European Cell Death Organization (ECDO)

UNIVERSITÄT BERN

16th ECDO Euroconference on Apoptosis

September 6-9, 2008, Bern, Switzerland

Programme and Book of Abstracts

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

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VIB/Ghent University,Ghent, Belgium
Boris **Zhivotovsky**
Karolinska Institutet, Stockholm, Sweden

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Welcome

Dear participants,

we welcome you to the 16th Euroconference on Apoptosis that is organized together with the 5th Swiss Apoptosis Meeting. Since the 8th Euroconference on Apoptosis took place in Davos, we are proud and honoured to have again the chance to organize this meeting in Switzerland. All Euroconferences on Apoptosis are initiatives of the European Cell Death Organization (ECDO). Over the last 4 years, our conferences were supported by EC within the framework of the Marie Curie Conferences and Training Courses. Unfortunately, this support is no longer available and ECDO was forced to find other financial sources. Therefore, the organization of this meeting was rather difficult and we had to reduce costs. In spite of the financial limits, we believe that we organized a very attractive meeting in terms of both science and social events.

The purpose of the ECDO is to advance the field of experimental and clinical cell death research in Europe. Scientific meetings like this certainly help to achieve this goal. We have tried to establish a program, which is something for basic researchers, clinician-scientists, as well as individuals working in the industry. We have purposely built into the program plenty of time for poster viewing and hope that this will greatly accelerate the scientific discussion. Moreover, we newly included a cutting edge young investigator session in the program.

Our goal is to provide a platform to encourage discussion among the different groups of researchers. Only when we work together, we will advance the field of cell death research and will be successful to bring important developments into the clinics to the benefit of our patients. We greatly appreciate that the two existing European networks in cell death research (Apo-Sys and ApopTrain) selected this meeting as a platform to meet.

We are thrilled at the level of interest in cell death research, as reflected by the attendance at this meeting of world-class scientists who have come together to review recent advances in the field. We are also thrilled by the many junior and other basic, clinical and industrial investigators who will present talks and posters. This meeting has more than 300 participants. This number was a real surprise to us, since we were unable to provide any travel support.

We wish to gratefully acknowledge the hard work of Veronique Vandevoorde (ECDO secretariat, Ghent) and the secretaries of the Institute of Pharmacology of the University of Bern (Sandra Suter and Anita Dähler). Moreover, we particularly thank our corporate, institution, and foundation sponsors for their generous financial support. Without them, this outstanding meeting would not be possible. On the other hand, the support we received demonstrates the need and interest in such events and should encourage us as a society to continue in this direction.

On behalf of the organizers, sponsors, and exhibitors, welcome again to ***ECDO 2008*** in Bern!

Hans-Uwe Simon
Chairman of the conference,
President of ECDO

Thomas Brunner
Chairman of the Conference

CONFERENCE VENUE:

Auditorium ETTORE ROSSI
University Hospital Bern (Inselspital)
Freiburgerstrasse
CH-3010 Bern
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REGISTRATION

To collect the conference materials you are kindly requested to register at the Conference Secretariat at the entrance of the Auditorium ETTORE ROSSI.

Opening hours of the registration desk:

September 6 (Saturday)	12:00 – 18.00
September 7 (Sunday)	08.00 – 18.00
September 8 (Monday)	08.00 – 18.00
September 9 (Tuesday)	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, lunches,..

CONFERENCE SECRETARIAT

If you need any help during the conference you can find the local organisers 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 +41-79-595-1281 (Hans-Uwe Simon)

LECTURE ROOM

All lectures will be given in the **Auditorium ETTORE ROSSI, University Hospital Bern (Inselspital).** Poster presentation will take place next to the meeting room. Please check the information on the poster sessions on the conference website.

INTERNET CONNECTION

Please contact the registration desk. A few PCs with internet access will be available to the conference delegates at the Institute of Pharmacology, University of Bern.

MEALS AND REFRESHMENT

All meals/refreshments below are included in the conference registration fee. Please wear your name badge at all times when attending these events.

The Welcome reception on Saturday evening September 6 will take place at the **House of the University**, which is in walking distance from the congress center.

Lunches on Sunday, Monday and Tuesday will be served at the congress center. Lunch tickets for accompanying persons can be bought at the registration desk (CHF 60 for all lunches).

Dinner on Sunday evening September 7 will be served in the **Kornhauskeller.** Please **pick up your voucher at the registration desk on Saturday**. There is a little map on the voucher, which describes the location of the Kornhauskeller.

The **gala dinner** on Tuesday evening September 9 will take place at the restaurant **Dählhölzli (Tierpark).** Please **pick up your voucher at the registration desk on Sunday**. There is a little map on the voucher, which describes the location of the restaurant Dählhölzli.

During lunch and coffee breaks there will be opportunity to visit the exhibitions.

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

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

Poster session 1: Sunday, September 7: 16:30-18:00 : Posters 1-93
Poster session 2: Monday, September 8: 16:30-18:00 : Posters 94-184

Poster should be made in portrait, posterboard measurements are **120 cm (height) x 90 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.

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

Posters should be posted on the day of presentation and immediately be removed by the end of each poster session. We cannot take responsibility for the posters not removed.

SMOKING

Smoking is NOT allowed in the Auditorium ETTORE ROSSI, University Hospital Bern (Inselspital).

TRANSPORT TO/FROM THE AIRPORT

Bern can be reached from the airports in Zurich, Basel, or Geneva by train. From the Bern main train station, take Bus No. 11 direction of “Inselspital” to Insel Hospital (two stops only). Participants arriving at the airport in Bern reach the city by bus or taxi. If you come by car, take freeway exit Bern Forsthaus and follow the Murtenstrasse until you enter the Hospital area on the right.

TELEPHONE SERVICE

Public telephones are available at the University Hospital Bern (Inselspital).

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 Swiss currency is Swiss Franc (CHF). Currency exchange booths are available at the airport terminals and in a large number of hotels. Usual opening hours of Swiss banks are: Monday-Friday: 9:00-12:00 and 14:00-17:00.

Participants are expected to pay for the hotel room upon check-out.

SPONSORS

The Local Organisers and ECDO wish to thank the **SPONSORS** listed below for their financial support.

Gold

- Kontaktgruppe für Forschungsfragen (Novartis, Roche, Merck-Serono), Basel
- Max and Elsa Beer-Brawand-Fonds, Bern
- Pfizer AG, Zurich

Silver

- Carl Zeiss AG, Feldbach
- Union of the Swiss Societies of Experimental Biology (USSBE/USGEB)

Bronze

- Institute of Pharmacology, University of Bern
- Institute of Pathology, University of Bern

Welcome Reception

- City of Bern
- Canton of Bern

Training Course

- Graduate School (University of Bern)

gcb Graduate School for Cellular and Biomedical Sciences

Poster Prize

- Nature Reviews Molecular Cell Biology

Congress bags

- AstraZeneca AG, Zug

Publicity

- Cell Death and Differentiation, Nature Publishing Group
- Apoptosis, Springer

EXHIBITIONS

The Local Organisers and ECDO wish to thank the **EXHIBITORS** listed below for their active contribution.

5th Training Course on

Concepts and Methods in Programmed Cell Death

Bern, Switzerland, September 6, 2008

Programme

Chair: Seamus Martin (Dublin, Ireland)

14:00 – 14:45	**Adi Kimchi (Rehovot, Israel)** Many ways to die – the different forms of programmed cell death and their analysis
14:45 – 15:30	**Andreas Villunger (Innsbruck, Austria)** Death signaling pathways leading to apoptosis and their analysis
15:30 – 16:00	**Coffee break**
16:00 – 16:45	**Peter Vandenabeele (Ghent, Belgium)** Mechanisms of dead cell removal
16:45 – 17:30	**Klaus Schulze-Osthoff (Tübingen, Germany)** Cell death and disease
17:30 – 18:15	**Peter Krammer (Heidelberg, Germany)** Milestones and future perspectives in cell death research
18:15 – 18:30	**Discussion**

Conference Programme

16th EUROCONFERENCE ON APOPTOSIS and 5th SWISS APOPTOSIS MEETING

September 6-9, 2008, Bern, Switzerland

Saturday evening September 6

Welcome and ECDO Honorary Lecture
Chairs: Hans-Uwe Simon (Bern, CH) & Boris Zhivotovsky (Stockholm, Sweden)

19:30 – 19:40	**Alexander Tschäppät (President of the city, Bern, CH):** Welcome
19:40 – 19:50	**Peter Eggli (Dean of the Medical Faculty, Bern, CH):** Welcome
19:50 – 20:00	**Hans-Uwe Simon (Bern, CH):** Welcome
20:00 – 20:05	**Harald Reuter (Bern, CH):** Israeli-Palestinian Science Organization – background and goals
20:05 – 20:15	**Boris Zhivotovsky (Stockholm, Sweden):** Laudatio
20:15 – 21:00	**ECDO honorary lecture** **Pierre Golstein (Marseille, France)** Cell deaths from mouse to Dictyostelium
21:00	**Reception – House of the University**

Sunday, September 7, 2008

Session 1: System biology approaches in cell death research
Chair – Boris Zhivotovsky (Stockholm, Sweden)

09:00 – 9:30 **Inna Lavrik (Heidelberg, Germany)**
Promises and challenges of systems biology: Life/Death decisions in the CD95 signaling pathway

09:30 – 9:45 **Aswin Pyakurel (Geneva, CH)**
The role of Mek1 in controlling mitochondrial morphology

09:45 – 10:00 **Syam Prakash Somasekharan (Geneva, CH**)
The large dynamin GTPase Drp1 stimulates Bax activation

10:00 – 10:30 **Jochen Prehn (Dublin, Ireland)**
Real-time imaging and mathematical systems analysis of mitochondrial outer membrane permeabilisation and caspase activation during apoptosis: Is very little already too much?

10:30 – 11:00 Coffee break (with exhibition viewing)

Session 2: Cell death signalling
Chair – Simone Fulda (Ulm, Germany)

11:00 – 11:30 **Douglas R. Green (Memphis, TN, USA**)
Mitochondria, ER, and the greasy path to apoptosis

11:30 – 11:45 **Christoph Borner (Freiburg, Germany)**
Lysosomal proteases: Triggers or amplifiers of apoptosis?

