to the phospholipid-binding domains of blood clotting factors V and VIII. Our experiments have shown that lactadherin can act as a link between two surfaces by binding to integrin receptors ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) through its N-terminus and to phospholipids through its C-terminus. Additional studies have shown that lactadherin showed preferential binding to phosphatidylserine. This binding was shown to take place in a Ca++-independent manner, to be stereoselective for the L-form, and that lactadherin binding is enhanced by membrane curvature.
During the past decade the amount of knowledge about the biological activity of lactadherin is growing. It turns out that lactadherin might be a multifunctional protein involving opsonising clearance of apoptotic cells in situations like macrophage phagocytosis, inflammation, adult onset-autoimmune lupus-like pathology, spenomegaly, and defective germinal center formation. Relations for lactadherin to sickle cell anemia, Alzheimers's disease, neovascularization and angiogenesis have also been reported. When it comes to the mammary gland, lactadherin have been suggested to be important for secretion of milk fat globules and the removal of these during involution. Finally, milk lactadherin has been shown to hold attenuating activities against rotavirus and enterotoxic E. coli.
The ability to bind phospholipids is an important feature for the function of lactadherin. We are engaged in several scientific collaborations in order to investigate the manner in which lactadherin can be utilized to detect and/or measure the exposed phosphatidyl serine on platelets and apoptotic cells both *in vitro* and *in vivo*. This presentation will summarize some of the data we have gathered until now.

Keywords: PS exposure, lactadherin, apoptosis

Presenting author: Matthias Regner

Unlike granzymes A and B, the granzyme activating enzyme, dipeptidyl peptidase I, is not required for mice to recover from the natural mouse pathogen ectromelia

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Dipeptidyl peptidase I, also designated cathepsin C (CatC), is thought to be essential for the cleavage of a di-peptide from pro-granzymes (gzm)A and B, a process required for their activation. Thus, lack of CatC should bestow a gzm AxB null phenotype. Deficiency in the two principal gzm, gzmA and gzmB renders resistant C57BL/6 mice highly sensitive to infection with ectromelia virus, the virus causing mousepox. Surprisingly, mice lacking CatC (B6.CatC$^{-/-}$) do not exhibit a gzmAxB null phenotype in response to ectromelia virus infections but exhibit lower viral load, less pathology and no mortality. We have investigated the B6.CatC$^{-/-}$ phenotype with regard to enzymatic activities of gzmB and expression of “orphan” granzymes (gzm C, D, E, F, K, M, N).

Keywords: cathepsin C, dipeptidyl peptidase I, granzymes, ectromelia virus

Presenting author: Santiago Rello-Varona

Mitotic catastrophe induced by different cytotoxic treatments on HeLa cells

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Cell cycle alterations are closely linked with both cell death triggering and resistance, especially in tumour progression and anti-cancer therapy. The process in which a cell is subjected to cell cycle arrest, commonly at metaphase, with final aneuploid interphase restitution or/and delayed apoptosis triggering is often referred to mitotic catastrophe.

Mitotic catastrophe and other cell cycle alterations are related with p53 or its pathway dysfunctions that explain why the repair and apoptosis machinery do not become active properly, allowing the cell to pass the checkpoints until metaphase or even aneuploid interphase.

The well-known human cervix carcinoma HeLa cell line lacks functional p53. Here we present the results of different experiments in which different approaches to induce mitotic catastrophe on HeLa cells have been used. The first method was a short, pulsed, 3 h incubation with 50 µM etoposide (VP-16, a genotoxic drug which causes DNA double-strand breaks). The second stimulus used was photodynamic sensitisation with different photosensitisers (the porphyrin derivative, CF3, and zinc(II)-phthalocyanine, ZnPc); photodynamic sensitisation causes reactive oxygen species (ROS) accumulation at the photosensitiser subcellular placement. ROS are damaging agents due to their high reactivity with the cellular content.

Our results show a high correspondence between the morphological and biochemical events (analysed by fluorescence microscopy, flow cytometry and western blotting) provoked by the experimental treatments, showing cell cycle arrest with metaphase blockage, aneuplodisation, appearance of giant multinucleated cells and final triggering of an apoptosis-like cell death process with chromatin condensation, positive TUNEL staining, PARP cleavage and cell detachment.

Keywords: Mitotic catastrophe, HeLa cells, cancer, etoposide, photodynamic therapy, apoptosis

Presenting author: Benjamin Renouf

P53 activation by MDM2 antagonists has differential effects in EBV-negative and EBV-positive Burkitt's lymphoma cells

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Burkitt's lymphoma (BL), is a B cell neoplasm characterized by chromosomal translocations which affect the c-myc locus and result in constitutive activation of this oncogene. Furthermore, alterations in the p53 tumor suppressor pathway are also often involved in BL development. BL is very closely associated with the Epstein-Barr virus (EBV), most notably in its endemic form where more than 95% of cases are EBV-positive. In infected cells, the viral DNA persists in the nucleus and a limited number of viral proteins are produced, including six EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C and EBNA-LP) and three latent membrane proteins (LMP1, 2A and 2B). Differential expression of these viral proteins define three patterns of EBV latency. Most BL cases are in latency I (expression of EBNA I only) but some are in latency III (expression of the full spectrum of viral proteins).
In a previous study, we have shown that among 20 BL cell lines, 4 contained a wild-type p53 which was inactivated by overexpression of MDM2. To further investigate, the role of the p53/MDM2 complex in these BL cells, we used, nutlin, a potent and selective antagonist of MDM2. This cis-imidazoline compound, by preventing molecular interactions between MDM2 and p53, induces activation of the p53 pathway. We showed that nutlin induced activation of p53 in all BL cell lines, independently of their EBV status. However, nutlin strongly induced apoptosis of EBV(-) or latency I EBV(+) cells whereas it has almost no toxic effect on latency III EBV(+) cells. We then tested if activation of p53 by nutlin could sensitize BL cells to genotoxic drugs. We observed that pre-activation of p53 by nutlin, indeed sensitized EBV(-) or latency I EBV(+) cells to etoposide-induced apoptosis but surprisingly protected latency III EBV (+) cells against this apoptotic agent. Preliminary results indicate that the p21 increase, observed in latency III EBV (+) cells treated by nutlin, could be responsible for their resistance to etoposide.

Keywords: Apoptosis, Burkitt, p53, EBV, MDM2

Presenting author: Urska Repnik

Scaffolding proteins at cell junctions, the MAGUKs, as substrates for proteases during apoptosis

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Membrane-associated guanylate kinases (MAGUKs) are often found in different cell junction structures, where they have a general role in creating and maintaining specialized membrane domains and act as molecular scaffolds for signaling pathway components. MAGUK proteins have a common multidomain organization including several PDZ domains, an SH3 or WW domain, and one guanylate kinase (GUK) domain. All these domains act as a protein-protein interacting domains enabling MAGUKs to assemble a combination of cell adhesion molecules, cytoskeletal proteins, receptors, ion channels and their associate signaling components at specific membrane sites. Since loss of the cell-cell contacts and isolation of a dying cell is one of the important morphological features of apoptosis, it is believed that in order to ensure a fast and efficient detachment of a dying cell, components of cell-cell contacts have to be cleaved, among them the MAGUKs.
Our in vitro experiments showed that all MAGUKs tested were cleaved by at least one of the executioner caspases and degraded by several cathepsins. Serine protease Omi/HtrA2 did not cleave any of the MAGUKs tested. By inducing the apoptosis with UV and staurosporine in different epithelial cell lines, we showed that caspases can also cleave MAGUKs in different cell models. This cleavage could be prevented by zVAD-fmk, a pan-caspase inhibitor. Using a selective lysosomal disrupting agent LeuLeuOMe, which induces apoptosis through lysosomal pathway, we have further shown that MAGUKs are cleaved and/or degraded in HaCaT and CaCo-2 cells. This cleavage/degradation could be prevented by E-64d, an inhibitor of papain-like cysteine proteases, but not by zVAD-fmk. Immunohistological stainings in HaCaT, MDCK and CaCo-2 cells showed that MAGUKs are localized to cell membrane and that after induction of apoptosis with various agents this localization is disrupted, which correlates with the loss of cell-cell contacts. Furthermore, cells pretreated with zVAD-fmk were also dying (relatively high percentage of annexin V positive cells and compromised morphology under light microscopy), but had no DEVDase activity, suggesting that these cells undergo some kind of caspase-independent cell death. TEM pictures showed increased amount of double-membrane vesicles in these cells, a key feature of autophagy. In contrast to apoptotic cells, majority of these cells were still attached and MAGUKs were intact, further supporting the idea that cleavage of MAGUKs is an important step in fast and efficient cell detachment.

Keywords: MAGUK, apoptosis, cell junctions, caspases, cathepsins

Presenting author: Cecilia M. P. Rodrigues

Role of apoptosis in neurodegeneration of transgenic mice expressing the P301L mutated form of human tau

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder leading to dementia. Its hallmark pathologic features include extracellular plaques of amyloid β (Aβ) and intracellular aggregations of tau. The rTg4510 mouse model is characterized by massive neurodegeneration in AD-relevant cortical and limbic structures, deficits in spatial reference memory, and progression of neurofibrillary tangles (NFT). In this study, we examined the role of apoptosis in neuronal loss and associated tau pathology. The results showed that DNA fragmentation and caspase-3 activation are common in the hippocampus ($p < 0.01$) and frontal cortex ($p < 0.05$) of young rTg4510 mice. These changes were associated with cleavage of tau into smaller intermediate fragments, which persist with age. Interestingly, active caspase-3 was often co-localized with cleaved tau. *In vitro*, fibrillar $A\beta_{1-42}$ resulted in nuclear fragmentation, caspase activation, and caspase-3-induced cleavage of tau ($p < 0.05$). Notably, incubation with the antiapoptotic bile acid tauroursodeoxycholic acid abrogated apoptosis-mediated cleavage of tau in rat cortical neurons. In conclusion, caspase-3-cleaved intermediate tau species precede cell loss in rTg54510 brains and Aβ-exposed cultured neurons. These results underscore the role of apoptosis in neurodegeneration.

(Supported by POCTI/SAU-MMO/57936/04 from FCT, Portugal)

Keywords: Abeta; Apoptosis; Caspase-3-cleaved tau; Neuroprotection; NFT; TUDCA

Presenting author: Salvador Rodriguez-Nieto

Identification and characterization of genes sensitizing the non-small cell lung cancer cells to combined treatment with radiation and PKC412

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Resistance to radiotherapy is a major dilemma in the treatment of cancer, in general, and of non-small cell lung cancer (NSCLC), in particular. We have previously reported that combination of low LET radiation with protein kinase inhibitor, PKC 412, sensitizes resistant NSCLC cells to radiation and increases pro-apoptotic response via activation of apoptotic cascade. In contrast, a combination of the PKC inhibitor Ro 31-8220 and low LET radiation did not cause induction of apoptosis in the same radioresistant lung cancer cells, suggesting involvement of different mechanisms. Here using Affymetrix approach we have analyzed possible candidate genes whose change in expression is involved in the observed radiosensitizing effect of PKC 412. Analysis of global changes in gene expression upon combined treatment with either radiation and PKC 412 or radiation and Ro 31-8220 revealed in NSCLC cell line U1810 a striking downregulation of the gene coding for the ephrin-B3 ligand (EFNB3) in response to combination of low LET radiation with PKC 412. This observation was validated by RT-PCR. To further confirm the relevance of EFNB3 downregulation for resistance of these cells to treatment, we have silenced its expression by specific siRNA targeting EFNB3. Obtained data show that, indeed, downregulation of EFNB3 increases the sensitivity of U1810 cells to combined treatment with low LET radiation and PKC 412. Mechanisms of this effect will be discussed.

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Keywords: NSCLC, cell death, radiation, PKC412, sensitivity, ephrin-B3

Presenting author: Juan Rodríguez-Vita

Tranforming growth factor-β is a mediator of 3-hydroxy-3-methylglutaryl CoA reductase inhibitors induced apoptosis of Vascular Smooth Muscle Cells.

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The role of transforming growth factor-β (TGF-β) in atherosclerosis is unclear. TGF-β after binding to specific receptors, type I (TRI) and type II (TRII), activate the Smad pathway, leading to different responses, including apoptosis. The 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors (statins) exert beneficial effects on atherosclerosis, besides its control on circulating cholesterol levels. It has been reported that statins can induce apoptosis of vascular smooth muscle cells (VSMCs). Our aim was to investigate whether the statin-induced apoptosis could be mediated by modulation of TGF-β/Smad pathway. In rat aortic VSMCs, preincubation with Simvastatin or Atorvastatin for 48 hours in medium without fetal bovin serum (FBS) did not induce apoptosis; however coincubation with TGF-β increased VSMC apoptosis. In VSMCs incubated with 10%FBS and treated with statins, apoptosis was induced. TGF-β blockade with either a TRI inhibitor or removing TGF-β present in FBS diminished statin-induced VSMC-apoptosis, indicating that TGF-β present in FBS was necessary for statin induced VSMC apoptosis. Preincubation of VSMCs with statins for 48h increased TGF-β-induced Smad2/3 phosphorylation and Smad-dependent transcription. After 48h of incubation both statins increased TRII protein expression and TGF-β secretion. Statin induced VSMC apoptosis and TRII expresion were reverted by mevalonate and GGP. Inhibition of RhoA/ROCK pathway (by C3 pretreatment or Overexpression of a dominant negative form of RhoA (DNRhoA), ROCK inhibition with Y-27632) increased VSMC apotosis, TGF-β-mediated Smad activation and overexpression of TRII and TGF-β, mimicking statins effects and showing the involvement of RhoA/ROCK inhibition. Overexpresion of Smad7 inhibited statin and DNRhoA-induced VSMC apoptosis. Statins increased TGF-β-dependent CTGF production. Blockade of endogenous CTGF expression by an antisense oligonucleotide abolished statin/TGF-β- induced apoptosis, suggesting that CTGF production was involved in this process. In the experimental model of atherosclerosis in ApoE knockout mice, ATV increased TRII, and CTGF expression in VSMC from injuried vessels correlating with caspase-3 activation. In summary, Statins makes VSMC more susceptible to TGF-β induced apoptosis. Our data described a close relation between statin and TGF-β in apoptosis, and suggests that this effect could be mediated by modulation of TGF-β/Smad pathway.

Keywords: VSMC, statins, TGF-beta, Smad, RhoA

Presenting author: Gael Roue

The multifunctional chemotherapeutic agent bendamustine hydrochloride (TREANDA™) activates caspase-dependent and –independent cell death in B-cell non-Hodgkin's lymphomas (B-NHLs) by ROS generation and p53-independent up-regulation of Noxa

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Chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL) are two different types of mature B-cell non-Hodgkin's lymphoma (B-NHL) characterized respectively by an indolent natural history with inevitable relapse, and an aggressive course with rapid disease progression. Both entities suffer from the absence of a specific therapeutic regimen, mainly due to frequent mutations in p53 gene and recurrent defects in the DNA damage response pathway. Bendamustine hydrochloride (TREANDATM) is a multifunctional, alkylating agent with a purine-like ring system that exhibits activity in multiple cancer models, but whose mechanism is as yet unknown. The present study shows that bendamustine exerts a cytotoxic effect on most primary CLL and MCL cells as well as in cell lines, irrespective of clinical prognostic factors and p53 status. Bendamustine cytotoxicity was mediated by the generation of reactive oxygen species (ROS) and p53-independent triggering of the mitochondrial apoptotic pathway, involving up-regulation of the BH3-only protein Noxa, conformational activation of Bax and Bak, and cytosolic release of the mitochondrial apoptogenic factors cytochrome c, Smac/DIABLO and AIF. Subsequent caspase-dependent signalling was observed by cytofluorimetric quantification of caspase-3 processing and partial protective effect of the pan-caspase inhibitor z.VAD.fmk. The contribution of AIF to caspase-independent signalling was then confirmed by morphological observation of apoptotic-like nuclei and intracellular introduction of a neutralizing anti-AIF antibody that significantly decreased bendamustine cytotoxicity. Importantly, bendamustine was active as a single agent in p53 mutated CLL and MCL cases resistant to conventional chemotherapy, and exerted synergistic effects in combination with the nucleoside analogues fludarabine and gemcitabine currently used in the clinical practice for the treatment of CLL and MCL. These findings support the use of bendamustine as a therapeutic agent for CLL and MCL and establish the molecular basis for its combination with conventional genotoxic agents whose activity depends on p53 functionality.