11:45 – 12:00 **Margaret E. Harley (Dundee, UK)**
The regulation of Mcl-1 during mitosis

12:00 – 12:15 **Dagmar Kulms (Stuttgart, Germany)**
Sensitization of melanoma cells to TRAIL by UVB-induced and NK-κB-mediated downregulation of XIAP

12:15 – 12:30 **Leobaldo Solorzano (Philadelphia, PA, USA)**
XIAP regulation by asparagine deamidation and PIMT

12:30 – 13:00 **Seamus Martin (Dublin, Ireland**):
A role for the Bcl-2 family in mitochondrial fission-fusion dynamics unrelated to apoptosis

13:00 – 14:30 **Lunch (with exhibition viewing)**

Session 3: Cell death and inflammation
Chair – Hans-Uwe Simon (Bern, CH)

14:30 – 15:00	**Jürg Tschopp (Lausanne, CH)** Inflammasomes: danger sensing complexes involved in inflammatory diseases
15:00 – 15:15	**Clemens A. Dahinden (Bern, CH)** IL-3 induces a Pim1-dependent anti-apoptotic pathway in primary human basophils
15:15 – 15:30	**Alexander U. Lüthi (Dublin, Ireland)** Suppression of IL-33 bioactivity through proteolysis by apoptotic caspases
15:30 – 15:45	**Sara Y. Demiroglu (Göttingen, Germany)** Analysis of the role of heat shock protein 70 in granzyme B – induced apoptosis
15:45 – 16:00	**Claudia Manzl (Innsbruck, Austria)** Is the PIDDosome required for caspase-2 processing?
16:00 – 16:30	**Shigekazu Nagata (Kyoto, Japan)** Engulfment of apoptotic cells
16:30 – 18:00	**Poster Viewing Session #1, posters 1-93 (with refreshments)**

20:00 – 23:00	**Dinner (Kornhauskeller)**

Monday, September 8, 2008

Session 4: Cell death in model organisms
Chair – Pierre Golstein (Marseille, France)

09:00 – 09:30 **Michael Hengartner (Zurich, CH)**
Death, destruction and mayhem: regulation of apoptosis in the nematode C. elegans

09:30 – 09:45 **Daniele Bano (Leicester/Zurich, UK/CH)**
Alteration of nucleocytoplasmic transport occurs during calcium-mediated cell death

09:45 – 10:00 **Brigitte Galliot (Geneva, CH)**
Apoptosis, a driving force for head regeneration in hydra

10:00 – 10:30 **Shai Shaham (New York, NY, USA)**
A novel morphologically-conserved C. elegans cell death program

10:30 – 11:00 Coffee break (with exhibition viewing)

Session 5: Cell death in experimental mouse systems
Chair – Gerry Melino (Rome/Leicester, Italy/UK)

11:00 – 11:30 **Philippe Bouillet (Melbourne, Australia**)
Mouse models to study the function of the BH3-only members of the Bcl-2 family

11:30 – 11:45 **Wim Declercq (Ghent, Belgium)**
Caspase-14 is a crucial protease in proper stratum corneum formation

11:45 – 12:00 **Silke F. Fischer (Melbourne/Ulm, Australia/Germany)**
Proapoptotic BH3-only proteins Bim and Puma are essential for apoptosis of germinal center-derived memory B cells and antibody-forming cells

12:00 – 12:15 **Lars Joeckel (Freiburg, Germany)**
Biological role of “orphan” granzymes in LCMV-WE infection

12:15 – 12:30 **Oksana Voloshanenko (Heidelberg, Germany)**
Detachment-induced sensitization to TRAIL explains the metastasis-specific role of the TRAIL/TRAIL-R system in epithelial tumorigenesis

12:30 – 13:00 **Thomas Brunner (Bern, CH)**
Interactions between death receptor and mitochondrial apoptosis pathway in hepatocyte cell death

13:00 – 14:30 Lunch (with exhibition viewing)

Session 6: Autophagic cell death
Chair – Laszlo Fesus (Debrecen, Hungary)

14:30 – 15:00	**Guido Kroemer (Villejuif, France)** Autophagy control by transcription factors: the example of p53
15:00 – 15:30	**Eric Baehrecke (Worcester, MA, USA)** Genetic regulation of autophagic programmed cell death
15:30 – 15:45	**Laura Bonapace (Zurich, CH)** The BH3 mimetic obatoclax overcomes glucocorticoid-resistance by activating autophagic cell death
15:45 – 16:00	**Patricia Boya (Madrid, Spain)** The autophagic machinery is necessary for removal of cell corpses during the development of the nervous system
16:00 – 16:30	**Mauro Piacentini (Rome, Italy)** Regulation of autophagy in mammals by AMBRA1 and its partners
16:30 – 18:00	**Poster Viewing Session #2, posters 94-184 (with refreshments)**
18:00 -20:00	**ECDO Board meeting** (seminar room, Institute of Pharmacology)

Free evening

Tuesday, September 9, 2008

Session 7: Cell death and cancer
Chair – Guido Kroemer (Villejuif, France)

09:00 – 09:30 **Gerard Evan (San Francisco, CA, USA)**
The ins and outs of oncogene-induced tumor suppression

09:30 – 09:45 **Arun Dharmarajan (Perth, Australia)**
Pro-apoptotic, anti-proliferative, and anti-angiogenic therapeutics in cancer

09:45 – 10:00 **Nina Gebhardt (Berlin, Germany)**
Bax/Bak independent induction of apoptosis by BH3-mimetic small molecule compounds

10:00 – 10:30 **Gerry Melino (Rome/Leicester, Italy/UK)**
miR, "stemness" & skin

10:30 – 11:00 Coffee break (with exhibition viewing)

Session 8: Cell death biomarkers
Chair – Marie-Lise Gougeon (Paris, France)

11:00 – 11:30 **Klaus-Michael Debatin (Ulm, Germany)**
Cell death and cancer therapy in the clinic

11:30 – 11:45 **Sharon Leong (Sydney, Australia)**
Proteomic profiling for apoptotic biomarkers of breast cancer response to chemotherapy

11:45 – 12:00 **Dieter Demon (Ghent, Belgium)**
Proteome analysis reveals different cleavage site preferences for caspase-3 and -7

12:00 – 12:15 **Bonafsheh Mirnikjoo (Houston, TX, USA)**
The redistribution of lysosomal phosphatidylserine to the plasma membrane outer membrane leaflet during apoptosis

12:15 – 12:30 **Gabriela Brumatti (Parkville, Australia)**
Regulated Hox gene expression reveals a novel role for Hox genes in myeloid cell proliferation and survival

12:30 – 13:00 **Heike Bantel (Hannover, Germany)**
Apoptosis biomarkers in liver diseases

13:00 – 14:30 Lunch (with exhibition viewing)

Session 9: ECDO Keynote Lecture
Chair – Thomas Brunner (Bern, CH)

14:30 – 15:30 **Josef Penninger (Vienna, Austria)**
Title to be announced

15:30 – 16:00 Coffee break

Session 10: Cutting Edge Young Investigator Session
Chair – Boris Turk (Ljubljana, Slovenia)

16:00 – 16:20 **Thomas Kaufmann (Melbourne, Australia/Bern, CH)**
Fatal hepatitis mediated by secreted TNFα requires caspase-8 and the two BH3-only proteins Bid and Bim

16:20 – 16:40 **Guillermo Velasco (Madrid, Spain)**
Cannabinoid antitumoral action: inducing ER stress and autophagy to promote cell death

16:45 – 17:00 Concluding remarks

17:00 – 17:45 ECDO General Assembly

19:30 – 24:00 Apéro, Gala dinner and Disco (Dählhölzli, Tierpark)

Dinners and coffee breaks are sponsored by pharmaceutical companies.

Lecture Abstracts

Invited speakers

Abstracts in this section are listed 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/bern/index.html.

Genetic regulation of autophagic programmed cell death.

Eric H. Baehrecke

Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01615, USA.

Little is known about the mechanisms that mediate autophagic cell death. A proteomic screen resulted in the identification of factors that are expressed during autophagic cell death of salivary glands during Drosophila development, including the large tumor suppressor Lats (Wts) that is in the Hpo (Ste-20 kinase) signaling pathway. Loss-of-function mutations in wts, and RNAi knockdown of hpo, prevent destruction of salivary glands. wts mutant salivary glands possess markers of caspase activity, but fail to arrest growth and exhibit impaired autophagy. Surprisingly, expression Yki (Yap) in salivary glands fails to phenocopy wts mutants in this tissue, even though expression of Yki in other tissues does phenocopy wts. Expression of genes that are downstream of Yki is sufficient to alter salivary gland death, indicating that wts functions in a Yki-independent manner in this tissue. wts mutant salivary glands have defects in class I PI3K signaling, and their cell death phenotype depends on the function of chico (IRS) and TOR in the PI3K signaling pathway. Although previous studies have shown that the Wts/Hpo pathway influences the cell cycle and apoptosis, it has not been linked to defects in cell growth arrest and autophagy that are mediated by the class I PI3K pathway.

Apoptosis Biomarkers in Liver Diseases

Heike Bantel

Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany

Histological evaluation of liver biopsies is presently the gold standard for determining liver disease activity, although it remains associated with sampling errors and with the risk of serious clinical complications. Measurement of serum aminotransferases which is commonly used to detect liver injury does not correlate with disease activity. Moreover, 25% of patients with chronic hepatitis C virus (HCV) infection show normal aminotransferase levels despite histological evidence of progressed disease activity. Thus, there is a strong need for better non-invasive methods to assess early liver injury.
Apoptosis has been implicated to play an important role in the pathogenesis of liver diseases. Using activation-specific antibodies for caspases as well as novel antibodies for caspase-generated neoepitopes of caspase substrates such as cytokeratin (CK)-18 which is expressed by hepatocytes, we have demonstrated that caspase activation is increased in liver biopsies of patients with chronic liver diseases and correlate with disease activity. Based on these observations, we have recently demonstrated that caspase-mediated CK-18 fragments of apoptotic hepatocytes can also be detected in the serum of patients with liver diseases. For quantification of caspase-generated CK-18 fragments in serum, we established an ELISA (M30) that selectively detects a neo-epitope of a caspase-generated CK-18 cleavage fragment. By using this ELISA we demonstrated that hepatic caspase activity is significantly increased in sera of patients with liver diseases such as chronic HCV infection or liver steatosis and correlated with disease activity. Moreover, elevated serum caspase activity could even be detected in patients with normal aminotransferase levels but progressive fibrosis. Measurement of caspase activity in serum therefore represents a sensitive non-invasive biomarker for detection of apoptotic liver injury and for monitoring of disease activity.
Having demonstrated that caspase activation is considerably elevated in chronic HCV infection, we investigated whether caspase activation is required for apoptotic HCV clearance. In addition to the assessment of hepatocyte apoptosis by the M30-ELISA, we established a luminometric substrate assay which detects caspase activity in serum independently of the cell-type. Compared to non-responders, patients responding to an antiviral therapy showed significantly elevated caspase activity in the serum that closely correlated with virus load. Both, the immunologic and the enzymatic assay provided very similar results, underlining the reliability of our caspase measurements. Since TRAIL has been suggested to play a pivotal role in the apoptotic elimination of virus-infected cells, we further analyzed this apoptosis-marker in the serum of HCV-patients. Similarly to caspase-activation, patients responding to antiviral therapy showed significantly higher serum levels of TRAIL during the course of antiviral treatment compared to non-responding patients. Thus, the measurement of caspase- and TRAIL-activities in serum represents sensitive biomarkers to predict treatment response.
In addition to the M30-ELISA, we have recently established a pan-specific CK18 (M65) ELISA that detects the total release of CK18 (i.e. the cleaved and uncleaved form) into serum. When this assay is used in combination with the caspase-specific ELISA, even different forms of cell death, such as necrosis and apoptosis, can be distinguished. By using this method we found that in patients with spontaneous recovery from acute liver failure (ALF) apoptotic cell death and in non-spontaneously recovered ALF patients necrotic cell death is predominant.

In summary, these novel assays enable us to non-invasively assess early liver cell death and apoptosis in serum samples of patients with different liver diseases.

Mouse models to study the function of the BH3-only members of the Bcl-2 family

Philippe Bouillet P, Delphine Mérino, Maybelline Giam.

Molecular Genetics of Cancer Division, The Walter and Eliza Hall Institute, Parkville, VIC3050, Australia

BH3-only proteins are crucial initiators of the apoptotic cascade regulated by the Bcl-2 family. Interactions between the members of this family leads to the activation of Bax/Bak and damage to the mitochondria.
Of all BH3-only proteins, Bim has certain characteristics that make it an interesting tool to study the role of BH3-only proteins in Bax/Bak activation. The hemopoietic phenotype of Bim-null mice is well defined (excessive numbers of lymphoid and myeloid cells, anomalies in the deletion of autoreactive thymocytes and B cells and survival advantages of many sub-populations).
To determine how changing its binding specificity affects the biological activity of Bim in vivo, we have modified the mouse Bim gene in situ by replacing its BH3 domain with that of Bad, Noxa or Puma. We have then tested whether these mutants could rescue the polycystic kidney disease and hematopoietic phenotypes of the Bcl-2-KO mice. We conclude that Bim BH3 has abilities that neither Bad, Noxa nor Puma BH3s have.
Analysis of many mutant mice deficient for two members of the Bcl-2 family indicates that Bim, Bmf, Bax and Bak are responsible for the removal of interdigital mesenchyme by apoptosis during development. Subsequent considerations have led us to propose that non-apoptotic cell death can be triggerred by the Bcl-2 family members when the apoptosis process is incapacitated.

Interactions between death receptor and mitochondrial apoptosis pathway in hepatocyte cell death

Thomi Brunner, Nadia Corazza, Sabine Jakob, Anastasia Badmann

Institute of Pathology, University of Bern, Switzerland

In most cell types the death receptor signaling pathway, as mediated by TRAIL Fas ligand and TNFα, and the mitochondrial pathway represent distinct apoptosis signaling processes. In so-called type II cells, however, death receptor signaling is amplified via the mitochondrial pathway to efficiently induce apoptotic cell death. This is particularly obvious in hepatocytes where Fas-induced apoptosis requires the cleavage and activation of the BH3-only protein Bid to amplify the signal via the mitochondrial apoptosis pathway. We have recently defined a novel interaction of the death receptor signaling pathway with the mitochondrial pathway, which seems to have important implications in a variety of apoptosis signaling events in hepatocytes but also in liver-derived tumors. While TRAIL receptor activation fails to directly induce apoptosis in primary hepatocytes, it amplifies the mitochondrial apoptosis pathways induced by other triggers. This TRAIL-induced sensitization involves the activation of Jun kinase and Jun kinase-mediated activation of the BH3-only protein Bim. The TRAIL-Jun kinase-Bim axis is capable of amplifying a variety of cell death signals and seems to represent a conserved apoptosis modulation pathway in the liver. Recent findings in hepatocytes and liver tumor cells will be discussed.

Cell death and cancer therapy in the clinic

Klaus-Michael Debatin

University of Ulm, Department of Pediatrics and Adolescent Medicine, Eythstrasse 24, Ulm, Germany

The ins and outs of oncogene-induced tumor suppression

Laura Soucek, Daniel Murphy, Melissa Junttila, Laurent Pouyet, Lamorna Brown Swigart and Gerard I. Evan

Dept. Pathology and Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, California 94143, USA

Myc is a highly pleiotropic transcription factor that coordinates the mechanistically diverse intracellular and extracellular biological programs required for orderly and efficient expansion of somatic cells. Although such properties indicate that Myc would make an excellent therapeutic target, there are several problems. First, Myc is deemed “undruggable” by the pharmaceutical industry. Second, while Myc is deregulated and elevated in most human cancers, this is rarely due to mutations in the Myc genes themselves but usually a consequence of their relentless induction by upstream oncogenic signals. It has been shown that experimental Myc-driven transgenic tumors remain dependent upon sustained Myc activity for their maintenance: whether normal Myc, acting as a client of upstream oncogenes, fulfils the same pivotal function is unknown. Third, Myc function is required for proliferation of all normal tissues, intimating that systemic Myc inhibition might be accompanied by devastating side effects. Sidestepping the issue of Myc’s “druggability,” we have now modeled the both the therapeutic efficacy and index of a Myc-inhibitory drug in the mouse against KRas and SV40 T-induced tumors using a novel genetic strategy. Our studies indicate that Myc inhibition has potent and selective anti-tumor activity. We now know that one of the major mechanisms restraining the oncogenic activities of deregulated Myc is its obligate coupling to growth-inhibitory programs like apoptosis and replicative senescence – a phenomenon dubbed intrinsic tumor suppression. Thus, Myc induces cell proliferation but also apoptosis and senescence. Clearly, cells need to be able to discriminate between normally activated Myc, where tumor suppression is suppressed, and oncogenic Myc, where it is activated. How cells do this is unknown although the unusual persistence of oncogenic Myc, its activation outside the context of other growth signals, and its elevated expression have all been suggested as mechanisms. To investigate this, we have used a novel model of Myc oncogenesis in which Myc is deregulated but without over-expression. Through analysis of the *in vivo* oncogenic and tumor suppressor properties of Myc in this model we have now identified the key attribute of oncogenic Myc that cells recognise. These data have important implications for how Myc appears to select from amongst its very different biological outputs.

Invited speaker: Pierre Golstein
ECDO Honorary Lecture

Cell deaths from Mouse to Dictyostelium.

Pierre Golstein

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To study cell death, we shifted from Mouse to the protist Dictyostelium discoideum. The latter turns out to be a very favorable, genetically tractable model to study non-apoptotic (eg autophagic, necrotic) cell death. I'll first describe this model, then I'll list several advantages of Dictyostelium to study the induction of non-apoptotic cell death in vitro. First, its small, sequenced and haploid genome facilitates genetic especially mutational approaches. Second, the Dictyostelium genome does not encode the main protein families at play in apoptotic cell death, namely the caspase (except an irrelevant paracaspase) and the bcl-2 families. Thus, the autophagic and necrotic cell death in Dictyostelium can take place with no interference from the apoptosis machinery.
Third, induction of autophagic cell death follows in this case a two-step process, namely starvation-induced sensitization leading to autophagy but not to death, followed by a DIF-1-induced pathway leading to cell death proper. The latter, DIF-1-induced pathway is defined experimentally through sequential additions, and most important also genetically through random mutagenesis leading to the preparation and study of several mutants. Further study of the DIF-1 pathway should shed further light on the induction of autophagic cell death (as opposed to that of just autophagy) in Dictyostelium and by extension perhaps in other organisms. Similar approaches and conclusions also hold for an atg1 mutation and a two-step induction of necrotic cell death.
These and other approaches and results will be described.

Mitochondria, ER, and the greasy path to apoptosis

Douglas Green

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Death, destruction and mayhem: regulation of apoptosis in the nematode C. elegans

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Fatal Hepatitis Mediated by Secreted TNFα Requires Caspase-8 and the two BH3-only Proteins Bid and Bim

Thomas Kaufmann (1,5,6), Philipp J Jost (1,7), Marc Pellegrini (2,7), Hamsa Puthalakath1 (3), Raffi Gugasyan (1), Steve Gerondakis (1), Razq Hakem (2), Philippe Bouillet (1), Tak W Mak (2), Vishva M Dixit (4) and Andreas Strasser (1,5)

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Apoptotic death of hepatocytes, a feature of many chronic and acute liver diseases, can be a consequence of over-activation of the immune system and is caused by pro-inflammatory cytokines, such as TNFα. Injection of mice with lipopolysaccharide (LPS) plus the transcriptional inhibitor D(+)-galactosamine (GalN) results in rapid hepatocyte apoptosis. This is mediated by secreted TNFα signaling via TNF-R1, but the effector mechanisms are unclear. Our analysis of gene-targeted mice showed that caspase-8 is essential for this hepatocyte killing. Loss of Bid, the pro-apoptotic BH3-only protein activated by caspase-8, and essential for Fas ligand-induced hepatocyte killing, resulted only in a minor reduction of liver damage and mortality. Remarkably, combined loss of Bid and another BH3-only protein, Bim, activated by JNK, protected mice from fatal LPS/GalN-induced hepatitis. These observations identify caspase-8 and the BH3-only proteins Bid and Bim as potential therapeutic targets for treatment of inflammatory liver diseases.

Autophagy control by transcription factors: the example of p53

Ezgi Tasdemir (1-3), M. Chiara Maiuri (1-4), Eugenia Morselli (1-3), Alfredo Criollo (1-3), Marcello d'Amelio (5), Francesco Cecconi (5), Nektarios Tavernarakis (6), and Guido Kroemer (1-3)

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It is known that genotoxic stress can induce autophagy in a p53-dependent fashion and that p53 can transactivate autophagy-inducing genes. We have observed that, in addition, inactivation of p53 by deletion, depletion or inhibition can trigger autophagy. Thus, human and mouse cells subjected to knockout, knockdown or pharmacological inhibition of p53 manifest signs of autophagy such as depletion of p62/SQSTM1, LC3 lipidation, redistribution of GFP-LC3 in cytoplasmic puncta, and accumulation of autophagosomes and autolysosomes, both *in vitro* and *in vivo*. Inhibition of p53 caused autophagy in enucleated cells, indicating that the cytoplasmic, non-nuclear pool of p53 can regulate autophagy. Accordingly, retransfection of *p53-/-* cells with wild type p53 as well as a p53 mutant that is excluded from the nucleus (due to the deletion of the nuclear localization sequence) could inhibit autophagy, while retransfection with a nucleus-restricted p53 mutant (in which the nuclear localization sequence has been deleted) did not inhibit autophagy. Several distinct autophagy inducers (e.g. starvation, rapamycin, lithium, tunicamycin and thapsigargin) stimulated the rapid degradation of p53. In these conditions, inhibition of the p53-specificE3 ubiquitin ligase HDM2 could avoid p53 depletion and simultaneously prevented the activation of autophagy. Moreover, a p53 mutant that lacks the HDM2 ubiquitinylation site and hence is more stable than wild type p53 was particularly efficient in suppressing autophagy. In conclusion, p53 plays a dual role in the control of autophagy. On one hand, nuclear p53 can induce autophagy through transcriptional effects. On the other hand, cytoplasmic p53 may act as a master repressor of autophagy.

Promises and challenges of systems biology: Life/Death decisions in the CD95 signaling pathway

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Our study explores a highly significant dilemma in cell biology, namely how triggering of CD95 (Fas/APO-1) in some situations results in cell death and in others circumstances leads to the activation of NF-kB resulting in cell growth. We established an integrated kinetic mathematical model of CD95-mediated life and death signalling. Systematic model reduction resulted in a surprisingly simple model well approximating experimentally observed dynamics. The model postulates a novel link between c-$FLIP_L$ cleavage in the CD95 Death-Inducing Signalling Complex (DISC) and the NF-kB pathway. We validated experimentally that CD95 stimulation results in binding of p43-FLIP to the IKK complex followed by its activation. Furthermore, we demonstrated that the apoptotic and NF-kB pathway diverge already at the DISC. Model and experimental analysis of DISC formation showed that a subtle balance of c-$FLIP_L$ and procaspase-8 determines life/death decisions in a non-linear way. This is the first mathematical model explaining the complex dynamics of CD95-mediated apoptosis and survival signalling.

A role for the Bcl-2 family in mitochondrial fission-fusion dynamics unrelated to apoptosis

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Bax and Bak promote apoptosis by perturbing the permeability of the mitochondrial outer membrane and facilitating the release of cytochrome c by a mechanism that is still poorly defined. During apoptosis, Bax and Bak also promote fragmentation of the mitochondrial network, possibly by interfering with the mitochondrial fusion machinery. It has been proposed that Bax/Bak-induced mitochondrial fission may be required for release of cytochrome c from the mitochondrial intermembrane space. Here we show that Bcl-xL, as well as other members of the apoptosis-inhibitory subset of the Bcl-2 family, antagonized Bax and/or Bak-induced cytochrome c release but failed to block mitochondrial fragmentation associated with activation of these proteins. These data suggest that Bax/Bak-induced fragmentation of mitochondrial networks and cytochrome c release are separable events.

miR, "stemness" & skin

Gerry Melino

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Although p63, similarly to its homologues p73 and p53, regulate apoptosis during DNA damage, shows a crucial role in regulating epidermal development. The activity of p63 depends on its steady state protein levels, and a number of evidence suggest that post-transcriptional regulation rather than transcriptional control plays a major role in p63 function. While p63 is important for epithelia formation (Candi et al. CDD 2006. 13: 1037), including the thymus (Candi et al. PNAS 2007. 104: 11999), p73 is also involved in neurodegeneration and immune responses. Although the tumor suppressor protein PML modulates p63 half-life by recruiting them to the PML-nuclear bodies to regulate their transcriptional activities and thus inhibiting their degradation, the molecular mechanisms underlying the regulation of p63 protein stability remain largely unknown.
We have identified the Hect-containing E3 (Nedd-4-like) ubiquitin-protein ligase Aip4/ITCH as responsible for the proteosomal degradation of both p73 (Rossi et al. Embo J 2005. 24: 836) and p63 (Rossi et al. PNAS 2006. 103: 12753). The PY motif-containing C-terminal region of p73/p63 binds to the WW domain of Itch, resulting in ubiquitination and degradation of p73/p63. We have also identified a regulator of ITCH activity, N4BP1, able to regulate the function of ITCH's substrates (Oberst et al. PNAS 2007. 104: 11280). On these bases, we are developing small molecular inhibitor of Itch able to regulate p73/p63 degradation and therefore their function in DNA damage, finely regulating apoptosis and chemosensitivity.
Here, we describe the ability of p63 mutants, found in genetic diseases, to be regulated by Itch. We also report the identification of miR-203 able to target and repress p63, thus repressing "stemness" and allowing epithelial differentiation. During keratinocyte's differentiation the sudden upregulation of miR-203 causes the drastic reduction of DNp63 proteins, as miR-203 binds the 3'UTR of the *trp63* transcript to regulate its translation. This relationship is concerved in ES cell commitment in vitro. miR203 inhibit ES clonogenicity, while antagomiR-203 enhances clonogenicity, in keeping with a role of DNp63 in "stemness" proliferation potential (Candi et al. PNAS 2007. 104: 11999). miR-203 seems to be at the interfece regulating the transit of epithelial cells from the "stemness" compartment to the differentiation compartment.