Keywords: DNA damage, p53, BH3-only, caspase-independent, lymphoproliferative disorder

Presenting author: Anita Rudy

Cephalostatin mediates apoptosis via Smac/Diablo and induces the formation of the PIDDosome.

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The cephalostatins are a promising group of compounds with marine origin, which show a unique growth inhibitory profile in the NCI-60 cell line screen.
Cephalostatin 1 induces apoptosis in tumor cells *via* an apoptosome-independent but caspase-9-dependent pathway and via an endoplasmatic reticulum stress response that is accompanied by caspase-4 activation (Cancer Res. 2003 Dec 15, 63(24): 8869-76, J Biol Chem. Nov 3, 281(44): 33078-86)
Here we show by western blot analysis and confocal microscopy that cephalostatin evokes mitochondrial Smac (second mitochondria-derived activator of caspases) but not cytochrome *c* release in various carcinoma cell lines. We also show that Smac is critically involved in caspase-9 but not caspase-4 activation as evidenced by gene silencing experiments. Remarkably, caspase-2 seems to be a major target for cephalostatin-induced cytosolic Smac. Using biochemical and genetic inhibition experiments we demonstrate that caspase-2 plays an important role in the apoptotic machinery induced by cephalostatin. Cephalostatin-activated caspase-2 acts as initiator caspase and does not seem to be involved in the activation of caspase-9. Importantly, experiments immunoprecipitating PIDD (p53-induced protein with a DD), RAIDD (RIP associated ICH-1/CED-3-homologous protein with DD) and caspase-2 identify cephalostatin as an experimental drug that induces recruitment of the PIDDosome, the macromolecular complex described to be responsible for caspase-2 activation. Therefore, the *bis*-steroid cephalostatin proves to be both a helpful tool to investigate apoptotic signaling as well as a promising chemotherapeutic agent.

Keywords: cephalostatin, smac, piddosome, apoptosis

Presenting author: Veronika Sackova

Caspase-independent apoptosis induced by hypericin in HT-29 cells is associated with increased acid and neutral sphingomyelinase activity

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Photodynamic therapy is a selective cancer treatment mode based on the light activation of a photosensitizer preferentially localized in tumor tissue with subsequent cell death induction both *in vitro* and *in vivo*.
Hypericin is a photosensitive agent effective in induction of active cell death in many cancer cell types. In the present study we partially demonstrate the apoptotic response mechanisms of hypericin treated HT-29 colon adenocarcinoma cell line. No elevated caspase activity (3, 8, 9) was detected in cells incubated with apoptotic concentrations (100 nM) of photoactivated drug at indicated time periods. Western blotting revealed the translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus. In contrast, release of further mitochondrial intermembrane proteins, cytochrome c and the other caspase-independent apoptotic factor, endonuclease G (EndoG), were not detected. The activity of both acid (aSMase) and neutral (nSMase) sphingomyelinase was analyzed at the same conditions of photodynamic protocol. None of tested sphingomyelinases were activated at short time intervals after hypericin activation (10 min, 30 min, 1 h) but longer time period analysis (24 h after photosensitization) revealed significant increase of both aSMase and nSMase activities in cells treated with apoptotic hypericin concentrations. This is the first report about the caspase-independent apoptosis via the AIF and activation of acid and neutral SMases induced by hypericin-mediated photodynamic therapy.

Acknowledgments. This work was supported by the Science and Technology Assistance Agency under Contract APVT-20-003704 and by the grant agency VEGA under Contract 1/2329/05.

Keywords: hypericin, HT-29, sphingomyelinase, AIF, EndoG,

Presenting author: Hela Saidi

Activated NK cells inhibit Apoptosis of HIV-1 infected Dendritic cells while inducing their Maturation by an HMGB1-dependent mechanism. Implication for the establishment of HIV-1 reservoirs

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Background: HIV infection is characterized by quantitative and qualitative alterations of dendritic cells (DC), an essential subset for both antigen-presentation and activation of naïve $CD4^+$ T cells. Recently, it was found that DC differentiation requires a cross talk with natural killer (NK) cells, leading to the maturation of both DC and NK cells. In this study, we analyzed the impact of HIV on NK-DC cross-talk and we addressed the question of the influence of R5 and X4-HIV-1 strains on the proapoptotic activity of NK cells on infected autologous DC.
Methods: PBMCs were isolated over Ficoll-Hypaque gradients. Cells were incubated at 37℃ for 45min and non-adherent cells were kept for subsequent NK cellsisolation whereas the adherent cell population was used to isolate monocytes. NK cells were freshly isolated by negative selection. Non-activated NK cells (naNK cells) were cultivated for 6 days in the presence of suboptimal dose of IL-2 whereas activated NK cells (aNK cells) were stimulated for 3 d with PHA and rhIL-2 at 10 ng/ml and with IL-2 for 2 additional days. To generate immatures dendritic cells (iDC), monocytes were isolated by negative selection and then cultured in complete media plus IL-4 and GM-CSF. After 5 days iDC were incubated with NK cells at NK:DC ratios of 1:5, 1:1 or 5:1. In some cases, iDC were induced to undergo maturation by stimulation with LPS (DC0), sCD40 (DC1) or LPS + PGE2 (DC2) for 24 h. They gave rise to mature DC (mDC). In other cases, iDC were infected by either R5-HIV-1_{JR-CSF} or X4-HIV-1_{NDK} for 24 h. Subsequently, infected DC were incubated with NK cells.
Results: aNK showed opposite effects on DC, depending on their maturation state: they induced apoptotis of iDC, while increasing the survival of mDC, such as DC0, DC1 or DC2. Following infection of iDC with R5 or X4 HIV-1 strains, aNK-dependent apoptosis was dramatically decreased (by 70-80%), as observed for uninfected mDC. Interestingly, HIV-infection of iDC did not induce their maturation, as evaluated by the expression of specific markers, such as CD80, CD86, CD83 and HLA-DR, while their coculture with aNK cells led to their maturation. The addition of polyclonal antibodies specific for HMGB1 molecule strongly inhibited NK-induced maturation of DC, suggesting that the pro-inflammatory cytokine HMGB1 is involved in this cross-talk.
Conclusion: Altogether, our results show that aNK cells inhibit apoptosis of infected DC by inducing their maturation, a process that allows the resulting mDC to migrate from peripheral tissue, the site of HIV-1 capture, to secondary lymphoid tissues, where they likely transfer efficiently the captured virus to T cells. Thus, NK cells would facilitate *in vivo* both HIV-1 transmission mediated by DC and persistence of the virus at a latent stage in infected DC.

Keywords: HIV-1 apoptosis maturation DC NK

Presenting author: Yolanda Sánchez

The soy isoflavone genistein selectively potentiates apoptosis induction by the antileukemic drug arsenic trioxide in human leukemia cells via reactive oxygen species generation

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The reported capacity genistein to cause intracellular oxidation lead us to check the ability of this isoflavone to modulate the toxicity of the reactive oxygen species (ROS)-sensitive antileukemic agent arsenic trioxide (ATO, Trisenox™) and, for comparison, other antitumour drugs. Co-treatment with 20-50 μM genistein potentiated apoptosis induction by 0.5-6 μM ATO in U937 promonocytes and other human leukemic cell lines (HL60, THP-1, Jurkatt, and RPMI 8866), but not in phytohemaglutinnin-stimulated, non-tumour peripheral blood lymphocytes. Genistein, alone and with ATO, transiently increased ROS accumulation in all cell types, and apoptosis was attenuated by the antioxidant agent N-acetyl-L-cysteine. Co-treatment with the polyphenolic agent epigallocatechin-3 gallate instead of genistein also increased ROS accumulation and potentiated ATO-toxicity; and the addition of exogenous H_2O_2 mimicked the capacity genistein to potentiate ATO-provoked apoptosis in leukemic cells, but not in non-tumour lymphocytes. On the other hand, neither genistein nor H_2O_2 potentiated the toxicity of other antitumour agents, namely the DNA-targeting drugs cisplatin and doxorubicin, the DNA deacetylase inhibitor MS-275, the proteasome inhibitor MG-132, and the anion superoxide-increasing agent 2-methoxioestradiol. Genistein, alone or with ATO, failed to affect the basal NF-κB binding activity, Akt and JNK phosphorylation, and intracellular reduced glutathione (GSH) content. However, it elicited N-acetyl-L-cysteine-inhibitable phosphorylation of the oxidant-inducible kinases AMPK and p38-MAPK, and apoptosis was attenuated by pharmacologic inhibitors against these kinases. It is concluded that genistein selectively potentiates ATO-provoked apoptosis via intracellular ROS production, a property which could be used to improve the clinical efficacy of this and perhaps other ROS-sensitive anti-leukemic drugs.

Keywords: Genistein, arsenic trioxide, apoptosis

Presenting author: Beatriz Santamaria

Regulation of mesothelial cell apoptosis during peritoneal dialysis (PD)

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During PD mesothelial cells are progressively lost and this limits the survival of the technique. Cell death by apoptosis contributes to the loss of mesothelial cells. We now report the modulation of mesothelial cell death by PD solutions, the glucose degradation product (GDP) present in PD solutions 3,4-dideoxyglucosone-3-ene (3,4-DGE), and the influence of an inflammatory milieu, such as that observed during bacterial peritonitis.

Mesothelial cells were grown from the effluent of PD patients.

PD solutions with a high content of both glucose and GDPs induced mesothelial cell apoptosis and loss of cell viability. 3,4-DGE also induced mesothelial cell apoptosis. Apoptosis induced by high-GDP PD solutions or 3,4-DGE was associated with aggregation of Bax at mitochondria, and activation of caspases and was prevented by Ku-70-derived peptide targeting Bax or the pan-caspase inhibitor zVAD. Taken together the data suggests that high GDP PD solutions and 3,4-DGE share the pathways for apoptosis induction and that 3,4-DGE is the main toxic metabolite in PD solutions.

The proinflammatory cytokines TNFα or IFNγ do not induce apoptosis in human mesothelial cells. However, their combination promotes apoptotic cell death in human mesothelium. The combination of high GDP PD solutions (but not low -GDP PD solutions) with in a proinflammatory millieu further increased the rate of cell death. The pancaspase inhibitor zVAD-fmk and specific caspase-8 and caspase-3 inhibitors prevented apoptosis. Co-incubation with both cytokines also impaired mesothelial wound healing in an in vitro model. However, inhibition of caspases did not prevent this effect.

In conclusion, both endogenous (cytokines secreted during peritonitis) and exogenous factors (high-GDP PD solutions) cooperate to induce mesothelial cell apoptosis. 3,4-DGE is the main proapoptotic factor in high GDP PD solutions. The use of such solutions should be avoided during peritonitis. If they cannot be avoided, the use of inhibitors of apoptosis in vivo should be studied.

Keywords: Peritoneal dialysis,glucose degradation products,cytokines,caspases, mesothelium

Presenting author: Lucília Saraiva

Yeast as a new approach to study the regulation of p53 activity by individual protein kinase C isoforms

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Cancer represents an imminent human health problem and the tumour suppressor p53 one of the most relevant protein in carcinogenesis [1]. p53 suppresses tumours by induction of growth arrest or apoptosis, responses that protect a cell from uncontrolled proliferation and neoplastic transformation. Consequently, elucidation of p53-signalling pathway and the identification of key components of its pathway that regulate p53 activity, is a major challenge in cancer biology [1].
Several studies revealed that post-translational modification, by phosphorylation, of p53 constitutes an important mechanism of p53 regulation, and that protein kinase C (PKC) represents an important family of kinases involved in the phosphorylation and consequent activation of p53 [2].
In order to elucidate the role of each PKC isoform in p53 regulation, the human wild-type p53 protein was co-expressed, in the yeast *Saccharomyces cerevisiae,* with the individual PKC isoforms: PKC-alpha (a classical PKC), PKC-delta, PKC-epsilon (novel PKCs) or PKC-zeta (an atypical PKC). Firstly, we confirmed that, as reported [3], expression of p53 induces a marked yeast growth inhibition, without causing cell death. Afterwards, we detected that, among the isoforms tested, only the novel PKC-delta and PKC-epsilon, significantly increase p53-induced growth inhibition and cell cycle arrest. The effect of PKC isoforms on p53 activity was also analysed in yeast treated with the cell death inducer H_2O_2. The results obtained showed that p53 stimulates H_2O_2-induced cell death and that only PKC-delta and PKC-epsilon significantly increase p53-stimulation of cell death, an effect accompanied by preservation of cell membrane integrity and by an increase on DNA fragmentation and reactive oxygen species production. p53 phosphorylation by PKC-delta and PKC-epsilon, both in the absence and in the presence of the cell death inducer H_2O_2 is being assessed to further confirm these results.
This work reinforces the use of yeast as a new approach to study p53-signalling pathway and contributes to the elucidation of the role of individual PKC isoforms in the regulation of p53 activity.

We thank to REQUIMTE/CEQUP, to Universidade do Porto and Caixa Geral de Depósitos for financial support.

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Keywords: p53-signalling pathway, PKC isoforms, yeast

Presenting author: Andrew Schile

Regulation of Apoptosis by XIAP Ubiquitin-Ligase Activity

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Tight negative regulation of the apoptotic machinery prevents inappropriate activation of cell death pathways. Inhibitor-of-apoptosis proteins (IAPs) prevent the induction and activity of caspases. The X-linked inhibitor-of-apoptosis protein (XIAP) is considered the principle IAP in mammalian cells. XIAP regulates apoptosis with a combination of BIR motifs that bind and inhibit active caspases, as well as a RING domain that functions as an E3 ubiquitin-ligase. Prior evidence suggests that XIAP RING can promote polyubiquitination and degradation of XIAP itself, as well as binding partners like caspase-3 and antagonists like ARTS and Smac. Structural, biochemical and overexpression studies offer many insights into possible modes of XIAP activity, but how endogenous XIAP functions in physiological situations remains an open question. We used gene targeting in the mouse to remove the RING and generate a truncated protein. This has allowed us to study the relative contribution of XIAP-directed ubiquitination to regulating apoptosis. RING-deleted mice were fertile, born at Mendelian ratios for gender and genotype, and displayed no overt phenotypes or susceptibility to disease. Removal of the RING in cultured embryonic stem cells and fibroblasts sensitized these cells to diverse apoptotic stimuli. Wild-type and RING-less XIAP were expressed comparably throughout apoptosis in embryonic cells, but caspase activity was significantly elevated in RING-less cells. TUNEL staining increased accordingly in RING-less cells. This result correlated with an increased abundance of active caspase-3 subunits and provided genetic evidence for XIAP RING-dependent regulation of caspase stability in embryonic cells. Thymocyte apoptosis is characterized by IAP degradation, but the significance of this phenomenon is uncertain. Removal of the RING stabilized XIAP during thymocyte apoptosis, whereas full-length XIAP was degraded by the proteasome in wild-type cells. A slight decrease in RING-less XIAP expression was attributed mostly to caspase proteolysis. Removal of the RING did not alter the degradation of Smac or c-IAP1 during thymocyte apoptosis. The persistence of stabilized XIAP in apoptotic thymocytes did not affect the induction or activity of caspases, however, and cell death occurred normally. Moreover, deletion of the XIAP RING did not affect thymus composition or cellularity.