Engulfment of apoptotic cells

Shigekazu Nagata

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Apoptosis is triggered by a variety of stimuli, and dying cells are swiftly phagocytosed by macrophages. Apoptosis is mediated by a cascade of caspases, which eventually activates a specific DNase (CAD: caspase-activated DNase). *CAD*-deficient cells do not undergo apoptotic DNA fragmentation, but DNA of dead cells is degraded by DNase II in macrophages after they are engulfed. Using this knowledge, we established an assay for engulfment of apoptotic cells, and identified several hamster monoclonal antibodies that inhibit the engulfment of apoptotic cells. Characterization of the antigens that are recognized by these mAbs indicated that different subsets of macrophages use different molecules (MFG-E8 and Tim-4) to recognize phosphatidylserine on apoptotic cells for engulfment. MFG-E8 is a soluble protein present on a subset of macrophages such as tingible-body macrophages in the spleen, and thioglycollate-elicited peritoneal macrophages. Lack or excess of MFG-E8 blocks the engulfment of apoptotic cells *in vitro* and *in vivo*, and causes SLE-type autoimmune disease. Using ELISA for human MFG-E8, we found that some SLE patients carry a significant level of MFG-E8 in their blood samples, suggesting an involvement of aberrant expression of MFG-E8 in the pathoetiology of human SLE in some cases. With the MFG-E8-mediated engulfment system, we monitored the engulfment of apoptotic cells in a single cell level, and found that apoptotic cells are engulfed successively at the specific location of the phagocytes, suggesting the presence of portals for apoptotic cells.

Invited speaker: Josef Penninger
ECDO Keynote Lecture

Josef Penninger

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Regulation of autophagy in mammals by AMBRA1 and its partners

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Autophagy is a self-degradative process involved in the basal turnover of cellular components and plays a key role in response to nutrient starvation or cellular stress in a wide range of eukaryotes. Autophagy is considered the cellular major regulated mechanism for degrading long-lived proteins and organelles In fact, during autophagy, portions of the cytoplasm are sequestered by double-membraned vesicles called autophagosomes, and are degraded after fusion with lysosomes for subsequent recycling. In mammals, autophagy, acting as a pro-survival or pro-death mechanism, is involved in different pathological conditions, such as neurodegeneration and cancer. We have recently identified Ambra1 (activating molecule in Beclin1-regulated autophagy), a large (~130 KDa protein), previously unknown protein bearing a WD40 domain at its amino terminus, as a key player in the regulation of autophagy and in embryogenesis. We demonstrated that Ambra1 is a positive regulator of the Beclin1-dependent programme of autophagy. Notably, Ambra1 functional deficiency in mouse leads to death during gestation of embryos due to accumulation of undegraded ubiquitinated proteins, unbalanced cell proliferation and excessive apoptotic cell death. In order to get further insight on the regulation of autophagy by the Beclin1/Ambra1 complex, we used a yeast two-hybrid strategy to identify other Ambra-1 interactors. We found that Ambra-1 interacts with Dynein Light Chain -1 and -2 (DLC1 and DLC2). It is well known that Dynein Complex is involved in minus-end transport upon microtubules and plays a role in the autolysosomes formation. Furthermore, it is reported that DLC1 and DLC2 are also involved in apoptosis by interaction with BimEL and Bmf, respectively. Therefore, DLC1 and DLC2 have probably a dual role in apoptotic and autophagic pathways. To unveil the physiological significance of dyneins interactions with Ambra-1, we down-regulated the expression of DLC1, DLC2 or both DLC1 and Ambra-1 by siRNA. Our results indicate that: a) DLC1 regulates Ambra-1 ability to induce autophagy by binding to microtubules; b) DLC1 down-regulatin is "per se" able to induce autophagy indicanting a key role for the microtubules integrity in the regulation of the autophagic programme. Our results highlight a key role for Ambra1 as an essential element of the core machinery regulating autophagy and provide important evidence for the existence of a complex interplay in the regulation of autophagy, cell growth and cell death.

Real-time imaging and mathematical systems analysis of mitochondrial outer membrane permeabilisation and caspase activation during apoptosis: Is very little already too much?

Jochen Prehn

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A novel morphologically-conserved C. elegans cell death program

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Programmed cell death is an essential process during metazoan development. During wildtype C. elegans development, nearly all cells slated to die activate caspases and undergo stereotypical morphological changes including chromatin compaction and cell shrinkage. The male-specific linker cell (LC), however, is an exception. The LC leads the migration of the developing gonad to the cloaca, and once migration is complete at the L4-to-adult transition, the LC dies using a cell-autonomous death program. Strong loss-of-function alleles of canonical cell-death pathway genes, including ced-3, ced-4, and egl-1, as well as a ced-9(gf) mutation, do not block LC death, indicating that LC death must be controlled by a novel program (1). Indeed, electron micrographs of dying LCs reveal non-apoptotic features, including nuclear crenellation, absence of chromatin condensation, organelle swelling, and accumulation of cytoplasmic membrane-bound structures. Remarkably, similar features are seen during developmental deaths of neurons in the vertebrate spinal cord and ciliary ganglia, suggesting that LC death is morphologically conserved.

Previous studies revealed that LC death requires the developmental timing genes let-7 and lin-29, and that these genes act within the LC to promote cell death. To identify additional components required for LC death, we performed a genome-wide RNAi screen aimed at identifying genes whose loss prevents LC death. We identified several candidate genes, and have begun to verify these. One of the genes we identified is tir-1: 30% of tir-1(RNAi) and 10% of tir-1(qd4) mutants exhibit LC survival. tir-1 encodes a Toll-like/interleukin-1 receptor adaptor protein that functions as a signaling protein in innate immunity and during neuronal cell-fate establishment. In these processes, tir-1 functions upstream of nsy-1/MAPKKK and sek-1/MAPKK. We reasoned that tir-1 could be activating a similar MAPKcascade in the LC and tested whether strong loss-of-function alleles of nsy-1 and sek-1 also block LC death. While the nsy-1(ky397) and nsy-1(ky542) alleles do not affect LC death, the sek-1(km4) allele prevents LC death in 50% of males. These results suggest that a MAPK cascade may control genes essential for LC death. Downstream targets of this cascade may emerge from studies of the other RNAi candidates we identified and/or from a genetic screen currently in progress (E.B. and R. Razi, unpublished).

1) Abraham et al., Dev. Cell 12:73-86, 2007.

Inflammasomes: danger sensing complexes involved in inflammatory diseases

Jurg Tschopp

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The innate immune system relies on its capacity to rapidly detect invading pathogenic microbes as foreign and to eliminate them. The discovery of Toll-like receptors (TLRs) provided a class of membrane receptors that sense extracellular microbes and trigger anti-pathogen signaling cascades. More recently, intracellular microbial sensors have been identified, including Nod-like receptors (NLRs). Some of the NLRs also sense non-microbial danger signals and form large cytoplasmic complexes called inflammasomes that link the sensing of microbial products and metabolic stress to the proteolytic activation of the proinflammatory cytokine Il-1β and IL-18. The NALP3 inflammasome has been associated with several autoinflammatory conditions including gout. Likewise, the NALP3 inflammasome is a crucial element in the adjuvant effect of aluminium and can direct a humoral adaptive immune response. I will discuss the role of NLRs and in particular the inflammasomes in the recognition of microbial and danger components and their role in health and disease.

Cannabinoid antitumoral action: inducing ER stress and autophagy to promote cell death

Guillermo Velasco

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Cannabinoid administration curbs the growth of several types of tumor xenografts in rats and mice including gliomas. In this talk, I will discuss our last findings on the mechanism through which cannabinoids mediate these actions. Thus, our data unravel that cannabinoids - via stimulation of *de novo* synthesis of ceramide - activate an endoplasmic reticulum (ER) stress-evoked response [involving the up-regulation of the transcriptional co-activator p8 and its target the pseudo-kinase Tribbles homologue 3 (TRB3)] that leads to induction of autophagy and promotion of cancer cell death by activating apoptosis. Our findings support that stimulation of this route constitutes a potential therapeutic strategy for inhibiting tumor growth.

Lecture Abstracts

Short Oral Communications

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

Presenting Author: Daniele Bano

Alteration of nucleocytoplasmic transport occurs during calcium-mediated cell death

Daniele Bano (1,4), David Dinsdale (1), Barry Mccoll (2), Elisa Ferrando-May (3), Michael Hengartner (4) and Pierluigi Nicotera (1)

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In the past two decades considerable effort has been directed to elucidating the cross-talk between organelles during cell death. The death signalling cascades that involve mitochondria, ER and the plasma membrane have been extensively studied. However, probably because nuclear deregulation is considered to be a downstream event in cellular disassembly, only a handful of studies have examined functional and structural alterations of nucleocytoplasmic transport during cell death.
We can now show the temporal and spatial changes in nucleocytoplasmic transport during cell death induced by excitotoxic stimuli in rodent neurons and by the analogous calcium-dependent neuronal demise in C.elegans deg-3 mutants. Through a combination of fluorescent confocal imaging and biochemical studies, we have ordered the events that promote alterations in nucleocytoplasmic transport followed by disassembly of the nucleus. We can prove that an initial Ca2+ signal promotes permeability changes to small reporter molecules, whereas activation of the Ca2+ - dependent proteases, calpains triggers a subsequent leakiness of the nuclear permeability barrier to larger molecules followed by disruption of the nuclear pore architecture. Our studies suggest that these events are a common step in different death paradigms.

Keywords: AIF, calcium, calpains, neuronal degeneration, C elegans, nucleocytoplasmic transport

The BH3 mimetic obatoclax overcomes glucocorticoid-resistance by activating autophagic cell death

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Glucocorticoid (GC) -resistance is a common feature of refractory lymphoid malignancy. Leukemic cells from highly chemoresistant, very high risk acute lymphoblastic leukemia (VHR-ALL) patients demonstrate complete blockade of mitochondrial apoptosis despite exposure to high-dose dexamethasone. Reactivating cell death by targeting the BCL-2 family represents an attractive strategy for subverting drug resistance. We tested the capacity of the broad spectrum BH3 mimetic obatoclax (GX15-070) to restore the glucocorticoid response in GC-resistant childhood lymphoblast leukemia (ALL). Indeed, low doses of obatoclax (10% IC50) induced a glucocorticoid response in GC-resistant ALL cell lines and in primary cells from T-cell and precursor B-cell ALL patients with poor in vivo responses to prednisone. Stapled α-helices of BCL-2 domains (SAHBs) recapitulate this effect, underscoring the importance of the BH3 functionality for GC-resensitization. We further found that obatoclax triggered the death of cells harboring a genetic blockade of mitochondrial apoptosis, such as caspase 9-deficient Jurkat leukemia cells and Bax-/-Bak-/- mouse embryo fibroblasts, revealing that, in this context, obatoclax can activate a non-apoptotic cell death. Using the GFP-LC3 reporter assay combined with viability assays we demonstrate that obatoclax-induced GC-resensitization of refractory leukemia cells operates by triggering autophagic cell death rather than by reactivating mitochondrial apoptosis. The specificity of action is highlighted by abrogation of the obatoclax effect by the pharmacologic inhibitors of autophagy 3-methyladenine and bafilomycin, as well as by Beclin-1 RNA interference. Furthermore, mouse embryonic fibroblasts deficient for the autophagy protein ATG5 were insensitive to obatoclax. In a mouse xenograft model of primary VHR-ALL, the combination of obatoclax and dexamethasone halted leukemic progression, while the single agents were ineffective as monotherapy. These data provide a compelling rationale for clinical application of obatoclax-glucocorticoid combination therapy in GC-resistant ALL and suggest that activating autophagic cell death may offer an alternative therapeutic strategy when mitochondrial apoptosis is blocked.

Keywords: Acute Leukemia, steroid resistance, BH3-mimetic, autophagic cell death

Lysosomal proteases: Triggers or amplifiers of apoptosis?

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Certain apoptotic stimuli trigger lysosomal membrane permeability (LMP), leading to the release of cathepsins, which activate death signalling pathways in the cytosol. An unresolved issue is whether this process is required for apoptosis and an initiating or amplifying event. Here we used mouse monocytes and embryo fibroblasts exposed to etoposide or UV or deprived of cytokines to show that LMP and the cytosolic release of cathepsin B, L and D consistently depends on Bax/Bak and components of the apoptosome. Neither Bax nor Bak resided on lysosomes indicating that lysosomes were not directly perforated by Bax/Bak but by effectors downstream of the apoptosome. Various cathepsin knock-out cells were resistant to apoptosis but did not regrow after stress removal, as seen for Bax/Bak DKO. Our findings indicate that cathepsins enhance rather than initiate apoptosis in various cellular systems exposed to different apoptotic stimuli.

Keywords: lysosomes, proteases, DNA damage, mitochondria, Bax/Bak

The autophagic machinery is necessary for removal of cell corpses during the development of the nervous system

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Autophagy is a lysosomal degradative pathway necessary for the clearance of damaged or proteins and organelles. It allows for the recycling of intracellular constituents, providing energy during periods of metabolic stress and thus contributing to cell viability. In addition, disruption of autophagic machinery interferes with embryonic development in several species, although the underlying cellular processes affected remain unclear. Here, we investigate the role of autophagy during the early stages of development of the nervous system by using the chick retina as a model. During these stages of development the retinal neuroepithelium proliferates and starts to generate the first neurons, the retinal ganglion cells. These two developmental processes are accompanied by programmed cell death. We have inhibited the autophagy by using 3-methyladenine and found that retinas accumulated numerous TUNEL-positive cells that correlated with a lack of the “eat-me” signal phosphatidyl-serine. In consequence, neighbouring cells did not engulf apoptotic bodies and persisted as individual cell corpses, a phenotype that was also observed after blockade of phagocytosis with phospho-L-serine. Supplying the retinas with methylpyruvate, a cell-permeable substrate for ATP production, restored ATP levels, phosphatidyl-serine presentation at the cell surface, engulfment and lysosomal degradation of apoptotic bodies. Thus during neurogenesis, the authophagic machinery provides the retina with the energy required for proper cell corpse removal and further degradation of apoptotic cells.

Keywords: autophagy, development, programmed cell death, retina

Regulated Hox gene expression reveals a novel role for Hox genes in myeloid cell proliferation and survival.

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The Hox family of homeodomain transcription factors are essential for the regulation of hematopoiesis and deregulated expression of some Hox gene is associated with the development of myeloproliferative disorders and leukaemia. In mammals, 39 Hox genes are organized into four clusters (A, B, C or D) and the expression of these genes is tightly regulated at particular differentiation points in haematopoiesis. Importantly, overexpression of Hox genes, in particular HoxA9 and HoxA10 are associated with poor prognosis of acute myeloid leukaemia. Overexpression of murine HoxB8 together with IL-3 results in myeloid leukaemia in mice. The mechanisms of action of Hox proteins in leukaemogenesis remain to be determined but are thought to result from a block in myeloid differentiation. We aimed to determine the molecular mechanisms by which Hox genes, in particular HoxB8 and HoxA9, contribute to leukaemic transformation. Using a lentiviral inducible expression system, we generated primary, growth factor dependent myeloid cells in which we are able to regulate the expression of HoxB8 and HoxA9 by administration of agent 4 hydroxy-tamoxifen (4HT) to explore the biological effects of deregulated expression of Hox genes. Conditional (growth-factor dependent) immortalisation of myeloid progenitors was possible only in the presence of induced Hox gene expression and surprisingly, withdrawal of Hox gene expression did not result in differentiation. Instead, loss of Hox expression by 4HT withdrawal, even in the presence of IL-3, induced Go/G1 cell cycle arrest and caspase-dependent cell death. Our results also suggest HoxB8 regulates expression of the pro-apoptotic Bcl-2 family member Bim, since loss of Hox resulted in substantially increased Bim expression and the cell death induced by loss of Hox expression was partially inhibited in Bim-deficient cells. Re-addition of 4HT to cell cultures restored Hox expression and resulted in an increase in cell viability, cell proliferation and decrease of Bim expression. Therefore, our results suggest that overexpression of Hox genes contributes to myeloid transformation by coupling a growth factor signal such as IL-3 to proliferation and the regulation of the apoptotic machinery.

Keywords: leukaemia, Hox genes, proliferation, Bcl-2 family, apoptosis

IL-3 induces a Pim1-dependent anti-apoptotic pathway in primary human basophils

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The contribution of basophils in allergic disease and other Th2-type immune responses depends on their persistence at sites of inflammation, but the ligands and molecular pathways supporting basophil survival are largely unknown. The comparison of rates of apoptosis and of the expression of anti-apoptotic proteins in different human granulocyte types revealed that basophils have a considerably longer spontaneous live span than neutrophils and eosinophils consistent with high levels of constitutive Bcl-2 expression. IL-3 is the only ligand that efficiently protects basophils from apoptosis as evidenced by screening a large number of stimuli. IL-3 up-regulates the expression of the anti-apoptotic proteins cIAP2, Mcl-1 and Bcl-XL, and induces a rapid and sustained de novo expression of the serine/threonine kinase Pim1 that closely correlates with cytokine-enhanced survival. Inhibitor studies and protein transduction of primary basophils using wild-type and kinase-dead Pim1-Tat fusion-proteins demonstrate the functional importance of Pim1 induction in the IL-3-enhanced survival. Our data further indicate that the anti-apoptotic Pim1-mediated pathway operates independently of PI3-kinase but involves the activation of p38 MAPK. The induction of Pim1 leading to PI3-kinase-independent survival as described here for basophils may also be a relevant anti-apoptotic mechanism in other terminally differentiated leukocyte types.

Keywords: Inflammation, Allergy, granulocytes, anti-apoptotic pathway, kinase

Caspase-14 is a crucial protease in proper stratum corneum formation

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Caspase-14 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. 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 characterized by an altered profilaggrin-processing pattern and by reduced skin hydration levels and increased water loss. In vitro, caspase-14 can directly cleave profilaggrin, a member of the S100-fused proteins. In skin, the levels of caspase-14 activity are associated with the strength of the epidermal barrier and the levels of filaggrin-derived moisturizing factors. We identified hornerin as a second member of the S100 fused gene family which is a direct caspase-14 substrate. Immunoblotting revealed that the processing of hornerin is affected in caspase-14 deficient epidermis. The functional role of hornerin is currently not clear. Apparently, caspase-14 is a crucial protease in the correct processing and/or degradation process of S100-fused proteins such as filaggrin and hornerin.
We further analyzed the defective barrier function of the caspase-14 deficient skin by electron microscopy and dye based penetration assays. We demonstrate that there is premature compensatory hypersecretion of lamellar bodies in caspase-14-/- epidermis. In addition, cornified envelopes either failed to form or appeared highly attenuated in transitional cells in caspase-14-/- epidermis after acute barrier disruption.
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 enhances caspase-14 expression and activity in the skin.

Keywords: caspase, differentiation

Analysis of the role of heat shock protein 70 in granzyme B-induced apoptosis

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Heat shock proteins, including HSP70, are part of the cellular stress response system and have been demonstrated to protect cells against various cytotoxic stimuli. Intracellular HSP70 inhibits apoptotic pathways at specific points of the respective cascades. Extracellular HSP70 on the other hand, is an immunological danger signal which can induce the antigen-specific activity of cytotoxic T lymphocytes (CTL) and improve the effector function of natural killer cells. Interestingly, HSP70 does not appear to protect cells against cell death mediated by CTL. Acute overexpression of HSP70 might even increase the susceptibility of target cells to CTL, which use the perforin and granzyme-dependent granule-exocytosis pathway to induce apoptosis (Dressel et al. Eur J Immunol 29: 3925; Dressel et al. J Immunol. 166: 3957; Dressel et al. Cancer Res 63: 8212). Granzyme B is one of the most important effector proteases of cytotoxic granules. We analysed now the effect of acute overexpression of HSP70 on granzyme B-induced apoptosis.
HSP70 was expressed in the human melanoma cell line Ge under the control of a tetracycline-inducible promoter (Ge-tet). Cells containing only the transactivator construct (Ge-tra) served as control. HSP70 was induced in the Ge-tet cells by addition of doxycycline for 24 hours. This treatment improved previously the lysis of the Ge-tet cells by alloreactive CTL. The effect of HSP70 induction on granzyme B-mediated apoptosis was now analysed in flow cytometry by subG1-peak measurements after delivery of granzyme B into the cytosol of the target cells by the endosomolytic activity of adenovirus type 5. HSP70 did not protect the target cells against granzyme B-mediated apoptosis. However, we did also not observe a significant pro-apoptotic effect of HSP70. When apoptosis was induced by the kinase inhibitor staurosporine, a significant protection of the Ge-tet cells by HSP70 was observed. In a first attempt to further elucidate the molecular alterations caused by acute HSP70 overexpression, we performed a micoarray analysis on Ge-tra and Ge-tet cells using a whole human genome microarray. Bioinformatic analysis revealed that only 0.16% of the 44000 sequence tags on the array were regulated significantly by acute HSP70 overexpression. The most significant alteration was found for HSP70 itself and this result, in contrast to others, could be confirmed by quantitative real-time PCR analysis.
Acute overexpression of HSP70 does not seem to protect tumour cells from granzyme B-induced apoptosis despite its ability to increase resistance to other apoptotic means such as staurosporine. The results of the microarray analysis suggest that the acute overexpression of HSP70 itself does not lead to major alterations of the transcriptome although HSP70 is known to interact also with transcription factors, such as the heat shock factors. The different effects of HSP70 on CTL, granzyme B, and staurosporine-induced apoptosis are therefore likely regulated only at the protein level. The analysis of the major apoptotic events after exposure of Ge-tet cells to the different cytotoxic means might help to further elucidate pro- and anti-apoptotic effects of HSP70 at the molecular level.

Acknowledgements: The work has been supported by the Deutsche Forschungsgemeinschaft through the Research Training Group GRK 1034 and the grant DR 394/2-3. The authors thank Prof. C. J. Froelich (Evanston Healthcare Research Institute, Feinberg School of Medicine, Northwestern University, Evanston, USA) for the granzyme B, Dr. T. Seidler (Dept. of Cardiology and Pneumology, University of Göttingen) for the adenoviruses, L. Opitz and Dr. G. Salinas-Richter (DNA Microarray Facility, University of Göttingen), for help with the microarray analysis.