Keywords: ubiquitin xiap ring

Presenting author: Jan Schmidt-Mende

Hypomethylation and apoptosis in 5-ayacytidine treated myeloid cells

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INTRODUCTION: Although the hypomethylating nucleoside analogue 5-azacytidine (5-aza) is the first drug approved for the treatment of the Myelodysplastic Syndromes, its mode of action is still unclear. In the present study, mechanisms for 5-aza-induced apoptosis were investigated. These results were compared to 5-aza-induced alterations in DNA methylation.
MATERIALS AND METHODS: Myeloid P39 and HL-60 cells were treated with 5-aza in different apoptosis-inducing concentrations. Subsequently, specific apoptotic pathways were investigated. Global DNA methylation was assessed by a Luminometric Methylation Assay (LUMA).
RESULTS: 5-aza induced dose-dependently apoptosis. In parallel, global DNA hypomethylation was dose-dependently increased. When sorting non-apoptotic and apoptotic cells, hypomethylation was solely observed in the apoptotic cell fraction, while non-apoptotic cells retained a methylation pattern similar to untreated cells.
5-aza-induced apoptosis involves several pathways: cleavage of Bcl-2 family proteins, activation of caspase-2 and -3. Mitochondrial changes were characterized by loss of the transmembrane potential and the release of cytochrome c. Furthermore, the cytosolic pH shifted towards acidic pH in 5-aza-treated cells. Selective inhibition of caspase-3,-2,-8,-9 and general caspase inhibitors as well as stabilization of cytosolic pH by monensin completely failed to block apoptosis. Interestingly, Poly (ADP-ribose)polymerase (PARP) inhibitors partially blocked apoptosis and caspase-2 activity. This indicates that PARP activation upstream of caspase-2 might be an important mechanism in 5-aza-induced apoptosis.
The non-hypomethylation drug cytosine arabinoside induced a similar degree of apoptosis, while leaving methylation status mainly unaffected.
CONCLUSION: 5-azacytidine acts via multiple and separately regulated pathways, including parallel induction of hypomethylation and a complex pattern of apoptotic mechanisms. The broad action of 5-azacytidine may explain its therapeutic effects, even in poor-prognostic MDS.

Keywords: DNA methylation, leukemia, apoptosis, 5-azacytidine

Presenting author: Uta Monika Schneiders

Spongistatin 1 induces apoptosis involving the BH3-only protein Bim and the endonuclease G

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Spongistatin 1 is a remarkable natural compound, isolated from the sponges *Spirastrella spinispirulifera* and *Hyrtios erecta*, that exhibits a strong cytotoxic potence in the human tumour cell line screen of the National Cancer Institute. However the apoptotic mechanisms involved remain unknown. In our study, we examine the apoptosis-inducing potential and the underlying apoptotic signalling pathway of spongistatin 1 in MCF-7 cells, a epithelial breast cancer cell line deficient in caspase-3.
Our results show that spongistatin 1 induces apoptosis in a time- and dose-dependent manner and exerts also long term effects on the clonogenic survival of MCF-7 cells. Interestingly, the spongistatin 1 induced apoptosis is largely caspase-independent as shown by the use of the pan-caspase-inhibitor zVAD.fmk. Spongistatin 1 triggers the release of the proapoptotic proteins from the intermembrane space of mitochondria to the cytosol, such as AIF and EndoG, important factors acting independendly of caspases during apoptosis. Indeed AIF and EndoG translocate to the nucleus after stimulation with spongistatin 1 and they play a functional role in the induction of apoptosis shown by silencing the respective genes. The next step was to indentify upstream effectors in spongistatin 1 induced apoptosis. Since spongistatin 1 acts as a tubulin depolymerizing agent, microtubule interacting factors could play an important role in the induction of apoptosis by spongistatin 1. Therefore, the proapoptotic Bcl-2 family member Bim (Bcl-2 interacting mediator of cell death) is of particular interest to our work. Silencing of Bim by siRNA infact lead to diminished release of mitochondrial proteins as well as to decreased translocation from AIF and EndoG to the nucleus resulting in a decreased DNA fragmentation and apoptosis. Therefore Bim is regarded to be a major proapoptotic regulator targeted by spongistatin 1 upstream of mitochondria.

Keywords: apoptosis, spongistatin 1, Bim, EndoG

Presenting author: Elisa Schulze Schleithoff

The status of the p53 family network determines induction of apoptosis and treatment response in hepatocellular carcinoma

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Molecular links between apoptosis, tumourigenesis and drug resistance provide the foundation for new therapeutic approaches and for a targeted cancer therapy. Recent studies show that p53 is not the only component in predicting prognosis and response to chemotherapy, but instead the status of a network that contains p53 and the “younger” p53 family members p63 and p73. We investigated the mechanisms by which TAp73/TAp63. isoforms and dominant-negative p73/p63 isoforms (deltaNp73/deltaNp63) regulate apoptosis and influence sensitivity to anticancer therapy in hepatocellular carcinoma (HCC). The ratio of TA isoforms / deltaN isoforms of the p53 family is a critical component in tumorigenesis, tumor progression and treatment sensitivity. Overexpression of deltaNp73 in HCC patients correlates with reduced survival (Gressner et al., EMBO J, 2005; Müller et al., Cell Death Differ 2005, Kern et al., Cancer Res, 2006). Furthermore, we investigated the impact of different p53 mutants on the induction of apoptosis and their interaction with TAp63 and TAp73. Different p53 mutants have specific effects on the induction of apoptosis in HCC. We could show that p53 hot spot mutants (175H, 248W and 273H) inhibit death receptor-mediated apoptosis signalling pathways and thus acquire so called “gain-of-function” effects.In addition and of clinical importance, we found that these p53 hot spot mutants inhibit TAp63- and TAp73-mediated transactivation of the CD95 gene. Thus, p53 “gain-of-function” may in part be explained by interference with TAp63- and TAp73-mediated transcriptional activation of apoptosis target genes.
Therapeutic modulation of the ratiosTAp63/deltaNp63 and TAp73/deltaNp73 and additional efforts aimed at liberating TAp63/TAp73 from blockade by mutant p53 may constitute new strategies for the treatment of hepatocellular carcinoma.

Keywords: p53, p63, p73, chemosensitivity, hepatocellular carcinoma

In vivo modulation of apoptotic proteins in female rat CA1 with long-term estrogen or tamoxifen therapy

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Estrogen-mediated neuroprotection has been elucidated by several *in vivo* and *in vitro* studies. Although several direct estrogenic effects on neurons are known, the cellular mechanisms behind the neuroprotective efficacy of the steroid are only beginning to be elucidated. Moreover, the synthetic ligand of estrogen, tamoxifen (TAM) is a first generation selective estrogen receptor modulator (SERM) and its exact biocharacter in the CNS is yet to be fully unraveled.

In our laboratory, the ovariectomized rats constitute an interesting *in vivo* model to study the effects of hormone and the SERM. To begin with the OVX rats were either induced with E2 or TAM for a period of four weeks and thereafter the immunoexpressions of Bcl2 and Bax were analyzed on fixed brain tissue sections. The results revealed that with ovariectomy the apoptotic repressor protein Bcl-2 was downregulated while, the pro-apoptotic protein Bax was upregulated in the CA1 subregion of hippocampus. The Bcl2:Bax ratio revealed a shift towards pro-apoptotic pathway. Subsequent E2 (0.1 mg/kg bw) or TAM (0.05 mg/kg bw) therapy to the OVX rats (one s.c. inj/day), resulted in reversal of the results showing thus upregulation of immunoexpressions of the above molecules and a shift of Bcl2:Bax ratio towards the anti-apoptotic pathway. In conclusion, E2 and TAM are neuroprotective for CA1 neurons. Also, the SERM, TAM displays agonistic effects in CA1 of female rat hippocampus.

Keywords: ovariectomy, estradiol, SERM, apoptosis,neuroprotection

Presenting author: Manu Sharma

Mcl-1 is required for apoptosis resistance in *Chlamydia trachomatis* infected cells

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Chlamydia are obligate intracellular bacteria that cause a variety of human diseases. Host cells infected with *Chlamydia* are protected against many different apoptotic stimuli. The induction of apoptosis resistance is thought to be an important immune escape mechanism allowing *Chlamydia* to replicate inside the host cell. We performed an RNA interference screen to identify factors involved in the infection-induced inhibition of TNF-induced apoptosis. Among the factors whose ablation sensitized the infected cells, there were those which affected the Raf/MEK/ERK and the PI3K/AKT pathway and the Bcl-2 family member Mcl-1. Infection with *C. trachomatis* activates the Raf/MEK/ERK pathway and the PI3K/AKT pathway and the inhibition of these two pathways by chemical inhibitors sensitized *C. trachomatis* infected cells to TNF-, but also to granzyme B-mediated cell death. Infection leads to the Raf/MEK/ERK-mediated up-regulation and PI3K-dependent stabilization of the anti-apoptotic Bcl-2 family member Mcl-1. Consistently, interfering with Mcl-1 up-regulation sensitized infected cells for apoptosis induced via the TNF receptor, DNA damage and stress. Our data suggest that Mcl-1 up-regulation is primarily required to maintain apoptosis resistance in *C. trachomatis* infected cells.

Keywords: Chlamydia, apoptosis, Mcl-1, MAPK, Raf/MAPK, PI3K/AKT, mitochondria

Presenting author: Rui D Silva

Mammalian protein kinase C-α modulates Bax-induced cell death in yeast

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Mammalian protein kinase C (PKC) isoforms have been subject of intensive research due to their capability to modulate mammalian apoptotic proteins, namely Bcl-2 family members. Yet, the role displayed by each PKC isoform in apoptosis is still unclear due to the co-existence of several isoforms in mammalian cells [1]. Expression of pro-apoptotic Bcl-2 family members (e.g. Bax or Bak) in yeast induces cell death and this effect is prevented by co-expression with anti-apoptotic members (e.g. Bcl-2 or Bcl-xL) [2]. Recently, we showed that Bcl-xL can delay acetic acid-induced apoptosis in yeast and that distinct PKC isoforms can specifically modulate Bcl-xL anti-apoptotic effect [3]. This work supported our purpose to use yeast as a model system to study regulation of Bcl-2 family members by PKC isoforms.

In this line, and to study the role of PKC-α in Bax regulation, we co-expressed these two proteins in yeast. We observed that PKC-α co-expression increases Bax-induced cell death, an effect accompanied by preservation of membrane integrity and by an increased in the mitochondrial network fragmentation and reactive oxygen species production when compared to Bax expression alone. Consistent with these results, Western blot analysis showed a more than two fold increase on Bax expression in yeast co-expressing PKC-α what probably suggest that PKC-α leads to Bax accumulation in the cell and therefore to enhanced cell death. To confirm this hypothesis, further studies are being carried out in order to understand how PKC-α regulates the amount of Bax in yeast cells.

Together our findings indicate that PKC-α regulates Bax activity and further validate yeast as a tool to study regulation of Bcl-2 family members by PKC isoforms.

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Keywords: Protein Kinase C; Bax regulation; Yeast

Presenting author: Michelle Smith

Apoptosis Regulation in Developing Skeletal Muscle: Novel Pathways and Increased Control

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Apoptosis of myotubes not only occurs during normal muscle development and aging but it can lead to the destruction of skeletal muscles in many muscular dystrophies including Duchenne, and spinal muscular atrophy. Therefore, understanding the regulation of apoptosis in myotubes is important for developing novel strategies in the treatment of skeletal muscle loss.

Previous work in our lab had identified a unique level of apoptotic control in postmitotic neurons. While microinjection of cytosolic cytochrome *c* is sufficient to activate caspases in many mitotic cells, primary sympathetic neurons are markedly resistant. This resistance is due to the X-linked inhibitor of apoptosis protein (XIAP), which exerts strict control over this cytochrome *c* mediated caspase activation. Arguably, skeletal muscle would also benefit from an increased apoptotic regulation as these cells are postmitotic and their loss results in severe functional defects. In this study, we have investigated whether caspase activation in myotubes is regulated similar to neurons and whether there is an increase in the regulation of apoptosis as mitotic myoblasts are postmitotically differentiated into myotubes.

We found that differentiated C2C12 cells (skeletal muscle cell line) and cultured primary mouse myotubes are strikingly resistant to cytochrome c injection. Additionally, these postmitotic myotubes were capable of undergoing apoptosis in response to cytochrome c when XIAP was either functionally or physically inactived. Interestingly, this increased regulation was not due to a difference in the levels of XIAP as the protein levels are similar to that seen in other cell types.

Our examination of the apoptotic regulation of the mitotic precursor cells, myoblasts, revealed that as anticipated, mitotic C2C12 cells did not show any resistance to cytochrome c. Injection of tbid into cultured primary mouse myoblasts, to cause the release of mitochodrial factors, was sufficient to induce caspase dependent death. Surprisingly, Apaf-1 deficient myoblasts were not resistant to tbid expression, implying that caspase activation in primary myoblasts is Apaf-1 independent. This data suggests that there is a potentially unknown myoblast specific factor that can serve to activate the initiator caspase 9 independently of the apoptosome.

Keywords: XIAP, skeletal muscle, Apaf-1

Presenting author: Dominic Stadel

A combined sensitizer/inducer strategy of XIAP inhibition and TRAIL overcomes apoptosis resistance of pancreatic carcinoma cells and causes regression of established tumors *in vivo*

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Pancreatic cancer is one of the leading causes of cancer-related death highlighting the need for novel treatment strategies. Apoptosis resistance is a hallmark of pancreatic cancer and may be caused by high expression of anti-apoptotic proteins. Here, we establish a new paradigm for apoptosis-based therapies in pancreatic cancer by identifying XIAP as novel therapeutic target that critically determines apoptosis sensitivity. We found that XIAP is selectively overexpressed in pancreatic cancer compared to non-neoplastic pancreatic ducts. Strikingly, targeting XIAP by RNAi-mediated knockdown cooperates with TRAIL *in vivo* to suppress pancreatic cancer formation in a tumor establishment model in xenograft-bearing mice. Most importantly, XIAP knockdown and TRAIL also synergize to cause profound and sustained regression of established pancreatic carcinoma *in vivo* even after discontinuation of TRAIL treatment. Interestingly, the synergistic antitumor activity of XIAP knockdown and TRAIL is reflected at the cellular level by increased levels of active caspase-3 and apoptosis in tumor tissue *in vivo*. By demonstrating that targeting XIAP and TRAIL synergize to trigger apoptosis and to cause regression of established pancreatic carcinoma *in vivo*, our findings have important clinical implications. Thus, this combined sensitizer (XIAP inhibitors)/inducer (TRAIL) concept is a novel and effective antitumor strategy to overcome apoptosis resistance of pancreatic cancer that warrants further exploitation.

Keywords: XIAP, TRAIL, apoptosis, pancreatic carcionoma

Presenting author: Grzegorz Stasilojc

Distinct apoptotic phenotypes in two U937 cell variants

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The U937 histocytic leukemia cells is a widely studied model of apoptosis. We identified two variants of the cell line differing with respect to morphology of cells dying of apoptosis. In one of the variants, termed $U937_M$, we did not observe apoptotic body formation and disintegration of cells, while in another, $U937_{ATCC}$ cells, these two phenomena were evident. Different morphology of dying $U937_M$ cells was independent on stimuli used, as those activating extrinsic or intrinsic pathway of apoptosis induced similar phenotype. The main goal of the presented study was to identify a mechanism responsible for defective apoptosis occuring in $U937_M$ cells. We found that following apoptosis induction these cells in comparison to reference $U937_{ATCC}$ cells were characterized by faster and more pronounced decrease of mitochondrial membrane potential, more intense generation of reactive oxygen species and lower ability to reduce MTT to formazan. Additionally, pan-specific inhibitor of caspases z-VAD-fmk inhibited decrease of mitochondrial membrane potential only in the reference $U937_{ATCC}$ cells. Applying the caspase inhibitor in $U937_{ATCC}$ cells inhibited blebbing, formation of apoptotic bodies and cell disintegration. Applying Y27632, inhibitor of ROCK1 kinase, inhibited disintegration of $U937_{ATCC}$ cells stimulated by apoptosis inducers. In the $U937_M$ cells both caspase and ROCK1 inhibitors did not affect significantly morphology of dying cells. The lack of effect of caspases and ROCK1 inhibition in the $U937_M$ cells could indicate insufficient activation of executioner caspases needed for apoptotic bodies formation and cell disintegration. However, we found that in both cell lines inbibition of caspases by z-VAD-fmk decreased DNA degradation to the same extent. Obtained results indicate that defective apoptosis observed in the $U937_M$ variant cells is characterized by biochemical features which may reflect distinct mechanism of mitochondrial function and disturbed executioner phase of apoptosis, probably not dependent on direct of caspases activity.