Keywords: heat shock protein 70, granzyme B, cellular cytotoxicity, gene expression analysis

Proteome analysis reveals different cleavage site preferences for caspase-3 and -7

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Caspases constitute an evolutionary highly conserved family of endopeptidases with central functions in apoptotic and inflammatory signaling pathways. Due to identical cleavage specificity the executioner caspases-3 and -7 were considered as functionally redundant. Similar proteolytic activity of both caspases was observed on a variety of natural substrates such as Apaf-1, PARP-1, p43, ROCK I, ICAD and others. Moreover, screening of synthetic combinatorial tetrapeptide libraries revealed a similar proteolytic specificity for caspase-3 and -7. Despite this redundancy unique cleavage by caspase-7 was documented for ataxin-7, Nogo-B, TNFRI and kinectin. On the other hand specific proteolysis by caspase-3 was reported for CCDC47, SPARCL1, Synphilin-1, AR-12 and Unc5H2.
In order to document in a more systematic approach the unique and shared processing events of caspase-3 and -7 in native conditions, a proteome-wide analysis was performed using the COFRADIC N-terminal peptide sorting methodology (Gevaert et al, 2003). Cell extracts from untreated, caspase-3 or -7 treated macrophages were prepared after differential Stable Isotope Labeling with Amino acids in Cell culture (SILAC). Following LC-MS/MS analysis and based on aspartic acid specific cleavage, 57 potential cleavage sites in 50 potential caspase-3 and -7 substrates were found. Although with differential efficiency, 47 (82%) cleavage sites were shared by both caspase-3 and -7, 3 (5%) cleavage sites were identified as potential unique caspase-3 substrates and 7 (12%) as specific caspase-7 substrates.
Direct cleavage of the potential substrates was validated by treatment of in vitro translated substrates with recombinant caspase-3 or -7. Currently, peptides from the cleavage site region of unique protein substrates are synthesized to analyze the specificity of both caspases to peptide substrates. To obtain structural evidence for the specificity of both caspase-3 and -7, their novel identified specificity profiles are further analyzed by molecular modeling.

Gevaert K, Goethals M, Martens L, Van Damme J, Staes A, Thomas GR, Vandekerckhove J (2003) Exploring proteomes and analyzing protein processing by mass spectrometric identification of sorted N-terminal peptides. Nat Biotechnol 21: 566-569

Keywords: caspases, substrate, proteomics, specificity profile

Pro-apoptotic, anti-proliferative, and anti-angiogenic therapeutics in cancer

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Wnt signaling is involved in developmental processes, cell proliferation and cell migration. We have previously shown anti-proliferative and pro-apoptotic roles for the secreted frizzled protein 4 (sFRP4), a Wnt antagonist, during normal homeostasis in tissues such as ovary, corpus luteum, placenta and mammary gland as well as pathological states such as mesothelioma and colorectal carcinoma.
To date there is no published data demonstrating the involvement of sFRP4 in angiogenesis. In order to investigate the role of sFRP4 on endothelial cell physiology we utilized a variety of in vitro assays and examined its effect on physiological and tumor-associated angiogenesis using in vivo models.
Results from these studies demonstrated that endothelial cell migration, ring formation and ring stability are inhibited by antagonizing both the canonical Wnt/beta-catenin and non-canonical Wnt signaling pathways. sFRP4 also induces apoptotic events in endothelial cells in vitro by increasing cellular levels of reactive oxygen species, while tumor cells are unaffected. Furthermore, the addition of LiCl (in order to elevate levels of cellular beta-catenin) did not affect the ability of sFRP4 to antagonize endothelial cell migration in vitro. Importantly, sFRP4 restricts tumor growth in vivo by specifically interfering with endothelial cell function and markedly reducing the degree of angiogenesis to 40% of control levels.
These novel findings identify sFRP4 as a, so far unexplored, potent angiogenesis inhibitor.

Keywords: cancer, angiogenesis, sFRP4

Proapoptotic BH3-only proteins Bim and Puma are essential for apoptosis of germinal center-derived memory B cells and antibody-forming cells

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T cell-dependent B cell immune responses induce germinal centers that are sites for expansion, diversification and selection of antigen-specific B cells. During the immune response antigen-specific B cells are removed in a process that favours the retention of cells with improved affinity for antigen, a cell death process inhibited by excess Bcl-2. Here we examined the role of the BH3-only protein Bim, an initiator of apoptosis in the Bcl-2-regulated pathway, in the cell death accompanying an immune response. Following immunization, Bim-deficient mice showed persistence of both memory B cells lacking affinity-enhancing mutations in their Ig genes and antibody forming cells (AFC) secreting low affinity antibodies. This was accompanied by enhanced survival of both cell types in culture. In addition, we have currently identified the BH3-only protein Puma as a critical protein for the death of antigen-specific B cells. Our data strongly suggest that Puma has an impact on apoptosis of memory B cells. Unexpectedly however, Puma-deficient AFCs show unchanged numbers and normal affinity maturation. In addition, we have investigated the impact of Puma deficiency on Ig variable gene segment mutation. We could show that Puma-deficient cells have altered mutations in their complementarity-determining region leading us to propose that Puma may be involved in somatic hypermutation and has an effect on B cell affinity maturation. These results identify for the first time the physiological mechanisms for killing antigen-specific B cells in an immune response and show this to be dependent on the BH3-only proteins Bim and Puma.

Keywords: apoptosis, B cell immune response, BH3-only proteins

Apoptosis, a driving force for head regeneration in hydra

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The ability of an adult organism to undergo regeneration and functional repair is present in all multicellular phyla. However, many species, especially higher vertebrates do not respond to injury or tissue removal by re-growing missing body parts. Hydra, which provides a unique experimental model to address this question, was thought for long to regenerate through a rather specific type of regeneration, named “morphallactic” regeneration, i.e. relying on cell differentiation in the absence of any cell proliferation.
The recent results obtained in our laboratory show that this is not the case in wild-type hydra. The early cellular remodeling required for head regeneration in that context requires four concomittant processes 1) an immediate massive apoptosis that affects most differentiated cells of the interstitial cell lineage (neurons, mechano-receptor cells), 2) a simultaneous metaplasia of the endodermal myoepithelial digestive cells that engulf the apoptotic bodies, 3) the cell cycle re-entry of the interstitial stem cells in the region immediately underneath and 4) the migration of cycling interstitial progenitors towards the wound.
When apoptosis was inhibited either pharmacologically or genetically through RNA interference, this cellular remodelling did not take place and head regeneration was blocked. Conversely induction of apoptosis in foot-regenerating tips, where apoptosis normally remains very low after amputation, forced them to form a blastema and regenerate a head instead of a foot. How apoptotic cells drive cell proliferation? Gene silencing through RNA interference allowed us to identify one signalling molecule that is released by apoptotic cells during a very sharp time window early after amputation and activates the corresponding signalling pathway in adjacent cells to push them to synchronously divide.
Hence, these data show that apoptotic cells play a key role in the activation of stem cells and progenitor cells to form a blastema; the amplitude of the apoptotic process, which is reflected by the number of both apoptotic and apoptosis-responding cells, appears to be a critical parameter in the triggering of a complex regenerative programme.

Keywords: apoptosis, metaplasia, cell-cycle re-entry, blastema, hydra, regeneration, RNA interference,

Bax/Bak independent induction of apoptosis by BH3-mimetic small molecule compounds

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Gossypol, HA14-1 and BH3I-2´ are small organic compounds which are considered as antagonists to antiapoptotic Bcl-2 and Bcl-xL. Due to this activity they were suggested as suitable agents for the induction of apoptosis in Bcl-2 or Bcl-xL overexpressing cancer cells. Here, we show that Gossypol, HA14-1 and BH3I-2´ induce apoptosis independently from protein expression of Bcl-2 or Bcl-xL in human cancer cell lines Jurkat (T-lineage ALL), BJAB (Burkitt-like lymphoma) and HCT-116 (colon carcinoma). This indicated an off-target effect of these putative Bcl-2 and Bcl-xL antagonists. To functionally prove such an off-target effect we investigated whether induction of apoptosis by these compounds is dependent on the multidomain proapoptotic Bcl-2 homologues Bak and Bax. Notably, Gossypol, HA14-1 and BH3I-2´ all induced apoptosis independently of Bax and Bak in HCT-116 Bax/Bak single and double deficient cells. This indicates that induction of apoptosis by these compounds is at least partially independent of the Bcl-2 family and therefore indicates off-target activities.

Keywords: cancer, BH3-mimentics, apoptosis

The regulation of Mcl-1 during mitosis

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Controlling the balance between cell proliferation and cell death is essential in maintaining proper development and in avoiding diseases such as cancer. Regulation of apoptosis is required for protection against potentially cancerous defects during cell division and for responses to anti-cancer drugs that target dividing cells.

Control of the intrinsic mitochondrial pathway operating through caspase-9 is critical for the regulation of apoptosis during mitosis and in response to microtubule poisons, such as taxol and nocodazole, that cause mitotic arrest. We have demonstrated recently that phosphorylation of caspase-9 by CDK1-cyclin B1 delays the implementation of an upstream apoptotic signal generated during prolonged mitotic arrest [1]. We propose that a temporally controlled mechanism induces cytochrome c release from mitochondria and eventually activates caspase-9 if a cell fails to resolve mitosis. Elucidating the molecular control of this process is likely to be important for understanding both the defects causing carcinogenesis and what determines the sensitivity of cancer cells to anti-mitotic therapeutic drugs.

Myeloid cell leukaemia-1 (Mcl-1), an anti-apoptotic member of the Bcl-2-like family of proteins, is a likely candidate to control the initiation of apoptosis during mitotic arrest. We have found that the level of Mcl-1 is regulated during the cell cycle, increasing during G1-S-G2 and partially lost at mitosis. Mcl-1 is also subject to mitotic phosphorylation and is almost completely degraded in cells during a prolonged mitotic arrest. We are studying the regulation of Mcl-1 by phosphorylation and proteolysis during mitosis, and its role in apoptotic responses to drugs that arrest cells in mitosis. This work will increase understanding of the role of Mcl-1 in the apoptotic pathway and may lead to new strategies to improve anti-cancer therapy.

[1] Allan LA & Clarke PR (2007), Molecular Cell 26, 301-310.

Keywords: Mcl-1, apoptosis, mitosis, microtubule poisons.

Biological role of "orphan" granzymes in LCMV-WE infection

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The concerted action of the major granzymes (gzm) of cytotoxic lymphocytes, gzmA and gzmB together with perforin (perf) is critical for CTL-mediated recovery from many viral pathogens. However, it was recently found that certain viruses, like the lymphocytic choriomeningitis virus (LCMV), are readily controlled in mice deficient in gzmA and gzmB via a perf-mediated process. This observation suggests the involvement of still other CTL effector molecules in virus elimination. Possible candidates are additional gzms, termed orphan gzms, which have been described in mice and men, but not yet associated with a particular biological function(s).
We have monitored the course of LCMV-WE infection and disease in wild-type (wt), gzmAxB-/- mice, with major emphasis on the potential of the generated virus-specific CTL to induce proapoptotic processes and cell death in target cells in vitro. We found that CTL of gzmAxB-/- but not of wt mice expressed transcripts of orphan gzms like gzmD; and that both mouse strains expressed gzmK-specific mRNA. Most notably, wt and gzmAxB-/- mice were similarly able to control LCMV-WE infection in spite of the fact that their ex vivo-derived CTL exhibited strikingly different potentials to induce proapoptotic processes, when tested at various time points post infection. Our data indicate that the potential of CTL to eliminate LCMV in vivo does not necessarily correlate with their proapototic and cytolytic activities in vitro and suggest the involvement of orphan gzms and/or other factors in this process(es).

Keywords: CTL, Granzymes

Sensitization of Melanoma Cells to TRAIL by UVB-induced and NFkB-mediated downregulation of xIAP

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Effective treatment of malignant melanoma with the tumor selective death ligand, tumor necrosis related apoptosis inducing ligand (TRAIL) is curtailed by the fact that many melanoma cell lines are a priori resistant against TRAIL-induced apoptosis. Investigating 18 melanoma cell lines we show that TRAIL susceptibility is completely independent of the tumor progression stage but can be positively stimulated by co-exposure to a sublethal UVB-dose, providing an exellent tool to study the mechanism underlying TRAIL resistance. TRAIL resistance was shown to be independent of TRAIL receptor expression patterns as well as of FLIP expression levels, but could be linked to the ratio of xIAP and caspase-3 levels within the cell. UVB-induced sensitization coincides with enhanced xIAP degradation allowing full caspase-3 processing and activation. It is also accompanied by concomitant IκBα degradation resulting in NFκB-dependent transcriptional repression of xIAP. Loss of xIAP in turn was reduced upon overexpression of an IκBα super-repressor, thus NFκB activation appears to be responsible for differential regulation of xIAP and consequently determines TRAIL susceptibility. Since xIAP mediated blockade of apoptosis seems to be the dominant cause of TRAIL resistance of all melanoma cell lines investigated in this study, our data suggest that direct chemical xIAP inhibition or combination treatment with DNA damaging agents may offer new therapeutic strategies to generally sensitize melanoma towards TRAIL-induced apoptosis.

Keywords: cancer, DNA damage, NFκB, TRAIL

Proteomic profiling for apoptotic biomarkers of breast cancer response to chemotherapy

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Introduction
Breast cancer is a heterogenous disease and markers for therapy response remain poorly defined. Because some tumour cells respond poorly to these treatments, and to prevent unnecessary adjuvant chemotherapy in cancer patients, biomarkers that predict clinical responsiveness are needed. The main aim of the study is to discover novel biomarkers that predict drug/treatment efficacy in various breast cancer cell lines by employing two dimensional difference gel electrophoresis (2D-DIGE).
Methods
Several models of apoptosis (etoposide+TRAIL, doxorubicin (Dox), Dox+TRAIL), using a range of drug concentrations and time-courses, were generated in an array of breast cancer cell lines (MDA-MB-231, Hs578T, T47D, ZR-75-1) and a phenotypically normal breast epithelial cell line (MCF-10A). Caspase-3 activity assay and cell counting were used to monitor apoptosis (a key process in the response of tumour cells to chemotherapeutic drugs), and cell viability. Cell lysates were separated by 2D-DIGE using 17cm IPG strips of pH 4-7, spot detection and matching were performed using DeCyder software (GE Healthcare), and differentially-expressed proteins identified by tandem mass spectrometry.
Results
Over 3000 protein spots were detected on each gel, and over 50 proteins showed differential expression between drug-treated and untreated breast cancer cells. A subset of differentially-expressed proteins was identified, and we are in the process of validating these findings using Western blotting.
Conclusion
In conclusion, we have used a 2D-DIGE proteomic approach to identify potential markers of apoptosis in several breast cancer cell models. These candidate biomarkers may be useful in monitoring the efficacy of breast cancer response to drug treatment in a clinical setting.

Keywords: breast cancer, chemotherapy, apoptosis, biomarker, proteomics

Suppression of IL-33 bioactivity through proteolysis by apoptotic caspases

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IL-33 is the recently discovered ligand for the IL-1 receptor family member, ST2, and is involved in polarization of T cells towards a TH2 phenotype. It is widely believed that IL-33 is activated via caspase-1-dependent proteolysis, similar to the pro-inflammatory cytokines IL-1beta and IL-18, but this remains unproven.
Here we demonstrate that IL-33 is processed by caspases activated during apoptosis (caspases -3 and -7) but is not a physiological substrate for the inflammatory caspases (caspase -1, -4 and -5). Furthermore, proteolysis of IL-33 was not necessary for ST2 receptor binding or ST2-dependent NfkB activation. However, caspase-dependent proteolysis of IL-33 rendered this cytokine susceptible to degradation by serum proteases and dramatically attenuated IL-33 bioactivity in vitro and in vivo. These data suggest that IL-33 does not require proteolysis for activation, but rather, that IL-33 stability and bioactivity are diminished through caspase-dependent proteolysis within apoptotic cells. Thus, caspase mediated proteolysis acts as a switch to dampen the pro-inflammatory properties of IL-33.

Keywords: Apoptosis, caspases, cell death, inflammation, IL-33, Th2

Is the PIDDosome required for caspase-2 processing?

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The widely expressed p53-induced protein with a death domain (PIDD), together with the adaptor protein RAIDD, has been implicated in the activation of caspase-2 by the formation of a pro-apoptotic complex, called the PIDDosome. Furthermore, PIDD plays a role in DNA damage-induced NF-kB activation by generating a second multi-protein complex together with RIP-1 and NEMO. In order to investigate the physiological role of PIDD we have generated PIDD-deficient mice. To assess the importance of PIDD for DNA damage-induced apoptosis, thymocytes from wt, raidd-/- and pidd-/- mice were exposed to y-irradiation or were treated with etoposide, but no differences in apoptosis induction were observed. Surprisingly, activation of caspase-2 was readily detected in all these cells after DNA-damage, indicating that additional, PIDDosome-independent pathways for caspase-2 activation may exist in lymphocytes. Interestingly, in thymocytes, deficient for an additional, well-investigated p53-target, PUMA, caspase-2 processing was strongly delayed after stimulation, while caspase-3 cleavage products appear in the same time-frame as in the wt, raidd-/- and pidd-/- thymocytes. This result indicates that, at least in primary lymphocytes, caspase-2 processing occurs downstream of caspase-3 cleavage after DNA-damage. Since we could observe caspase-2 processing in both RAIDD- and PIDD-deficient cells, we investigated the spontaneous formation of the PIDDosome in the absence of RAIDD and PIDD by gel-filtration chromatography. In lysates from wt mouse embryonic fibroblasts a high-molecular weight complex containing caspase-2 (> 670 kDa) could be detected, but this complex also formed in the absence of RAIDD and PIDD. Overall we conclude that (1) an alternative, PIDDosome-independent mechanism of caspase-2 activation exists, (2) in non-transformed lymphocytes processing of caspase-2 occurs downstream of caspase-3 and (3) that assembly of a high-molecular weight complex containing caspase-2 in response to temperature shift, is formed spontaneously and does not require PIDDosome components.

Keywords: DNA damage, apoptosis, PIDD, Caspase-2

The redistribution of lysosomal phosphatidylserine to the plasma membrane outer membrane leaflet during apoptosis

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One of the earliest consequences of programmed cell death is the redistribution of phosphatidylserine (PS) from the inner-to-outer plasma membrane bilayer leaflet. Although it is assumed that the appearance of PS at the cell surface is a consequence of Ca2+-dependent membrane lipid scrambling, there is no evidence indicating that lipids from the inner membrane leaflet are the source of externalized PS. Since cells continuously internalize and externalize membrane through Ca2+-regulated endo- and exocytosis, we developed methods to study the fate and subcellular distribution of endosomes during apoptosis. The distribution of fluorescent labeled endosomes was monitored in murine embryonic fibroblasts labeled with rhodamine-phosphatidylethanolamine. We show that membrane lipid asymmetry was lost upon the formation of endosomal membranes resulting in the redistribution of PS to the luminal endosome lipid bilayer. During apoptosis, lysosome-endosome fusion products redistributed to- and fused with the plasma membrane resulting in the redistribution of PS from the luminal endosomal membrane to the outer leaflet of the plasma membrane.

Keywords: Apoptosis, Phosphatidylserine, membrane fusion, endosomes

The role of Mek1 in controlling mitochondrial morphology

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Mitochondria can exist as elongated network or fragmented structures according to the need of the cell. The shape and distribution of mitochondria depends on the balance between fusion and fission, regulated by a number of 'mitochondria shaping proteins'. Changes in mitochondrial shape are normally associated with the progression of the apoptotic cascade, as well as with cell proliferation, suggesting that fine mechanisms should exist to change the shape of mitochondria during life or death of the cell. However, very little is known about the signalling pathways that regulate mitochondria shaping proteins. MAP cascade of pleiotropic kinases is involved in the regulation of cell death. They phosphorylate and activate ERK1 and 2, which have been reported to be localized also at the mitochondrial level and to protect from apoptosis. We have found that a dominant negative form of MAP kinase kinase (MAPKK, also know as Mek1) causes elongation of mitochondria in mouse embryonic fibroblast (MEF). A genetic analysis proved that this depended on the mitochondria-shaping proteins mitofusin 1 and Opa1, but not on mitofusins 2. Our data point to a novel role for Mek and Erk in the modulation of mitochondrial shape.

Keywords: Mek1, mitochondria morphology

XIAP regulation by asparagine deamidation and PIMT

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X-linked IAP (XIAP) is an archetypal apoptosis protease inhibitor, with 3 conserved amino-terminal baculoviral inhibitor regions (BIR) and a carboxy-terminal RING domain. BIR 2 and 3 domains are largely recognized to directly inhibit pro-apoptotic proteases called caspases while the RING domain could also target caspases for ubiquitination. XIAP anti-apoptotic activity is expected to be down-regulated by proteasome degradation after XIAP self-ubiquitination and /or inhibited by a conserved interaction with mitochondrial proteins SMAC and /or Omi to favor apoptosis. However, this model has depicted XIAP as a monolithic protein that unalterably keeps unwanted caspase activity under control but probably over-simply XIAP biochemistry. This limitation arises after a lack of information about XIAP heterogeneity after using conventional one-dimensional electrophoresis.
In the present work we have showed using proteomic tools that deamidation of conserved asparagines in XIAP linker regions are a substantial source of ubiquitous post-translational modifications. Cell death induced by nocodazole or by inhibition of methyl-transferase activity with adenosine periodate oxidase (AdOx) alters XIAP isoforms resulting from asparagine deamidation. Mutation of asparagines to aspartic acid or accumulation of isoaspartic acid by AdOx treatment affects XIAP protein stability and NFKappaB activation by XIAP, concurring in an altered XIAP anti-apoptotic activity. We also found that XIAP self-ubiquitination is restricted to Ub-K63 chains and that asparagine deamidation alters the outcome. Further, protein isoaspartyl methyl transferase (PIMT) modified XIAP in vitro and in vivo, and also affect XIAP anti-apoptotic activity. We propose a novel two-stage mechanism in which asparagine deamidation prime XIAP for PIMT regulation.

Keywords: IAP, XIAP, regulation, asparagine deamidation, PIMT

Presenting Author: Syam Prakash Somasekharan Ramachandran

The large dynamin GTPase Drp1 stimulates Bax activation

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Apoptosis is a form of programmed cell death, which is essential for the development and maintenance of tissue homeostasis in multicellular organisms. The process of apoptosis is initiated by many cytotoxic stresses which lead to oligomerisation of proapoptotic proteins Bax or Bak and permeabilisation of the mitochondrial outer membrane (MOMP). Bax and Bak oligomerisation is essential for MOMP and the efflux of mitochondrial intermembrane space apoptogenic factors such as cytochrome c. The mechanism underlying Bax or Bak oligomerisation is not yet completely understood. Disintegration of the mitochondrial network with the involvement of Drp1, a large GTPase of the dynamin superfamily, is observed within the same time frame as Bax activation and MOMP in most cases studied. A delay in Bax mediated apoptosis has been reported in cells in which Drp1 is inhibited by overexpression of a dominant negative mutant Drp1 K38A, RNA interference, or the use of a chemical inhibitor. But the mechanism by which Drp1 could participate in apoptosis is unclear and poorly understood. Here we show using an in vitro assay that t-Bid induced oligomerisation of Bax is stimulated by Drp1. This function of Drp1 depends on arginine 247 present in the GTPase domain and is independent of its GTPase activity. In vivo, overexpression of Drp1 mutants (R247A/E) delays Bax oligomerisation and MOMP-related apoptosis. These findings suggest an important function of Drp1 in apoptosis and provide an insight into the mechanism of Bax activation.