Keywords: apoptosis, U937 variants, cell death morphology

Nuclear localized protein-1, a novel interactor of X-linked Inhibitor of Apoptosis Protein

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X-linked inhibitor of apoptosis protein (XIAP) is the most characterized member of the inhibitor of apoptosis protein family. XIAPs main mechanism of action is the direct binding and inhibition of processed caspase-3, -7 and -9, making XIAP a potent anti-apoptotic protein. Therefore, XIAP has been considered a therapeutically promising protein and as such it is of importance to fully characterize the physiological functions of the protein. A yeast-2-hybrid method was used to find novel interactors of XIAP, and the putative basic helix-loop-helix protein Nuclear localized protein-1 (Nulp1) was found to bind XIAP. In mammalian cells, staurosporine-induced apoptosis was required for the interaction of XIAP and Nulp1 to be detected. Exogenous expression of Nulp1 induced DNA-fragmentation in human Saos-2 cells and sensitized Neuro-2a cells to staurosporine-induced cell death. Co-transfection with XIAP in Saos-2 cells did not protect against the effect of Nulp1, neither did Nulp1 increase caspase-3 activity in staurosporine-treated N2a-cells. This suggests that the effect of Nulp1 is independent of caspase-3 and that XIAP is not able to protect against cell death induced by Nulp1 in tumour cells. The precise role of Nulp1 in apoptosis, and the functional implication of the XIAP-Nulp1-interaction, remains to be determined.

Keywords: XIAP, transcription factors, yeast-2-hybrid

Presenting author: Sandra Steinbrink

Genome-wide siRNA screens reveal new insights into death-receptor mediated apoptosis

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A systematic characterization of genes with unknown function remains a key challenge in modern biology. We are using genome-wide RNAi screens to systematically identify cellular signalling pathway components.

One of our approaches is to combine genome-wide siRNA knockdown experiments with death-receptor ligand treatment to identify components involved in receptor mediated cell death signalling pathways. The phenotype is measured by homogenous assays, such as luminescence or fluorescence that allow us to identify siRNAs that “rescue” the cell death phenotypes and are candidates for pro-apoptotic pathways. We have screened multiple independent pro-apoptotic pathways and compared the RNAi-phenotype profiles to identify specific and generic pathway components.

One of our candidates revealed a particular connection between death-receptor mediated apoptosis and evolutionary conserved signalling pathways important for development and several diseases. So far this protein was known as an important negative regulator of cell proliferation signals, but has not been linked to death receptor mediated apoptosis. At the moment, we are validating and characterizing this so far undiscovered connection to shed light on the biological mechanism.

Keywords: siRNA screens, death-receptor mediated apoptosis, signaling

Presenting author: Tatiana Storozhevykh

Cortexin protects rat cerebral neurons against glutamate-induced apoptosis

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The cerebral cortex lysate cortexin, a balanced composition of L-aminoacids, vitamins and microelements, was developed at Military Medical Academy of Saint-Petersburg, Russia. Cortexin has been shown to be effective in treatment of cerebral trauma, ischemic or hemorrhagic stroke, perinatal CNS disorders and others conditions that are commonly associated with release and impared uptake of glutamate and hyperstimulation of glutamate receptors. We studied the effects of cortexin on neurotoxicity induced by glutamate or growth factor B-27 withdrawal (with low 5 mM K+) in cultured cerebellar and cortical neurons. The cultures were double stained with two fluorescent dyes: the ethidium homodimer bromide for necrotic cells counting and the DNA-binding dye Hoechst 33342 for apoptotic nuclei visualization. The results were compared with MTT test. At 24 h after glutamate exposure (100 µM, 60 min) 22.9% of examined young (7-9 DIV) cerebellar neurons were apoptotic and 33.1% were necrotic. In cortical cultures only 9.9% of cells were alive. The B-27 withdrawal and low K+ induced the apoptotic death in 17.2 % of young cerebellar neurons and in 44,6% of old ones. Co-incubation of cultures with 100 ng/ml of cortexin during and 24 h after the glutamate exposure reduced the number of apoptotic cells in the young cerebellar and cortical cultures by 1.4 – 1.6-fold ($p < 0.01$). Protective effect of cortexin was less pronounced in old cerebellar and cortical neurons (5-7%, $p > 0.05$). Cortexin decreased neuronal apoptotic death caused by a B-27 withdrawal down to 11.8% in young cerebellar cells and to 27.4% in old ones. Necrotic death was not affected by cortexin at used concentration. The results suggest that clinical efficacy of cortexin may be partly due to inhibition of apoptosis intracellular signals. The work was supported by Geropharm Company (St.Petersburg, Russia).

Keywords: neuron, glutamate, cortexin, apoptosis

Effect of co-planar and non-planar hexa-chlorobiphenyls intoxication on development of male reproductive tract in rats

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Polychlorinated biphenyls (PCBs) are widespread and persistent environmental pollutants. Structural diversity of PCB congeners effects their residual levels, excretion and toxicity on animals and humans. The aim of our study was to examine effects of co-planar and non-planar hexachlorobiphenyls on testes development in male rat offspring exposed to PCBs during suckling period. 20 lactating Wistar rats were administered PCB congeners (PCB-169 and PCB-155) dissolved in olive oil or placebo (olive oil) by intraperitoneal injection. Young rats were sacrificed on 9th, 22nd and 42nd day, internal organs including the testes were collected, fixed and embedded in paraffin. Basic morphological parameters were measured on histological specimens in testes and proliferating and apoptotic indexes determined using the Ki-67, PCNA and caspase-3 antibodies and Klenow-FragELTM DNA fragmentation detection kit. Co-planar PCB-169 caused severe growth hindrance and liver damage, however the male offspring had seemingly normal development of the testes including ratio between proliferation and spontaneous apoptosis. However, in 6-week-old rats exposed to generally less toxic non-planar PCB-155 a significant amount of apoptotic Leydig cells was found. This suggests exposure to PCBs in early age can affect endocrine system and consecutive reproductive capacity in adult life.

Keywords: Polychlorinated biphenyls, apoptosis, Leydig cell, testis, rat

Presenting author: Yeu Su

Thymosin β_4 downregulation induces growth suppression and microfilament disruption in SW480 human colon carcinoma cells

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Colorectal carcinoma (CRC) is a highly fatal disease due mainly to its metastasis even though reduced tumor relapse could occasionally be accomplished by surgery and combined chemotherapy. Our recent studies have demonstrated that thymosin β_4 ($T\beta_4$), the major G-actin sequestering peptide in mammalian cells, not only is upregulated in the tumor tissues in the majority of metastatic CRC patients but also plays an important role in promoting the malignant progression of this deadly disease. Interestingly, downregulation of *$T\beta_4$* has been reported in apoptotic tumor cells triggered by various anticancer drugs. Moreover, siRNA-mediated knockdown of this gene in HeLa cells has been shown to increase their sensitivity to paclitaxel. To test whether $T\beta_4$ could be a therapeutic target for CRC, we generated recombinant adenoviruses carrying DNA fragments encoding two $T\beta_4$ shRNAs and used them to infect SW480 colon cancer cells. As expected, *$T\beta_4$* expression in these cells was dramatically reduced after infection. In the meantime, their growth was greatly suppressed and the number of dead cells was increased as the amounts of virus were increased. Intriguingly, while the population in G_0/G_1 phase of cell cycle was reduced upon *$T\beta_4$* knockdown, the number of aneuploid cells was markedly increased. Surprisingly, a disruption, rather than a stabilization, of F-actin microfilaments was detected in $T\beta_4$-downregulating cells. Together, our results warrant further investigations of using $T\beta_4$ as a target for developing novel therapeutic approaches for CRC.

Keywords: Thymosin â4, colon cancer, adenovirus, growth suppression

Presenting author: Vadim Sumbayev

Cross-talk of apoptosis signal-regulating kinase 1 (ASK1) downstream pathway and hypoxic signal transduction as a part of LPS-induced Toll-like receptor 4 signalling

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Toll-like receptor 4 (TLR4) is one of the most physiologically important TLRs required for recognition of lipopolysaccharide (LPS) of Gram-negative bacteria and induction of innate immune response to them. TLR4 is expressed in different cell types including myeloid cells, the key effectors of innate immune reactions. Though several aspects of TLR4 signalling are well investigated, involvement of some crucial pathways is not fully understood. We have found that LPS-induced TLR4 signalling triggers cross-talk of hypoxic signal transduction regulated by hypoxia inducible factor 1 alpha (HIF-1α) and ASK1 downstream pathway in THP-1 human myeloid monocytic leukaemia cells. Both pathways are activated via redox-dependent mechanism associated with tyrosine kinase/phospholipase C-1γ-mediated activation of protein kinase C α/β that activates NADPH oxidase and therefore production of reactive oxygen species that activate both HIF-1α and ASK1. ASK1 contributes to stabilisation of HIF-1α protein via activation of p38 MAP kinase that directly phosphorylates HIF-1α. TLR4-dependent activation of PI3-kinase promotes expression of HIF-1α but down-regulates activity of ASK1 protecting the cells against apoptosis. In addition, LPS-induced, HIF-1α-dependent, production of reactive nitrogen species leads to direct S-nitrosation and therefore down-regulation of ASK1 protecting cells against apoptosis. Furthermore, HIF-1α protein is known to activate ASK1-specific protein phosphatase (PP5), which dephosphorylates and therefore attenuates kinase activity of ASK1 protecting the cells from ASK1-induced apoptosis. Experiments with transfection of THP-1 cells with HIF-1α siRNA suggested that this protein is critical for regulation of energy metabolism and protection of LPS-stimulated cells against ASK1-induced programmed death. On the other hand HIF-1α protein and its cross-talk with the ASK1 downstream pathway appeared critical for TLR4-dependent cytokine production.

Keywords: Toll-like receptors, hypoxia, MAP kinases, apoptosis, inflammation and innate immunity

Presenting author: Xiao-Ming Sun

Bongkrekic acid –a cautionary tale!

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The mitochondrial permeability transition pore (PTP) plays a major role in both apoptosis and necrosis. The exact composition of the PTP is still debated although there is much data supporting adenine nucleotide translocator (ANT) in the inner mitochondrial membrane (IMM) and voltage dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM) as major components of the PTP. The normal physiological role of ANT is as an ADP/ATP carrier to transport ADP from the cytosol to the mitochondrial matrix and export ATP to the cytosol following its synthesis. ANT is a member of the mitochondrial carrier family of proteins. Bongkrekic acid (BKA), a complex fatty acid derivative, is secreted by the bacterium *Pseudomonas cocovenans* and binds to the matrix side of ANT. It is commonly used in many studies as apoptosis inhibitor due to its effects on inhibiting PTP. We have found that BKA is highly toxic to freshly isolated primary cells from patients with chronic lymphocytic leukemia (CLL) causing a concentration dependent toxicity in the sub-micromolar range. Sub-micromolar concentrations of BKA are also toxic to Jurkat cells. These results were particularly surprising as BKA has been used in micromolar concentrations in many studies of apoptosis. BKA (10-30 nM) was clearly affecting mitochondria in CLL cells as evidenced by a hyperpolarisation of mitochondria. We wished to assess the effects of BKA on induction of apoptosis by the novel Bcl-2 antagonist, ABT-737. ABT-737 caused a rapid induction of apoptosis in CLL cells. BKA (10-30 nM) inhibited ABT-737-induced apoptosis assessed by loss of mitochondrial membrane potential and by blocking the increase in phosphatidylserine externalisation. However BKA failed to inhibit release of cytochrome c and only partially inhibited activation. Ultrastructural examination of CLL cells exposed to BKA showed dramatic nuclear changes possibly related to a decreased production of ATP. These studies demonstrate a totally novel cellular effect of BKA.

Keywords: Bongkrekic acid PTP apoptosis

Presenting author: Aliz Szabo

PARP-1 inhibition-induced activation of PI-3-kinase-Akt pathway by treatment of Taxol

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Paclitaxel (Taxol) is an antineoplastic agent that specifically targets microtubules and arrests cells at the G2/M phase of the cell cycle. In addition, Taxol increases mitochondrial ROS production which can lead to PARP activation and consequently to apoptotic or necrotic cell death. To determine the role of PARP activation in the Taxol induced cell death process, PARP activation was suppressed by pharmacological inhibitor PJ34 and by the transdominant expression of the PARP-1 DNA binding domain. Both type of inhibition of PARP-1 activation lead to suppressed poly-ADP-ribosylation of nuclear proteins, prevention of NAD^+ depletion and a significant resistance to taxol induced cell death. PARP-1 inhibition abrogated the taxol-induced caspase-3 activation, indicating the prevention of taxol induced apoptotic cell death. Taxol induce a small increase of Akt activation which was augmented by PARP inhibition which is in accord to our previous observation that PARP inhibition induced AKT activation via Src-PI-3-kinase-Akt pathway. The prevention of the PARP inhibition induced activation of PI-3-kinase-Akt pathway by LY significantly reduced the cytoprotective effect of PARP inhibition. Since it is well documented that activation of PI-3-kinase-Akt cascade induced resistance against Taxol induced cell death, it is likely that PARP inhibition mediates it antiapoptotic effect through this pathway. These data show that the inhibition of PARP-1, beside its cell death promoting effect through the inhibition of DNA repair, causes resistance to antimicrotubule treatment likely through the activation of the PI-3-kinase-Akt pathway.

Keywords: Taxol, Akt, PARP, apoptosis

Presenting author: Zsuzsa Szondy

Roles of tissue transglutaminase in the phagocytosis of apoptotic cells

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Tissue transglutaminase (TG2) is a protein crosslinking enzyme with various additional biological functions including G protein activity, integrin receptor and fibronecting binding. We have reported that in $TG2^{-/-}$ mice the clearance of apoptotic cells is defective leading to autoimmunity. Here we investigated the role of TG2 in the phagocytosis of apoptotic cells and found that the loss of TG2 leads to abnormal rac activation and disturbances in the cytoskeletal rearrangements during phagocytosis. Rac activation during phagocytosis of apoptotic cells is regulated by the coordinated action of Mer family tyrosine kinase and alphav beta3 integrin receptors in macrophages. Both of these receptors recognize phosphatidylserine, an early “eat me” signal of apoptotic cells via bridging molecules through the opsonizing proteins Gas6, or MFG-E8, respectively. In TG2 null macrophages the Mer mRNA levels are unchanged, but the beta3 integrin mRNA and protein levels are increased and the receptor does not respond properly to extracellular stimulation. To investigate which biological role of TG2 is required for proper phagocytosis, various mutants of the enzyme was transported into TG2 null macrophages by adenoviral delivery. Crosslinking activity of TG2 was not required, but the cell surface secretion and the guanine nucleotide binding mutants were unable to increase phagocytosis of TG2 null cells. Since TG2 and integrin receptors are coexpressed on the cell surface in wild-type cells, our data suggest a role of TG2 in regulating integrin signaling participating in the phagocytosis process. The lack of TG2 also modulated the proinflammatory cytokine production by macrophages. The two phenomenon together might be responsible for the development of autoimmunity.

Keywords: transglutaminase, phagocytosis, signaling, proinflammatory cytokines

Presenting author: Katrin Tagscherer

Apoptosis-based treatment of glioblastomas with ABT-737, a novel small molecule inhibitor of Bcl-2 family proteins

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Defects in the apoptotic signaling cascades are one reason for the poor therapeutic response of malignant gliomas. As glioblastomas are characterized by high expression levels of anti-apoptotic Bcl-2 family proteins, we studied the effects of the novel Bcl-2 inhibitor, ABT-737, on malignant glioma cells. ABT-737 treatment released the pro-apoptotic BAX protein from its binding partner Bcl-2 and potently induced apoptotic cell death in glioblastoma cells *in vitro* and *in vivo.* Down-regulation of Mcl-1 and over-expression of Bcl-2 sensitized the cells to ABT-737-mediated apoptosis, whereas over-expression of survivin did not alter the sensitivity to ABT-737. Moreover, ABT-737 potentiated the cytotoxicity of the chemotherapeutic drugs vincristine and etoposide, and of the death ligand TRAIL. Since stem cell-like glioma cells might be crucial for the tumor progression and the resistance to treatment in glioblastomas, we investigated the effects of ABT-737 on the subpopulation of glioma cells exhibiting stem cell characteristics. Inhibition of proliferation and induction of apoptosis by ABT-737 was less efficient in stem cell-like glioma cells than in non-stem cell-like cells. Since the resistance of stem cell-like glioma cells was associated with high Mcl-1 expression levels, ABT-737 treatment combined with down-regulation of Mcl-1 might represent a promising novel approach in glioblastoma treatment.

Keywords: apoptosis, brain tumors, glioma, ABT-737, Bcl-2 inhibitor

Presenting author: Sei-ichi Tanuma

Structure-based Computational design of an agonistic peptide trimer targeting Fas

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A terta-peptide named FRAP (WEWT, Fas reactive peptide) that binds to the human Fas molecule was created using our computer screening strategies named the Interaction Domain Fragmentation (IDF) and the Amino acid Complement Wave (ACW) methods, which are based on the affinities and complementarities of interacting amino acids between comprehensive testing peptides and a target protein surface pocket. *In silico* docking studies demonstrated the specific interaction of FRAP with the main Fas ligand (FasL) binding domain in the Fas molecule. A unique trimer of this peptide produced by Coiled-Coil SARM (Self Assembling Regulatory Molecule), $(FRAP)_3$-SARM, effectively induced apoptosis in human ovarian cancer cell line NOS4 cells that was associated with the activation of caspases-8, -9 and -3, and the cleavage of PARP. Alanine substitution of the N-terminal W in FRAP resulted in complete loss of FasL-mimetic action of $(FRAP)_3$-SARM, suggesting that the aromatic functionality at the N-terminal position W appears to play an essentially important role in Fas binding ability. These observations indicate that the FasL-mimetic agonistic peptide should serve as an excellent starting point for the design of effective compounds with FasL-mimetic activity. Furthermore, the IDF and ACW methods for the structure-based design of optimized small peptides against receptor molecules such as Fas could open new avenues for the development of peptide mimetic and nonpeptidic organic forms to generate novel effective pharmaceuticals.

Keywords: Fas

Presenting author: Antal Tapodi

Role of MAP kinases and Akt in the cytoprotective effect of PARP-1 inhibition and the regulation of Oxidative Stress induced necrotic cell death.

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Many pathophysiological processes are connected to oxidative stress and progressive cell death. The reactive species formation induces nuclear poly/ADP-ribose) polymerase (PARP) enzyme activation through single-strand DNA breakage resulting in NAD^+ and ATP depletion, which can lead to cell death. Therefore, we have investigated the oxidative stress induced cytotoxicity in WRL-68 (human hepatocyta) cells, as well as the effect of different types of PARP inhibition on survival and signal transduction pathways in these cells.
Furthermore, we have compared cell survival in cells overexpressing the enzymatic inactive DNA-binding domain of PARP (PARP-DBD) with wild type cells in hydrogen peroxide induced oxidative stress. In a similar experiment we have dawn-regulated the expression of PARP enzyme by small interfering RNA (siRNA technique).
Cell survivals were determined by MTT^+ assay after 1-hour pre-treatment with PARP inhibitors followed by a 3-hour incubation of H_2O_2. We used a pEGFP expression vector for the overexpression of the PARP-DBD. We detected the activation level of the members of the ERK, p38-MAPK, c-Jun kinase and protein kinase B/Akt signal pathway by Western blotting.
The survival of the cells was significantly increased in all cases when the PARP enzyme was inhibited (1. PJ34, 2. overexpression of PARP-DBD, 3. PARP-siRNA) as compared to the control cells. PARP inhibition enhanced the activation of Akt/protein kinase B and GSK 3 β as well as MAP kinases. These data show that inhibition of PARP confers resistance against H_2O_2-induced oxidative stress in human hepatocyta, and activation of Akt, a well established pro-survival signal transduction pathway, significantly contributes to this protective effect, and furthermore, the basic activity of MAPKs is necessary to the cell survival in oxidative stress.

Keywords: PARP, oxidative stress, MAPK, Akt

Presenting author: Bram ten Cate

Acute Myeloid Leukemia (AML)-restricted apoptosis induction by an scFv:sTRAIL fusion protein with specificity for CD33

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Therapeutic application of Gemtuzumab Ozogamicin (GO), an immunotoxin comprising an anti-CD33 antibody chemically linked to the highly cytotoxic antibiotic calicheamicin, has improved the treatment outcome for a subgroup of AML patients. However, GO is associated with severe treatment-related toxicity and frequent relapses. Here, we report on a novel AML-selective therapeutic agent, designated scFvCD33:sTRAIL, designed to combine potent tumoricidal activity with a more favorable toxicity profile. Fusion protein scFvCD33:sTRAIL comprises a CD33-specific scFv antibody fragment genetically linked to human soluble TRAIL (sTRAIL). Treatment of AML cell lines with scFvCD33:sTRAIL resulted in potent apoptosis induction in CD33-positive tumor cells. Normal CD33-positive monocytes were fully resistant to prolonged treatment with scFvCD33:sTRAIL, whereas treatment with GO resulted in substantial toxicity. Strikingly, scFvCD33:sTRAIL was approximately 130-fold more potent than GO towards U-937 tumor cells. Moreover, scFvCD33:sTRAIL retained its CD33-restricted activity after prolonged storage at 37℃, whereas GO stored at 37℃ showed a rapid increase in CD33-independent toxicity. Ex vivo treatment of patient-derived AML tumor cells with scFvCD33:sTRAIL resulted in marked apoptosis induction that was synergized by valproic acid, a histone deacetylase inhibitor currently in phase II clinical trials for AML. Taken together, these promising preclinical data warrant further development of scFvCD33:sTRAIL for the treatment of AML.

Keywords: AML, TRAIL, targeting, CD33, apoptosis, Gemtuzumab Ozogamicin, Mylotarg

Presenting author: Katalin Tóth

Ligation of RARα enhances the glucocorticoid-induced apoptosis of T cells and promotes glucocorticoid receptor mediated transcriptional processes

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Previous works in our laboratory shown that all-trans retinoic acid and 9-cis retinoic acid enhanced apoptosis of mouse thymocytes. Previous experiments show that mouse thymocytes express RARα and γ but RARβ receptor not. We would like to know wether retinoids influence of glucocorticoids induced apoptosis of mouse thymocytes. If they do, what kind of retinoid receptors and which mechanism used.

We did experiments with agonists and antagonists of retinoid receptors and determined the percentage of apoptotic cells 6 hrs after the treatment by flow cytometrical analysis of the DNA content. All of RARα agonists enhanced dexamethasone induced apoptosis compared with RARβ and γ agonists. RXR agonists were inefficient too. These results suggest role of RARα receptor in enhancement of glucocorticoid induced apoptosis of the mouse thymocytes.

Whereas RARα antagonist was efficient and suggest that RARα might not function transcriptionally in this effect.

It is recognized that nuclear receptors interact together. We investigated that glucocorticoid and retinoid receptor bind together in a ligand-dependent manner and this interaction of the two receptors promotes the transcriptional activity of GR. Mammalian two-hybrid system reveals a direct interaction between RARα and GR.

Henceforth we studied whether dexamethasone induces GR translocation to the mitochondria or not. GR expression in the mitochondrial fraction of mouse thymocytes is increased after dexamethasone treatment indicating mitochondrial translocation of GR.

Keywords: apoptosis, glucocorticoid, retinoid, GILZ

Clusterin expression and apoptosis in STZ diabetic nephropathy.

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The aim of this study was to determine the roles of secreted clusterin (s-clu) and nuclear clusterin (n-clu) expressions in experimental diabetic nephropathy, and to investigate the effects of perindopril as an ACE inhibitor and irbesartan as an AT1 receptor blocker on clusterin expression and compare these results with apoptosis. In this study Wistar albino rats(5 groups) were used. (1) STZ-diabetic, (2) irbesartan treated diabetics (15 mg/kg/day, 30 days), (3) perindopril treated diabetics (6 mg/kg/day, 30 days), (4) combined treated diabetic group with perindopril and irbesartan (respectively, 3 mg/kg/day, 5 mg/kg/day, 30 days), (5) control group. During the experimental period blood glucose, microalbuminuria levels, body weights, kidney weight and amount of daily urine were measured. At the end of experiment, renal tissue samples were fixed in formaline and embedded in paraffin. TUNEL method for apoptosis and immunohistochemical staining for clusterin-β (s-clu) and clusterin-α/β (n-clu) antibodies were performed. Blood glucose levels of all STZ-diabetic groups were higher than the control group. The level of daily urine and microalbuminuria levels were decreased in the all treated diabetic groups. The number of apoptotic cells increased especially in the kidney tubules of STZ-diabetic group ($p<0,001$), whereas a significant decrease was observed in the combined drug treated group ($p<0,05$). The expression of clusterin-β was increased in the glomerules and tubules of the untreated diabetic group, although it was decreased in the treated diabetic groups. Immunopositivity of clusterin-α/β in the podocytes and mesangial cells and in the injured tubule cells of untreated diabetic group was found to be increased in their nuclei. The number of n-clu immunopositive cells was decreased in the treated diabetic groups, especially in the combined treated diabetic group ($p<0,05$). Our results show that the expression of s-clu was induced in the experimental diabetic nephropathy related to renal tissue damage and that the increase in the n-clu expression in the renal tubules was releated to apoptosis. Although irbesartan and perindopril prevented renal injury in the diabetes, low dose application of ACE inhibitor and AT-1 receptor blocker together revealed more efficient results in preventing renal damages.

Keywords: clusterin, apoptosis, nephropathy, STZ diabetes, rat

Presenting author: Agnieszka Turowska

EHV-1 and apoptosis

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Many reports have indicated that infection with alphaherpesviruses induces apoptosis in cultured cells. However, the cellular pathways involved in alphaherpesvirus- induced apoptosis remain poorly understood. The aim of this study was to investigate the influence of wild strain Jan-E and cell culture-adapted reference strain Rac-H of EHV-1 (equine herpesvirus 1) infection on caspase - 3 pathway activation in ED cells. Caspase-3 is a key protease that is activated during the early stages of apoptosis. Results were evaluated in scanning cytometer (Scan^R). Our preliminary data suggest that EHV-1 can modify cell death pathway, leading to the morphological features of apoptosis. In general, the ability of viral strains to induce apoptosis correlates with their virulence, but extact role of EHV-1 in apoptosis, however, is not understood in detail.

Keywords: equine herpesvirus 1, apoptosis

Presenting author: Alena Vaculova

Early Mcl-1 up-regulation induced by TRAIL and its role in modulation of colon cancer cell sensitivity to apoptosis

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As an inducer of tumour-specific cell death, TRAIL is considered to be a promising candidate for anticancer therapy. However, it has recently been shown that TRAIL is also capable of stimulating proliferation and survival of cancer cells of different origin. Thus, the signalling pathways triggered by TRAIL and their interactions need to be elucidated in order to avoid undesirable effects (survival of target cells) restricting its use in anticancer therapy.
We demonstrated that TRAIL induced a significant early increase in anti-apoptotic Mcl-1 protein level in HT-29 and HCT116 human colon adenocarcinoma cell lines that responded to its apoptotic effects (Western blotting). This was preceded by a significant induction of Mcl-1 mRNA expression (RT-PCR). On the other hand, substantially weaker or no response with regard to the changes in Mcl-1 protein level was observed in FHC normal foetal colon and SW620 colon cancer human cell lines, respectively, which were resistant to the apoptotic effects of TRAIL (100 ng/ml). Thus, we suggest that the early Mcl-1 up-regulation seems to be a more general phenomenon only in colon cancer cells that are responsive to TRAIL apoptotic effects. Next, we showed that ERK1/2 pathway was responsible for the up-regulation of both Mcl-1 mRNA and protein in these cells. This effect was completely abolished by U0126 (MEK1/2 inhibitor), which concomitantly potentiated TRAIL-induced apoptosis. Using Mcl-1 siRNA we confirmed that the transient Mcl-1 up-regulation stimulated by TRAIL is an important mechanism protecting the cells from its own acute apoptotic effects.
The dynamics of modulation of Mcl-1 level and the balance between TRAIL-induced pro- and anti-apoptotic signalling pathways were demonstrated as important factors determining the colon cancer cell sensitivity to apoptosis. It is therefore essential to consider them when predicting the cancer cell response and designing the effective therapy.

This study was supported by the GA CR 524/07/1178 and IGA AS CR 1QS500040507.

Keywords: TRAIL, Mcl-1, apoptosis, colon

Presenting author: Nele Vanlangenakker

Identification of novel potential kinases involved in necrotic cell death by an RNAi screen

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Necrosis has long time been described as accidental and uncontrolled cell death as a consequence of physico-chemical stress. Recently, it becomes clear that necrotic cell death is as well controlled and programmed as caspase-dependent apoptosis, and that it may be an important cell death mode with important pathological and physiological relevance. Many different stimuli such as TNF on certain cell lines, dsRNA and IFN-γ, ATP depletion, and ischemic conditions are able to elicit necrotic cell death. A crucial player in the initiation of the necrotic cell death process is receptor-interacting protein 1 (RIP1). To gather new insights in necrotic cell death pathways we developed an RNAi screen targeting the mouse kinome (Expression Arrest™ mouse kinase retroviral shRNA^{mir} library from Open Biosystems). The library consists of 721 constructs provided in 96-well plates containing cultures of E.Coli. An RNAi screen set-up was designed in which DNA preparation, transfection of retroviral packaging cell line (QNX-A cells), retroviral transduction, stimulation and biological read outs were optimized. DNA was prepared in 96-well plates by the BioRobot9600 from 4 ml bacterial culture. Four negative and positive controls were added to each DNA plate. Calcium phosphate transfection of QNX cells was performed manually in a 12-well format. Virus medium was collected 24 and 48 hours after transfection and used to infect L929sA fibrosarcoma cells in 96-wells. For optimal functioning of the positive controls (RIP1i and TNFR1i) L929sA cells were used overexpressing the viral serpin-like inhibitor of caspase-1 and -8. In the current screen setup the cells were stimulated (in duplicate) with two different concentrations of hTNF (10 and 1 IU/ml). Inhibition or sensitization of TNFR1-induced necrosis was analyzed by succinate dehydrogenase activity (MTT-assay) and release of lactate dehydrogenase in supernatant (LDH-assay) eighteen hours after stimulation of TNFR1. The moiety of the kinase library is already screened and revealed 21 possible regulators of necrosis so far. Hit selection was based on three different modes of statistical analyses. The potential candidates are now being validated in a secondary RNAi screen experiment.

Keywords: Necrotic kinases RNAi

Presenting author: Frederic Vanzembergh

Effect of LPS, dsRNA or interferons on the clearance by macrophages of apoptotic and necrotic cells and of virulent and attenuated strains of mycobacteria or rabies virus

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Under physiological and pathological conditions, cells may die via an ordered cellular process, called apoptosis, or via an alternative form of cell death, called necrosis. For example, L929hFas mouse fibrosarcoma cells infected with *vesicular stomatitis virus* (VSV) die by apoptosis, while the same cells infected with *encephalomyocarditis virus* (EMCV) die by necrosis.
We demonstrated before, using fluorescently labelled macrophages and dying cells in a flowcytometry-based phagocytosis assay, that although apoptotic and necrotic cells are recognized and phagocytosed by macrophages, uptake of apoptotic cells is more efficient both quantitatively and kinetically than that of necrotic cells. Our current results demonstrate that pre-treatment of macrophages with alarm signals including type I or II interferons or Toll-like receptor ligands, such as LPS or dsRNA, enhances the capacity of phagocytes to engulf both apoptotic and necrotic cells. Swift recognition and clearance of dying cells infected by bacteria or virus may help to contain these intracellular pathogens and is often the first step in the process leading to the development of acquired immunity. Thus, avoiding cell death may be beneficial for an intracellular pathogen, while inducing cell death could promote a protective immune response by an efficient attenuated vaccine. Therefore, we are currently exploring the effects on cell viability in macrophages infected with virulent strains of *Mycobacterium tuberculosis* or rabies virus, in comparison with those of corresponding attenuated vaccine strains, such as *M. bovis* BCG and ERA 2024 rabies virus. We are also examining the mode of cell death induced during infection and the effects of pre-treatment with interferons, LPS or dsRNA on the uptake of microbes by macrophages and on cellular, bacterial and viral viability.

Keywords: Macrophage/ Clearance/ Apoptotic and necrotic cell

Presenting author: Miroslav Varecha

Bioinformatic predictions and image analysis of localization and interactions of endonuclease G, AIF, and AMID in human cells

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During apoptosis several mitochondrial proteins are released. They participate in caspase-independent nuclear DNA degradation, namely apoptosis-inducing factor (AIF, also PDCD8 or AIFM1) and endonuclease G. Another interesting protein, which is not located inside mitochondria, was expected to act similarly as AIF due to high sequence homology with AIF. This protein is AIF-homologous mitochondrion-associated inducer of death (AMID, also PRG3 or AIFM2). We studied the cellular localization and colocalization of proteins AIF, endonuclease G and AMID experimentally using designed mammalian expression vectors, which carry genes encoding the proteins of interest fused to the fluorescent proteins and using bioinformatic predictions, that analyze the amino acid sequence of the proteins with various algorithms. We also designed and applied the novel method of single-cell image analysis of the translocation of the fluorescent proteins into the nucleus. We confirmed the colocalization of AIF and endonuclease G in the mitochondria of human cells. We also analyzed their translocation from mitochondria to the nucleus during apoptosis. AMID was found to be cytoplasmic protein, bound to various cellular surfaces from their cytoplasmic side. Overexpression of fusion protein AMID-HcRed-tandem was not lethal to the cells or mitochondria. We did not observe its translocation into the nucleus during staurosporine-induced apoptosis. The possible role of AMID in apoptosis was not observed. Bioinformatic predictions and time-lapse FRET experiments were conducted to analyze the interactions of the studied proteins in living and fixed human cells. Our results contribute to the comprehension of localization, interactions and functions of AMID, AIF, and endonuclease G in human cells.

This work was supported by the Grant Agency of the Czech Republic (grant number 204/05/P090) and by The Ministry of Education, Youth and Sports of the Czech Republic (projects number MSM0021622419 and LC535).

Keywords: AIF; endonuclease G; AMID; microscopy; bioinformatics; image analysis

Presenting author: Enrico Velardi

The Glucocorticoid-Induced Leucine Zipper (GILZ) protects against DNBS-induced colitis development

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Inflammatory Bowel Diseases (IBDs) are chronic inflammatory disorders widely diffused among children and adults in developed countries. IBDs are characterized by disfunction of mucosal immune response, NF-kB activation, abnormal cytokine production, with increase of TNFα and IL-1, cellular inflammation, with increase of adhesion molecules expression, and cell infiltrate that ultimately led to apoptosis and mucosal damage. Although the etiology of IBD remains unknown, there is circumstantial evidence supporting a central role for disregulation of T-cell and particularly of colonic CD4+ T helper (Th1) effectors cell responses, including IL-2 production, to the normal enteric bacterial flora as a common disease mechanism.
Among the effective drugs that are employed in IBD treatment, glucocorticoids (GCs) display their efficiency by inhibition of NF-kB activity. Unfortunately, GCs efficacy, as well as other immunosuppressive drugs, is limited by serious side effects occurrence and resistance development: as a consequence, new pharmacological strategies are needed.
GILZ is a small molecule known to mediate GCs effects: in particular, it inhibits NF-kB pathway by blocking its nuclear translocation and DNA binding through a direct protein-to-protein GILZ/NF-kB interaction and inhibition of Th1 phenotype.
By the use of the DNBS-induced colitis model, we investigated GILZ role during disease induction. We compared IBD induction efficiency and pathological and molecular markers in wild type mice with GILZ transgenic mice (GILZ-TG). Our results indicate that GILZ stable expression in T cells protects from IBD induction. In fact, disease was suppressed as evaluated by both macroscopic and histological observations, and expression of pathologic markers, such as CD4+ and CD8+ cell infiltrates. Moreover, IL-2, TNF-a, IL-1, FasL and P-Selectin expression were markedly reduced. Notably, all these pathological markers are regulated by NF-kB transcriptional activity. In this context, we investigated NF-kB activation, in WT and GILZ-TG mice, and we found that GILZ suppresses NF-kB activation and nuclear translocation in lamina propria T lymphocytes of DNBS-treated mice.
Overall, our data indicate that GILZ-mediated NF-kB pathway suppression mediates protective effects capable to counteract the disease development. Consequently, we speculate that the employment of small GILZ-based peptides could represent a new effective pharmacological tool against IBDs.

Keywords: GILZ, IBD, inflammation, mouse model of disease

Inhibition of XIAP sensitizes glioblastoma cells to γ-irradiation-induced apoptosis.

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Glioblastoma multiforme is the most common brain tumor. Resistance of this tumor to current therapies is at least in part due to overexpression of antiapoptotic proteins. Among antiapoptotic proteins the X-chromosome linked inhibitor of apoptosis protein (XIAP) is a potent inhibitor of apoptosis by blocking caspase9 and 3. Hence we reasoned that targeting XIAP by small molecule inhibitors could overcome apoptosis resistance of glioblastoma.
Here we report that XIAP inhibitors sensitized glioblastoma cells to γ- irradiation induced apoptosis. Analysis of signaling pathways revealed that XIAP inhibitors promoted loss of mitochondrial membrane potential, release of cytochrome c and activation of caspase 9 and 3 in response to γ-irradiation. Pan-caspase inhibitor zVAD abrogated the sensitization of XIAP inhibitors to γ-irradiation suggesting that apoptosis induction is caspase dependent. Importantly, XIAP inhibitors sensitized primary cultures derived from glioblastoma patients to γ-irradiation. By comparsion, XIAP inhibitors on their own were neither cytotoxic to normal neurons nor neural initiating cells.
Our data indicate that targeting XIAP is a promising approach to overcome the resistance of glioblastoma to radiation therapy.

Keywords: XIAP, glioblalstoma, gamma irradiation, apoptosis.

Presenting author: Mireille Verdier

The p53 status does delay mitochondrial apoptotic response kinetics of two neuroblastoma cell lines to Cobalt Chloride hypoxia-mimetic agent ?

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Cobalt Chloride ($CoCl_2$) is commonly used hypoxia-mimetic agent. Like physical hypoxia it induces the stabilization of Hypoxia-inducible factor-1alpha (HIF-1α) protein a major regulator for cellular response survival. It has been suggested that HIF-1α can cause p53 accumulation during hypoxia. Divergent data reports about the p53 implication in $CoCl_2$ hypoxia-mimetic cell death. We investigated p53 depending cell death into respectively two neuroblastoma cell lines, SHSY5Y (with wild type p53) and SKNBE(2c) with deficient p53 transcriptional activity. In first we observed that 500 µM $CoCl_2$ induced an significant toxicity in both two cell lines after 12h treatment which was correlated with cell shrinking, chromatin condense and nuclear fragmentation. Then, we focused $CoCL_2$ effects, looking for the up-stream events of the apoptotic process implying the p53-dependent pathways. The low p53 expression in control *SHSY5Y* cells started to increase as early 2 h ($p=0.09$ was nearly significant) under hypoxia treatment. The p53 level was strongly (about 50%) but transitorily enhanced at 6 h ($p=0.003$), then a slight decrease was observed from 12 h, after 24h the drastic disappearance of p53 was correlated to the main wave cell death. On the contrary, in *SKNBE(2c)* cells, p53 was detected at a high level in no-treated cells, it was significantly abolished in a manner time-dependent ($p<0.05$). To complete the data, we have investigated the phosphorylation status of p53 (focusing on serine 15 and Threonine 81), through its subcellular localization under hypoxia-mimetic treatment (12h) in both cell lines by immunofluorescence confocal microscopy. In wtp53-SHSY5Y cells and also in mutated p53-SKNBE(2c), p53 phosphorylated at threonine 81 was detected in nucleus. However, p53 phosphorylation at serine 15 was early (6 h) revealed in SHSY5Y cells and located to mitochondria, whereas no p53 phosphorylation at serine 15 was observed in SKNBE(2c) cells. No p53 phosphorylated at serine 15 was detected into nucleus for both cell lines. Moreover, mitochondrial death involving collapse of mitochondrial membrane potential, then subsequent caspase-3 activation was observed. We demonstrated that $CoCl_2$ hypoxia-mimetic agent induces apoptosis in 5Y and BE(2c) cells via p53 in a manner time dependent according to their sequential p53 phosphorylation pattern.

Keywords: p53 status, apoptotic pathway, mitochondria, neuroblastoma, hypoxia, CoCl2

Presenting author: Margreet Vissers

Neutrophil apoptosis and clearance are inhibited in ascorbate-deficiency due to up-regulation of hypoxia-inducible factor-1 alpha

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Neutrophils, the primary defence against micro-organisms, maintain high ascorbate concentrations, but its role in these cells has never been determined. In this study we have used neutrophils from *Gulo* -/- mice which lack the enzyme gulonolactone oxidase and are unable to synthesise ascorbate. The neutrophils were completely deficient in ascorbate and were unable to undergo spontaneous apoptosis. Initially there was an increase in cell survival, but within 24h cell death occurred by necrosis. Apoptosis by PMA-stimulated cells or cells phagocytosing bacteria was similarly affected. Neutrophils lacking ascorbate had elevated levels of hypoxia-inducible factor (HIF)-1α, the primary oxygen sensor that also inhibits neutrophil apoptosis under hypoxic conditions. This transcription factor is regulated by Fe^{2+}-dependent hydroxylases that require ascorbate for optimal activity. The results indicate that, in ascorbate-deficiency, up-regulation of HIF-1α blocks neutrophil apoptosis under normoxic conditions. That this affects neutrophil apoptosis *in vivo* was supported by finding that neutrophils persist in thioglycollate-induced inflammation in Gulo-/- mice, with the later appearance of necrotic cells. Promotion of neutrophil apoptosis therefore represents an essential function for vitamin C, and widespread necrosis in ascorbate deficiency could contribute significantly to the devastating symptoms of scurvy.

Keywords: neutrophil apoptosis, macrophage clearance, ascorbate, HIF-1

Presenting author: Oksana Voloshanenko

Identification of the apoptosis-inducing TRAIL receptor as a metastasis suppressor

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From the Divisions of [2]Apoptosis Regulation and [4]Molecular Biology of the Cell I of the German Cancer Research Center (DKFZ), Heidelberg, Germany and from the Divisions of [2]Human Biology and [3]Public Health Sciences, Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA, USA.

Initially, TRAIL (Apo2L) was found to specifically induce apoptosis in tumor cell lines but not in normal cell in vitro and in vivo. Then, endogenous TRAIL was shown to suppress experimental liver metastases in mice upon systemic injection of TRAIL-sensitive tumor cells. However, it now appears that cells freshly explanted from primary tumours are mostly TRAIL-resistant. Experiments with TRAIL- and TRAIL-R-deficient mice corroborate these findings as no role could be attributed to TRAIL and TRAIL-R in most models of primary tumorigenesis. Here, we show that TRAIL-R-deficient mice showed significantly elevated occurrence of lymph node metastases compared to wild-type mice, while they were not more susceptible to tumor initiation, promotion, and conversion of primary chemically-induced skin tumours. Thus, TRAIL-R specifically suppresses metastasis but not development of primary skin tumours.
We found that adherent TRAIL-R-expressing skin carcinoma cells were TRAIL-resistant in vitro. This is consistent with the observed lack-of-function of TRAIL-R in the prevention of initiation, promotion, and conversion of primary skin tumours. Pretreatment of carcinoma cells with the proteasome inhibitor PS-341 (Bortezomib) sensitized these cells for TRAIL-induced apoptosis, thus the TRAIL-R apoptosis pathway is principally still functional in these cells. An obligatory step in the development of metastasis is detachment of tumor cells from the primary tumor. We showed that upon detachment skin carcinomas cells are sensitized for TRAIL, consistent with the physiological role of TRAIL-R specifically during metastatic spread of primary skin tumours. Furthermore, combined treatment of detached carcinoma cells with TRAIL and the proteasome inhibitor PS-341 resulted in increased apoptosis. Therefore, our data suggest a therapeutic window for TRAIL-receptor agonists to specifically target metastasising tumor cells.

Keywords: TRAIL-R, metastasis

Presenting author: Lasse N. Waehrens

Apoptosis detection with fluorescent and radioactive lactadherin

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Early apoptosis is characterized by breakdown of the asymmetry of the plasma membrane lipid distribution. Phosphatidylserine (PS), normally maintained at the inner membrane leaflet, becomes available on the outer membrane leaflet. The secreted protein lactadherin is able to bind exposed PS with one domain and at the same time recognizing integrins $\alpha_V\beta_3$ or $\alpha_V\beta_5$ on macrophages. In this capacity lactadherin has been shown to operate as an opsonin.

To investigate and characterize the PS-binding ability we have purified lactadherin and conjugated it with either fluorescein isothiocyanate (FITC) or hydrazinonicotinamide (HYNIC) and ^{99m}Tc. Synthesis of ^{99m}Tc-HYNIC-lactadherin achieved a high radiochemical purity of 88 %. The radioactive conjugate was found to be stable over 5 hours if supplemented with 1.5 mg/mL fatty-acid-free BSA. Biological activity of labelled lactadherin was measured on etoposide induced apoptotic HL60 and K562 cell lines. Apoptotic HL60 cells demonstrated an ArgGlyAsp independent 3 to 4-fold higher binding for ^{99m}Tc-HYNIC-lactadherin compared to viable HL60 cells. Unlabelled lactadherin almost completely abrogated this binding indicating that the binding activity is specific for lactadherin.

Flow cytometry studies using FITC-lactadherin showed clear binding of lactadherin to etoposide-treated HL60 and K562 cells. This binding could be 95% inhibited by PS containing vesicles. The binding characteristics of lactadherin was compared with Annexin V, the benchmark PS probe, in biochemical and cell-staining experiments. Results indicated that a) PS was detected by lactadherin at lipid concentrations above 0.5%, whereas the benchmark PS probe, annexin V, holds a PS affinity at concentrations above 2.5% PS. b) Confocal microscopy on TUNEL positive K562 and HL60 cells revealed localized plasma membrane staining, then diffuse dim staining by lactadherin prior to bright generalized staining with both proteins. Condensed apoptotic bodies showed a diffuse ring staining of lactadherin, indicative of surface staining. In contrast, Annexin V showed a punctate staining with internalized bodies of annexin V as the primary location. At late stage apoptosis annexin V stained the plasma membrane. Thus, we suggest that lactadherin is a better probe for detection of PS exposure than Annexin V, as it enables detection at an earlier stage of apoptosis.

Keywords: lactadherin, fluorescent, radioactive, phosphatidylserine

Presenting author: Deborah Wallace

E2F-1 and p53 regulate Developmental expression of Apaf-1 and Caspase 3

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Apoptosis is a form of programmed cell death essential for both tissue development and maintenance of tissue homeostasis. Apoptosis protease activating factor-1 (Apaf-1) and the cysteine proteases known as caspases are genes central to the intrinsic apoptotic pathway. Apaf-1 is a core molecule in the formation of the apoptosome complex. Once activated, this complex can mediate the mitochondrial apoptotic pathway resulting in activation of caspase-9 and induction of a caspase cascade. We have previously demonstrated the down-regulation in expression of Apaf-1 and caspase 3 during postnatal development and have proposed a regulatory role for chromatin re-modelling in the developmental transcriptional regulation of Apaf-1 and caspase 3.
Here we endeavour to define the exact molecular mechanism involved in the transcriptional regulation of Apaf-1 and caspase 3. To this end, we employ both a cell line and retinal explants. We illustrate that Apaf-1 expression and processing of caspase 3 are up-regulated following inhibition of histone deacetylase activity by treatment with trichostatin A (TSA). We identify the mouse Apaf-1 promoter and locate putative E2F-1 and p53 binding sites within the Apaf-1 and caspase 3 promoters which are confirmed by chromatin immunoprecipitation assays. By performing siRNA in 661W cells we establish a link between E2F-1 / p53 and Apaf-1 / caspase 3. We find that both E2F-1 and p53 are down-regulated during development and up-regulated at early post-natal ages following TSA treatment. These data strongly suggest a role for chromatin-remodelling, E2F-1 and p53 in the developmental transcriptional regulation of the key apoptotic executioners Apaf-1 and caspase 3.

Keywords: Apaf-1 caspase 3 E2F-1 p53

Presenting author: Jing Wang

Ceramide induces apoptosis via a peroxisome proliferator-activated receptor *γ* -dependent pathway

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Induction of apoptosis in tumor cells is an appealing therapeutic approach. Although the biological mechanism of sphingolipids remains unclear, emerging evidence suggests that ceramide, as a main class of metabolites, may play an important role because of their proapoptotic activities. The mechanism that ceramide induce apoptotic cell death is an area of active investigation, but remains poorly understood. Previous studies showed ceramide activates proapoptotic mechanisms in three major ways: (a) ceramide acts indirectly to activate caspases, which leads to activation of caspase 3; (b) ceramide activates SAPK/JNK pathway, which then leads to activate caspase 3; and (c) ceramide up-regulates TNFR1 and FasR on the cell membrane and, therefore, may enhance the effects of antitumor T-lymphocytes and other Fas ligand and TNF-dependent processes. However, some studies indicate that other signal transduction pathways can't be excluded. On the other hand, PPARs are key players in lipid metabolism and belong to the nuclear receptor superfamily of transcription factors. Furthermore, some of the PPAR*γ* ligands can induce growth inhibition in many human cancer cells, which are attributed to induction of apoptosis by PPAR*γ*. Recent studies that PPAR*γ* expression is modulated by sphingomyelin and ceramide in 3T3-F442A and 3T3-L1 preadipocyte respectively, has shown certain relationship between PPAR*γ* and sphingolipid occurred. However, the roles of PPAR*γ* pathway, as well as cell signaling compounds played in ceramide-induced apoptotic process, needs more extensive study. In this study, these two observations converge, we here report that C2-ceramide induced apoptosis in colon cancer cells is mediated by activation of PPAR*γ* pathway. Treatment with ceramide in HT29 cells modulates PPAR*γ* expression level and its transcriptional activity, which lead to apoptosis. Administration of PPAR*γ* specific antagonist GW9662 partially prevents HT29 cells from apoptosis. Furthermore, MAP kinase pathway provided a potential modulation mechanism for PPAR*γ* pathway related with ceramide. Our results are the first to demonstrate that C2-ceramide induces apoptosis via a PPAR*γ* -dependent pathway.

Keywords: ceramide, PPAR gamma, apoptosis, MAPK signaling

Presenting author: Michal Wasilewski

N-Acylethanolamines affect mitochondria within HL-60 cells

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N-Acylethanolamines (NAEs) are common lipids present in various mammalian tissues in small amounts. Their content in heart and brain increases dramatically during ischemia. Mitochondria play a major role in ischemic tissue damage due to the production of reactive oxygen species (ROS) and release of proapoptotic molecules. NAEs have been shown to affect isolated mitochondria by inhibition of the respiratory chain and increase in the permeability of the inner mitochondrial membrane [1,2].

The present study was aimed to investigate whether NAEs can alter functions of mitochondria within intact HL-60 human leukemia cells. Within first few minutes after addition, NAEs induced inhibition of respiration. This effect was independent of both hydrolysis of NAEs to free fatty acids and activation of cannabinoid receptors, as the inhibition of respiration was not blocked by inhibitors of these processes. Detailed investigation of the respiratory chain revealed that the inhibition was localized manly within complex I. Decrease of the respiration rate was accompanied by an increase of NADH/NAD^+ ratio. In the presence of NAEs, the mitochondrial electrochemical potential (Δp) was dissipated by oligomycin indicating that, in these conditions, Δp was generated mainly by F_1F_O-ATPase. In a longer time scale these changes were followed by elevation of ROS production and eventually by cell death. These findings indicate that NAEs inhibit the respiratory chain and affect ROS production in living cells – critical processes for cell fate in ischemic tissue. More generally, NAEs could modulate ischemic damage by its influence on mitochondria.

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Keywords: mitochondria, respiratory chain, lipids

Presenting author: Anita Wichmann

Ionizing radiation induces caspase-dependent but Chk2- and p53-independent cell death in *Drosophila melanogaster*

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Ionizing radiation (IR) can induce apoptosis via p53, which is the most commonly mutated gene in human cancers. Loss of p53, however, can render cancer cells refractory to the therapeutic effects of IR. p53-independent pathways exist but are not as well understood as p53-dependent apoptosis. Studies of how IR induces p53-independent cell death could benefit from the existence of a genetically tractable model. In *Drosophila melanogaster*, IR induces apoptosis in the imaginal discs of larvae, typically assayed at 4-6 hours after exposure to a LD_{50} dose. In *Drosophila chk2* and *p53* mutants, apoptosis is not observed 4-6 hours after IR, leading to the widely-held belief that Chk2 and p53 are required for IR-induced apoptosis in *Drosophila*. However, when we look at later times after IR, such as 18 hours, we find that cell death does indeed occur in the imaginal discs of *chk2* and *p53* mutant larvae. We demonstrate that Chk2-and p53-independent cell death is a true apoptotic response, as it requires caspase activity and the pro-apoptotic Smac/DIABLO ortholog *hid*. Quantitative RT-PCR experiments show that *hid* transcripts increase following IR in *chk2* and *p53* mutants, but that the increase is delayed relative to that seen in wild-type. Recently we have found that *Drosophila E2F1* (*dE2F1*) is required for Chk2- and p53-independent apoptosis. We are currently testing whether dE2F1 induces apoptosis via transcriptional regulation of *hid*. We conclude that *Drosophila* has Chk2- and p53-independent pathways to activate caspases and induce apoptosis in response to IR. This work establishes *Drosophila* as a model for p53-independent apoptosis, which is of potential therapeutic importance for inducing cell death in p53-deficient cancer cells.

Keywords: p53-independent, ionizing radiation, Drosophila

Presenting author: Rachel Wilkie

A Functional NADPH oxidase promotes neutrophil apoptosis by a caspase-independent mechanism

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Neutrophils play a prominent role in host defense. Phagocytosis of bacteria leads to formation of an active NADPH oxidase complex that generates large amounts of reactive oxygen species to facilitate bacterial killing. After the infection is cleared neutrophils undergo apoptosis and are ingested by macrophages. It is proposed that oxidants derived from the NADPH oxidase also have a fundamental role in mediating apoptosis of phagocytic neutrophils. In this study we used diphenyleneiodonium an inhibitor of the NADPH oxidase, and neutrophils isolated from an X-linked gp91phox knockout chronic granulomatous disease (CGD) mice to determine what role NADPH derived oxidants have in the execution and resolution of apoptosis. We showed that the NADPH oxidase triggered exposure of phosphatidylserine and promoted the uptake of phagocytic neutrophils by human macrophages. The phosphatidylserine exposure and macrophage uptake was not caspase-mediated, indeed, we showed that NADPH-derived oxidants actually prevented caspase activation in phagocytic neutrophils. Caspases are redox-sensitive thiol enzymes, and inactivation could be associated with oxidative stress within the neutrophil cytoplasm. Phagocytosis resulted in protein carbonyl formation in the cytosol. We proposed that the carbonyls were derived from NADPH oxidase-dependent lipid peroxidation in the phagosomal membrane generated reactive aldehydes such as 4-hydroxynonenal that diffuse into the cytoplasm to target thiol proteins including caspases. Consistent with the hypothesis, inhibitors of lipid peroxidation inhibited carbonyl formation and caspase inactivation, while exogenous 4-hydroxynonenal inactivated caspases and generated a similar pattern of protein carbonylation as phagocytosis. We conclude that neutrophil oxidants have a key role in driving the clearance of phagocytic neutrophils, but this occurs via a novel mechanism because of excess lipid peroxidation in the phagosomal membrane producing an unfavourable environment for caspases.

Keywords: Neutrophil apoptosis, phagocytosis, caspases, oxidative stress

Presenting author: Chew Hooi Wong

Regulation of Autophagic and Apoptotic Cell Death by JNK

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Previously, classical programmed cell death has been exemplified by apoptosis, as it is a highly regulated and controlled form of cell death. However, more recent evidence indicates that programmed cell death is not exclusively confined to apoptosis. To that end, autophagy has been demonstrated as an alternative form of cell death; identified as Type II cell death in addition to the classical Type I apoptotic cell death. Despite the extensive studies under way, the exact mechanism of autophagy-induced cell death and the signaling pathways involved have not been fully established. JNK, a member of the MAP Kinase family has been implicated in autophagy. We are interested in identifying the role of JNK activation in the induction of autophagy. Furthermore, we aim to resolve the question of whether autophagy is an important cell death mechanism. In this study, we define a novel molecular pathway in autophagy involving JNK. In HCT116 colorectal carcinoma model, we employed a novel chemotherapeutic agent, C1 to characterize the molecular death pathways. JNK was activated at an early onset and its activation was sustained through out the course of cell death. Sustained JNK activation was found to be due to intracellular reactive oxygen species (ROS) production induced by C1, which suppresses the level of Map Kinase Phosphatase 1 (MKP1), the inactivating phosphatase of JNK. Activation of JNK was found to be an important mediator of autophagy, as it is a major regulator of MAP 1 LC3 expression. HCT116 p53+/- cells undergo early autophagic cell death, which is dependent on JNK and a delayed onset of apoptotic cell death. Onset of autophagy suppresses apoptotic cell death. Inhibition of JNK attenuates autophagic cell death and potentiates apoptotic death characterized by increased caspase processing and DNA fragmentation. In conclusion, JNK serves as a regulator of cell death in relation to autophagy and apoptosis.

Keywords: Autophagy, Apoptosis, JNK

Presenting author: Yick Fu Wong

Down regulation of GADD45G in advanced endometrioid endometrial cancer in Hong Kong women

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Endometrial cancer is the third most common gynecologic malignancy and the ninth most common malignancy for females overall in Hong Kong. Approximately 80% or more of these cancers are endometrioid endometrial adenocarcinomas. The aim of this study was to reveal genes contributing to the progression of endometrioid endometrial cancer, which may impact diagnosis, prognosis, and treatment of the disease. Whole-genome gene expression analysis was completed for a set of 55 microdissected sporadic endometrioid endometrial cancer (EEC) and 29 microdissected normal endometrium specimens using the Affymetrix Human U133 Plus 2.0 oligonucleotide microarray. Unsupervised hierarchical clustering displayed a distinct separation between the endometrioid adenocarcinomas and normal endometrium samples. Supervised analysis found expression of GADD45G was under-expressed for 4.04-fold in 11 late stage disease when compared to 44 early stage disease. To validate the gene change, real-time RT-PCR was used to determine GADD45G expression in another set of 56 EEC including 40 early stage disease and 16 late stage disease. It was shown that GADD45G was under-expressed for 5.59-fold in the independent set of late stage disease when compared to early stage disease. The results of this study indicate that down-regulation of apoptosis related GADD45G is involved in ECC progression. It might be as a candidate of prognostic marker and/or therapeutic target of ECC in Hong Kong women.

Keywords: Endometrioid endometrial cancer; Apoptosis; GADD45G

Presenting author: Fengfeng Xu

Cell death induced by a novel compound ---- disorazole C_1

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Disorazoles are a family of polyene macrodiolides that are isolated from the fermentation broth of myxobacterium. Disorazole C_1 is a synthetic form of the anti-cancer drug candidate. Our preliminary data demonstrates that disorazole C_1 disrupts microtubules from the plus end and this effect is partially reversible. Disorazole C_1 is shown to cause mitotic arrest and induce cell death in all cell lines tested. Retinal Pigmented Epithelial cells stably transfected with hTERT (RPE-hTERT) were chosen as the model cell line to study the mechanism through which disorazole C_1, as well as other microtubule inhibitors, induces cell death. After disorazole C_1 treatment, p53 expression is stabilized and nuclear fragmentation is observed; however, cytochrome C is not released and PARP is not cleaved. This suggests that disorazole C_1 may induce a cytochrome C-independent pathway leading to the cell death in RPE-hTERT cells. Interestingly, we also found that nuclear fragmentation can be prevented by cell cycle arrest (serum starvation), suggesting that cell cycling is required for the nuclear fragmentation. Currently, the questions we are aiming to address are what type of cell death disorazole C_1 induces and which pathways are involved. Furthermore, in comparison of other microtubule-inhibitors to disorazole C_1, we observed that Taxol, a microtubule hyper-stabilizer, induces nuclear fragmentation similarly to Disorazole C_1; while vinblastine, a microtubule disruptor, induces low levels of nuclear fragmentation in RPE-hTERT cells. This suggests that the nuclear fragmentation observed by disorazole C_1 treatment may be triggered by other pathways unrelated to microtubule disruption.

Keywords: cell death, disorazole C1, anti-cancer, microtubule

Presenting author: Ines Yacoubi

Overexpression of human p53 leads to apoptosis in *Saccharomyces cerevisiae*

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We expressed the human p53 cDNA, wild-type, three hot spot mutants (R248W, R249S, and R282W) and a double mutant (N268S::I332V) in *Sacchromyces cerevisiae*. Here, we show that wild type p53 and the majority of mutants dramatically affect yeast growth and survival specifically on minimal media. The double mutant (N268S::I332V) and the hot spot R282W p53 inhibit yeast growth as did the wild type. The R249S mutant also impairs growth and survival but less dramatically. However, the R248W mutant is completely inactive and had not effect on yeast grow. Through differential display analysis, we showed that p53 played its negative role by disturbing the transcription of several yeast genes. One of the p53 repressed genes was identified as the Thiordoxin and confirmed by Northen blotting to be strongly repressed in presence of p53. Moreover, the p53 wild type expressing yeast cells die on minimal media exhibiting apoptotic markers: exposition of phosphatidylserine and DNA strands cleavage as shown by Annexin V staining and TUNEL technique respectively...All these data suggest that human p53 does not simply acts as cytotoxic substances in *S.cerevisiae* but seems to activate cell death by apoptosis.

Keywords: p53; hot spot mutations; Saccharomyces cerevisiae; apoptosis, differential display

Presenting author: Angeline J. J Ye

Autophagic Cell Death in *Blastocystis* Induced by a Cytotoxic Monoclonal Antibody

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Previous studies from our laboratory (Tan and Nasirudeen 2005, Trends in Parasitology 21:547-50) demonstrated that a cytotoxic monoclonal antibody, MAb 1D5, elicits a programmed cell death (PCD) response in the protozoan parasite *Blastocystis* independent of caspases, mitochondria, or both. In this study, we investigated if MAb 1D5-treated *B. hominis* can undergo autophagic cell death. This form of PCD is caspase-independent, mitochondria-dependent and characterized by cytoplasmic vacuolation in the presence of DNA fragmentation. Flow cytometric analysis of caspase activation, mitochondrial membrane potential and *in situ* DNA fragmentation of MAb 1D5-treated *B. hominis* alone or in the presence of pan-caspase inhibitor zVAD.fmk, and/or a mitochondrial transition pore blocker, cyclosporin A (CA) showed that caspases and mitochondria were efficiently blocked by the inhibitors. The use of monodansylcadaverine (MDC) and 3-methyladenine, an autophagic marker and an inhibitor of autophagy respectively, showed the existence of autophagic cell death in MAb 1D5-treated *B. hominis*. Interestingly, autophagic cell death was intensified in MAb 1D5-treated cells in the presence of zVAD.fmk. Transmission electron microscopic studies also displayed morphological changes suggestive of autophagic cell death. MDC-labeling of *B. hominis* colonies suggest the presence of autophagy in the central regions, which is likely to be triggered by nutrient deprivation. However, MDC-accumulation was significantly reduced in the central regions of *B. hominis* colonies which were pre-treated with MAb 1D5. This suggests that MAb 1D5-resistant *B. hominis* do not undergo autophagy, even in the central regions, further confirming the role of MAb 1D5 in inducing autophagic cell death. This study showed, for the first time, that autophagic cell death occurs in an enteric protozoan parasite which is caspase-independent and likely to be mediated by mitochondria. This suggests that the seemingly simple Protozoa possess complex cell death machinery which may lead to the development of novel therapeutics targeting various modulators of the protozoan cell death pathway. (303 words)

Keywords: Autophagic Cell Death

Presenting author: Chiao-Hwa Yeh

Grouper iridovirus infection induces apoptosis in barramundi cells

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Iridoviruses are large icosahedral cytoplasmic DNA viruses, belong to the family Iridoviridae. Grouper iridovirus (GIV) has been isolated from diseased grouper *Epinephelus awoara* and characterized in our laboratory. Here we demonstrate that the GIV infection in the cell lines, BM (barramundi muscle) and BSB (barramundi swim bladder) induces apoptosis. The induction of apoptosis in response to GIV infection has been detected and confirmed through various methods. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay showed the inhibition of cell growth. Morphological changes such as shrinkage and rounding were observed in the induced apoptotic cells. The formation of apoptotic body-like vesicles was initially observed at 1 h post-infection (hpi). The DNA extracted from the GIV infected cells showed DNA laddering by DNA fragmentation assay at 2 hpi. This was further confirmed by TUNEL assay. The chromatin condensation was also observed at 2 hpi. By flowcytometry, the apoptotic cells were quantified through the detection of phosphotidylserine (PS) expressed on the apoptotic cell surface. The above results demonstrate that BM and BSB cell lines can be used as a tool for studying fish iridovirus triggered apoptosis and the molecular mechanisms of induced programmed cell death.

Keywords: grouper, iridovirus, apoptosis,barramundi cells

Presenting author: Jing Yin

Proteomic analysis of antibody- and metronidazole-induced programmed cell death in the protozoan parasite *Blastocystis*

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We have shown that the human intestinal protozoan parasite *Blastocystis* undergoes programmed cell death when treated with a surface reactive cytotoxic monoclonal antibody (1D5), or metronidazole, a nitroimidazole antibiotic used clinically to treat *Blastocystis* infections in humans. The morphological and biochemical observations indicated that the cell death mechanisms in *Blastocystis* and higher eukaryotes may be conserved at certain levels. The present study aims to identify in *Blastocystis* the molecular machinery involved in apoptotic mechanisms and regulation of this cell-death pathway by a proteomics approach. We have for the first time established the proteome map for *Blastocystis*. Over 1000 proteins were reproducibly separated by two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE). Using both 2D- and 1D-PAGE, the protein profile changes were studied after 3h, 6h, 9h or 12h exposure to $5x10^{-7}$ M metronidazole. Proteins were then identified by MALDI-TOF mass spectrometry. By employing rabbit anti-caspase 8 antibody, we also found the presence of caspase-8-like antigens in *Blastocystis*. The caspase-8-like protein appeared to be cleaved after metronidazole treatment, suggesting a role in cell death. The target of 1D5 was also revealed by 2D-PAGE and western blot, which was found to have a conserved peptidase C13 domain by bioinformatic analysis. Together these results demonstrate that proteomic tools are valuable for the study of *Blastocystis* cell death mechanisms and also present a framework for further studies.

Keywords: Blastocystis, proteomics, programmed cell death

Presenting author: Joanna Zarzynska

TGF-β_1 and somatotropic pathways proteins are involved in the control of apoptosis and autophagy in involuting bovine mammary gland

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In spite of a great interest in bovine mammary gland function and milk production, the mechanism of secretory tissue involution is still poorly understood. It has been shown that expression of TGF-β_1 and its receptors in mammary epithelial cells (MEC) increases during involution of mammary gland in mouse, goat and sow. It has been also shown that TGF-β_1 is an antiproliferative and apoptotic factor in bovine MEC. Our recent study revealed that expression of TGF-β_1 in bovine MEC undergoes complex endocrine and auto/paracrine regulation by hormones of the somatotropic axis.

The present study focused on the role of TGF-β_1 and somatotropic pathways proteins in control of the switch between survival and death of bovine mammary epithelial cells. Expression of TGF-β_1, TGF-βRII, IGF-IRα, IGF-IRβ, GH-R, IGFBP-3, -4, and -5 in mammary tissue sections taken from Holstein-Fresian heifers (n=7) and cows (n=23) in early lactation (1-100 day), late lactation (200-260 day) and drying off (280-340 day), were compared with biochemical indices of apoptosis (caspase 3, 89 kDa fragment of PARP) and autophagy (Beclin1). The results revealed that an increase in apoptosis during the dry period was accompanied by highly significant increases in TGF-β_1 and TGF-βRII expression. Beside biochemical markers, typical morphological features of apoptosis were observed. TGF-β_1 expression and induction of apoptosis was facilitated by the suppression of somatotropic pathway during drying off, manifested with down-regulation of GH-R and IGF-IRα, and up-regulation of IGFBP-4 and -5.

Similarly to apoptosis, the intensity of autophagy was the highest in the dry period, as shown by increased expression of Beclin1 and morphological features. Autophagy observed in the involuting mammary tissue could be the natural cell defense against transient undernourishment and action of apoptogenic peptides (e.g. TGF-β_1, IGFBPs), thus maintaining cellular homeostasis in the dry period.

Keywords: TGF-beta1 , MEC, somatotropic axis, bovine mammary gland

Presenting author: Shouting Zhang

Brief crosslinking of Fas/CD95 on T cells leads to rapid and efficient clearance by human macrophages in the absence of common markers of apoptosis

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Crosslinking of Fas (APO-1/CD95) on the surface of T cells, either by its natural ligand (FasL/CD95L) or by agonistic anti-Fas IgM, initiates a biochemical cascade leading to programmed cell death, with phosphatidylserine (PS) externalization and caspase activation. The subsequent efficient clearance of the dying cells is of importance to prevent the development of chronic inflammation and autoimmune responses. The loss of membrane phospholipid asymmetry and the exposure of PS on the outer surface of the apoptotic cells has been implicated to be a crucial recognition signal for macrophages (Kagan et al., J. Immunol., 2002). In the present study, we showed that brief treatment (15 min) of Jurkat T cells with anti-Fas IgM (CH11) resulted in efficient phagocytosis by human monocyte-derived macrophages (HMDM), prior to noticeable PS exposure and caspase-3 activation. The engulfing efficiency was comparable to that of Jurkat cells treated for 4 hours with CH11, which displayed pronounced PS exposure and caspase-3 activation. Moreover, short-term (15 min) crosslinking of Fas by recombinant Fas Ligand in Jurkat cells could also induce rapid clearance. Pretreatment of the short-term CH11-treated Jurkat cells with the pan-caspase inhibitor zVAD-fmk did not inhibit target cell engulfment by HMDMs, whereas the absence of serum during the co-cultivation of target cells with macrophages significantly suppressed the phagocytosis of both short and long-term CH11-treated target cells. Of interest, despite the comparable efficiency of engulfment, co-cultivation of macrophages with the short-term CH11-treated Jurkat cells induced much lower levels of the pro-inflammatory cytokine TNF-alpha when compared to co-cultivation with long-term CH11-treated target cells. On the contrary, production of the anti-inflammatory cytokine interleukin-10 (IL-10) by macrophages was shown to be more pronounced when engulfing short-term CH11-treated cells. Our study suggests that upon very brief Fas/CD95 crosslinking, target cells can be efficiently cleared by professional phagocytes prior to the development of common biomarkers of apoptosis including caspase activation, thus ensuring the safe and silent disposal of dying cells.

Keywords: Fas/CD95, cell clearance, apoptosis

Presenting author: Ralf Zwacka

MnSOD protects colorectal cancer cells from TRAIL-induced apoptosis by inhibition of Smac/DIABLO release

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The mitochondrial enzyme Manganese Superoxide Dismutase (MnSOD) has been shown to have two faces with regard to its role in tumor development. On the one side it is well documented that overexpression of MnSOD slows down cancer cell growth, whereas on the other side MnSOD also has a metastasis promoting activity. We set out to examine the role of MnSOD in TRAIL-induced apoptosis, thought to be a first-line tumor surveillance mechanism and failure to undergo apoptosis might contribute to metastasis formation.
We show that overexpression of MnSOD at moderate levels is able to protect cells from TRAIL-induced apoptosis. While caspase-8 activation and Bid cleavage were not affected by MnSOD, we detected a marked decrease in caspase-3 activation pointing to a mitochondrial resistance mechanism. Indeed, we found that MnSOD overexpressing cells showed reduced cytochrome-c and no Smac/DIABLO release into the cytosol. The resulting lack of XIAP inhibition by cytosolic Smac/DIABLO most likely caused the TRAIL resistance as RNAi against XIAP rescued caspase-3 activity and TRAIL sensitivity. Our results show that reactive oxygen species are involved in TRAIL-induced Smac/DIABLO release and in TRAIL-triggered apoptosis. Hence, high levels of MnSOD, which decompose and neutralize these reactive oxygen species, might contribute to metastasis formation by allowing disseminated tumor cells to escape from TRAIL-mediated tumor surveillance. As part of TRAIL regimens, adjuvant treatment with XIAP inhibitors in the form of Smac/DIABLO mimetics or MnSOD inhibitors might be able to break TRAIL resistance of malignant tumor cells.

Keywords: MnSOD, TRAIL, Colon Cancer, Smac/DIABLO

Presenting author: Cecilie Torp Andersen

EXTRA POSTER

Gliotoxin Mediated Apoptotic Cell Death in Jurkat cells

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Aspergillus fumigatus infections cause severe morbidity and high mortality in immunocompromised patients. The mycotoxin gliotoxin is secreted during infection and it has been suggested to be a factor in the pathogenesis of invasive aspergillosis. This toxin has been detected in the serum of patients with probable or proven invasive aspergillosis. Gliotoxin has been shown to inhibit multiple processes associated with activation, differentiation, and/or effector functions of immune cells in vitro.
We have initiated studies of cell death on the leukemic T cell line Jurkat E6 in the presence of Gliotoxin. Cell death was determined using Annexin V- Fluos and propidium iodide staining and flow cytometry. We find evidence of apoptotic cell death in a time- and concentration-dependent manner. Gliotoxin-stimulation in concentrations of 50-2000ng/ml shows a bell-shaped response curve with maximal apoptosis defined as annexin positive, PI negative cells with 500ng after 4 and 6 hours. The effect was almost completely inhibited using the pan caspase- inhibitor Z-VAD-FMK. Analysis of active caspase-3 using flow cytometry, shows an increase in caspase 3 activity from 3 % in the control group to 8, 21 to 40% after stimulation with 250ng Gliotoxin for 2, 3 and 4 hours respectively.

Our findings suggest that gliotoxin may modulate the T-cell response during invasive aspergillosis.

Keywords: Gliotoxin, apoptosis, Jurkat

Presenting author: Mirjana Djukic

EXTRA POSTER

SOD activity in striatum of Wistar rats pretreated with L-NAME and poisoned with paraquat

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Paraquat produces superoxide anion ($O_2^{\bullet-}$) during its metabolism. Developed oxidative stress (OS) interferes with cell signaling pathways and leads to apoptosis. The increased activity of antioxidative enzyme superoxide dismutase (SOD), scavenger of $O_2^{\bullet-}$, is anticipated in such events. N^G-nitro-L-arginine methyl ester (L-NAME), a competitive, nonspecific inhibitor of nitrogen oxide (NO) synthase, possible could restrain the development of OS and nitrosative stress in neuronal cells by reducing NO bioavailability.

The goal of the study is to investigate SOD activity in the striatum of Wistar rats, after *intrastriatally* (*i.s.*) acute poisoning with PQ and to reveal if the administration of L-NAME in the pretreatment of PQ poisoning interferes with SOD activity.

The animals were divided in five groups (24 animals in each): K_i - intact control group; K_{so} - sham operated (*i.s.* treated with 10 µl of 0.9% NaCl); L-NAME - *i.s.* treated with L-NAME (50 mg/kg); PQ - *i.s.* poisoned with PQ (50 mg/kg); and L-NAME+PQ - pretreated with L-NAME (50 mg/kg) 30 minutes before PQ administration.

Measurement of SOD activity was performed spectrophotometrically in mitochondria crude fraction of striatum, 30 minutes, 24 hours and 7 days after treatments.

Observed results indicate similar SOD activity in both side (ipsilateral and contralateral) of the striatum in all treatments and time points. Compared to Ki, SOD activity in Kf was 50% higher, what indicates oxidative injury induced by the way of application, although physiological saline was administrated. By itself, L-NAME decreased SOD activity for 60%. These results are in accordance with literature and approve neuroprotective effect of L-NAME, as well, involvement of NO in OS development after the act of substance administration. Interestingly, PQ did not enhance SOD activity. Obtained values were closed to controls with remarkable decline of SOD activity (25% of controls) at 24 hour. These results indicate that metabolic pathways of $O_2^{\bullet-}$ in brain tissues, differ from those seen in lung. Probably, spontaneous reaction between $O_2^{\bullet-}$ with NO takes advantage on dismutation by SOD. SOD activity in the group L-NAME+PQ are closed to controls in each time point.

Striatum respond to the act of a substance administration by OS development. Involvement of NO in OS induced by PQ is confirmed by SOD activity assessment and L-NAME applying, in our study. Greater ability of $O_2^{\bullet-}$ to react with NO than with SOD could be one of its possible metabolic pathways in PQ neurotoxicity.

Presenting author: Stein Ove Døskeland

EXTRA POSTER

Abolition of stress-induced protein synthesis sensitizes leukemia cells to anthracycline-induced death

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Anthracycline action has been thought to involve the neosynthesis of pro-apoptotic gene products, and therefore depend on protein synthesis for optimal effect. We found that inhibition of general, but not rapamycin-sensitive (cap-dependent), protein synthesis in the pre-apoptotic period enhanced anthracycline-induced AML cell death, both in vitro and in several animal AML models. Pre-apoptotic anthracycline-exposed AML cells had altered translational specificity, with enhanced synthesis of a subset of proteins, including ER chaperones. The altered translational specificity could be explained by perturbation (protein degradation, truncation or dephosphorylation) of the cap-dependent translation initiation machinery and of proteins controlling translation of specific mRNAs. We propose that judiciously timed inhibition of cap-independent translation is considered for combination therapy with anthracyclines in AML.

Keywords: Apoptosis; Daunorubicin; Idarubicin; BNML; AML; Proteomics; Phosphorylation; mTOR; Ribosomal protein; Elongation; Cycloheximide

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