Keywords: Apoptosis, Mitochondria, Bax, MOMP, Drp1

Presenting Author: Oksana Voloshanenko

Detachment-induced sensitisation to TRAIL explains the metastasis-specific role of the TRAIL/TRAIL-R system in epithelial tumorigenesis

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Initially, TRAIL was found to specifically induce apoptosis in tumour cell lines but not in normal cells in vitro and in vivo. It now appears that freshly explanted cells from primary tumours are mostly TRAIL-resistant, yet, they can be specifically sensitised by chemotherapy or radiation. Recently, we showed that TRAIL-R-deficient mice developed significantly more lymph node metastases from wild-type mice while they were not more susceptible to tumour initiation, promotion and conversion of chemically-induced skin tumours. Thus, TRAIL-R specifically suppresses metastasis but not development of primary skin tumours. We next studied the mechanism for this metastasis-specific property of the TRAIL/TRAIL-R system. An obligatory step in the development of metastasis is detachment of tumour cells from the primary tumour. We found that adherent TRAIL-R-expressing cells from skin and breast carcinomas were TRAIL-resistant in vitro. Detachment of the carcinoma cells sensitised them to both TRAIL- and CD95L-induced apoptosis. Sensitisation was specific as treatment of cells with different cellular stresses did not have the same effect. Interestingly, detachment did not sensitise cells to 5-FU and etoposide, two inducers of the intrinsic apoptosis pathway. Hence, detachment-induced sensitisation seems to be specific for the death receptor as compared to the mitochondrial pathway of apoptosis induction. We could exclude changes in the expression of TRAIL-R and cFLIP and in the activity of PI3K/Akt survival pathway as causative when examining cause for death receptor-specific apoptosis sensitisation. However, we found that detachment-induced inactivation of ERK sensitises breast and skin carcinoma cells to TRAIL- and CD95L-induced apoptosis.

Keywords: detachment, TRAIL, cancer

Poster Abstracts

Abstracts in this section are listed in **alphabetical** order by the name of the presenting author.

Presenting Author: Sonja Aits

HAMLET triggers autophagic cell death in tumor cells

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HAMLET, a complex of partially unfolded α-lactalbumin and oleic acid, kills a wide range of tumor cells but not healthy differentiated cells. This activity is retained in vivo seemingly without causing adverse effects as shown in animals and skin papilloma and bladder cancer patients. The mechanism of cell death is complex. HAMLET targets mitochondria, proteasomes and chromatin resulting in the activation of several, parallel death response pathways (1-3).
Here we investigated if HAMLET causes macroautophagy in tumor cells, and if this contributes to their death. During macroautophagy cytoplasmic material and organelles are enwrapped in double-membrane-enclosed vesicles called autophagosomes which fuse with lysosomes where the content is degraded. After HAMLET treatment we observed extensive cytoplasmic vacuolization and double-membrane-enclosed vesicles were formed. In addition, HAMLET changed LC3 staining in LC3-GFP-transfected cells from uniform (LC3-I) to granular (LC3-II) reflecting LC3 translocation to autophagosomal membranes during macroautophagy. LC3-II accumulation was confirmed by Western blot and LC3 translocation was blocked by 3-methyladenine which inhibits class III phosphatidylinositol-3 kinase and thereby macroautophagy. Macroautophagy by HAMLET may be due to mitochondrial damage and reduced levels of active mTOR.
To determine if macroautophagy contributed to cell death, we used RNA interference targeting Beclin-1 and Atg5, which are required for autophagosome formation. Suppression of Beclin-1 and Atg5 was confirmed by RT-PCR and Western blot and significantly reduced death of HAMLET-treated tumor cells. In parallel, the siRNAs inhibited the increase in granular LC3-GFP staining. The results show that HAMLET triggers macroautophagy in tumor cells and indicate that macroautophagy is essential for HAMLET-induced tumor cell death.

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Keywords: HAMLET, cell death, cancer therapy, macroautophagy, α-lactalbumin

Presenting Author: Gustavo Amarante-Mendes

Knocking-down of TRAIL in CML patients is mediated by Bcr-Abl-induced up-regulation of PRAME

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TRAIL, a member of the TNF-a family, was shown to selectively kill oncogenically transformed cells and, therefore, to participate in the cell-mediated immunity against tumors. However, TRAIL is down-regulated in a variety of tumor-bearing patients. On the other hand, PRAME (preferentially expressed antigen of melanoma) is frequently overexpressed in a wide variety of malignant diseases. It was recently shown that PRAME can function as a repressor of retinoic acid receptor and this transcriptional repression depends on the formation of a complex with the polycomb group EZH2 (enhancer of zeste homolog 2). Interestingly, trail expression can be positively regulated by retinoic acid. Unpublished data in our lab revealed that TRAIL is down-regulated by Bcr-Abl. Therefore, we decided to study the expression of prame and ezh2 in Chronic Myeloid Leukemia (CML) patients and to investigate the correlation between prame and trail in these patients. We analyzed the expression of prame and ezh2 in 31 patients in different phases, 9 in cytogenetic remission post-imatinib and 10 healthy individuals. CML patients showed a significant higher prame expression in the advanced phases of the disease with a significant down-regulation after complete cytogenetic remission (CCR). No differences were found in ezh2 levels in these patients. Furthermore, there was a significant, negative correlation between the expression of prame and trail in CML patients. Overexpression of Bcr-Abl in HL60 cells increased the levels of prame and decreased the levels of trail. To test whether PRAME could be responsible for the downregulation of TRAIL in CML cells, we generate a K562 CML cell line stably transfected with shRNAs against prame or ezh2. Knocking-down of either prame or ezh2 in these cell lines resulted in a dramatic up-regulation of trail. In conclusion, prame up-regulation seems to play an important role in CML´s pathogenesis by repressing retinoic acid signaling pathway and, consequently, down-regulating trail expression, a potent anti-cancer agent and an inducer of the extrinsic pathway of apoptosis.

Support: FAPESP, CNPq and CAPES

Keywords: BCR-ABL, TRAIL, PRAME, EZH2, CML, cancer

Presenting Author: Ivano Amelio

miR-203 controls the proliferative potential of epidermal progenitor cells by repressing DNp63

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The epidermis is a stratified epithelium that functions as a barrier to protect the organism from dehydration and external insults. The epidermis develops depending on of the transcription factor p63, a member of the p53 family of transcription factors. p63 is strongly expressed in the innermost basal layer where epithelial cells with high clonogenic and proliferative capacity reside. Deletion of p63 in mice results in a dramatic loss of all keratinocytes and loss of stratified epithelia, probably due to a premature proliferative rundown of the stem and transient amplifying cells. Here we report that miR-203 is induced in vitro in primary keratinocytes in parallel with differentiation. We found that miR-203 specifically targets human and mouse p63 3'-UTRs. We also show that miR-203 over-expression in proliferating keratincytes is not sufficient to induce full epidermal differentiation in vitro. In addition, we demonstrate that miR-203 is down regulated during the epithelial commitment of embryonic stem cells, and that over-expression of miR-203 in rapidly proliferating human primary keratinocytes significantly reduces their clonogenic capacity. The results suggest that miR-203, by regulating the DNp63 expression level, is a key molecule controlling the p63-dependent proliferative potential of epithelial precursor cells both during keratinocyte differentiation and in epithelial development. In addition, we have shown that miR-203 can regulate DNp63 levels upon genotoxic damage in head and neck squamous cell carcinoma cells, thus controlling cell survival.

Keywords: microRNA, mir-203, p63, proliferative potential, epidermis.

Presenting Author: Nicola Andina

Pim-1 but not PI3K is essential for IL-5 - mediated eosinophil survival

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Eosinophils are prominent effector cells in many allergic and parasitic inflammatory responses. Eosinophil differentiation, activation, and survival are largely regulated by interleukin-5 (IL-5). IL-5 - mediated transmembrane signal transduction involves both Lyn - mitogen-activated protein kinases (MAPK) and Janus kinase 2 (Jak2) – signal transduction and activator of transcription (STAT) pathways. The aim of this study was to detect additional signalling molecules/pathways that are critically involved in IL-5 - mediated eosinophil survival. Although pharmacological inhibition of phosphatidylinositol-3 kinase (PI3K) by LY294002, wortmannin, or the selective PI3K p110delta isoform inhibitor IC87114 was successful in each case, only LY294002, but not the other two PI3K inhibitors, blocked increased IL-5 - mediated eosinophil survival. This suggested that LY294002 inhibited, besides PI3K, another kinase, which is critically involved in this process. Indeed, LY294002 has previously shown to block Pim-1. Pim-1 was rapidly and strongly expressed in eosinophils following IL-5 stimulation in vitro and readily detected in eosinophils under inflammatory conditions in vivo. Both, Pim-1 expression and IL-5 - mediated eosinophil survival, were Jak2 dependent. Moreover, specific protein transfer in eosinophils, using HIV-TAT fusion proteins containing wild-type Pim-1 or a dominant-negative form of Pim-1, demonstrated the critical role of Pim-1 in IL-5 - mediated anti-apoptotic signalling. In conclusion, we show that Pim-1 but not PI3K plays a critical role in IL-5 - mediated eosinophil survival.

Keywords: Pim-1, PI3K, eosinophils, survival

Presenting Author: Christopher Andoniou

Inhibition of Bax in haematopoietic cells promotes viral dissemination

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The evolutionary survival of viruses relies on their ability to disseminate infectious progeny to sites of transmission. The capacity to subvert apoptosis is thought to be crucial for ensuring efficient viral replication in permissive cells, but its role in viral dissemination in vivo has not been considered. We show here that the murine cytomegalovirus (MCMV) m38.5 protein specifically counters the action of Bax and this is sufficient to prevent apoptosis. Surprisingly, m38.5 activity was not required for in vivo viral replication to occur in visceral organs. By contrast, MCMV replication in salivary glands, the major site of viral transmission, was attenuated by deletion of m38.5. We determined that m38.5 activity protects virally-infected hematopoietic cells from apoptosis, thereby ensuring efficient dissemination to the salivary glands, the main site of viral transmission. These results demonstrate that preferential activation of Bax can occur in vivo, and that CMV inhibits this process to ensure optimal viral dissemination occurs.

Keywords: MCMV, Bax, viral dissemination, m38.5

Presenting Author: Marita Angermeier

hUBC13 plays an important role in UV-induced apoptosis

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hUbc13 functions as ubiquitin-conjugating enzyme by catalysing the formation of lysine-63 polyubiquitin chains. It is known to be involved in the NFκB pathway and in DNA repair processes like homologous recombination in mammalian cells.
Our aim is to characterize cellular functions of hUbc13 after DNA damage induction by UV-irradiation. Therefore we examined human cells depleted for hUBC13 by RNAi and analyzed the roles of hUbc13 in TNFR1 signalling and NFκB activation after UV-irradiation and treatment with TNFα. Furthermore its influence on apoptosis in through the TNFR1 pathway is investigated.
We achieved an efficient knockdown of hUBC13 over a period of more than four days and found increased sensitivities towards UV-irradiation in hubc13 downregulated cells. Reduced survival after UV-irradiation could be shown to be due to increased apoptosis. As apoptosis is also increased after TNFα stimulation in hubc13 downregulated cells, we analysed the TNFR signalling pathway after UV-irradiation in more detail. The apoptotic phenotype after UV-irradiation was dependent on caspase-8 activity suggesting that receptor mediated apoptosis is influenced by hUbc13. Significantly reduced TNFR1 protein levels were found in soluble extracts in hubc13 knockdown cells as compared to control transfected cells. Additionally, hubc13 downregulated cells show increased TNFR1 receptor clustering.
In conclusion our results suggest a new role for hUBC13 in an early step of the TNFR1 pathway activation after UV irradiation.

Keywords: hUbc13, UV-irradiation, NFκB signalling, TNFR1

Role of proteoglycans in apoptosis induced by cationic liposomes

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Cationic liposomes are expected as candidates of non-viral vectors for gene delivery. However they showed cytotoxicity, which may deleteriously affect application as non-viral vectors. We have recently reported that cationic liposomes induced apoptosis in various types of cells. To clarify the mechanism of apoptosis exerted by cationic liposomes may provide important information for the use of cationic liposomes as non-viral vectors. In the mouse macrophage-like cell line RAW264.7, we have reported the following findings; (1)generation of reactive oxygen species (ROS), (2)ROS-mediated activation of p38 MAP kinase, (3)activation of caspase-8 by p38, and (4)cleavage of Bid, a member of bcl-2 associated with the release of cytochrome c from mitochondria. However, it is not yet clear how cationic liposomes interact with cells and induce the apoptosis. The major components of extracellular matrix (ECM) are proteoglycan, glycoprotein, and collagens. ECM not only acts as a physical framework, but also exerts a profound effect on cell shape and behavior, including cell adhesion, spreading, migration, proliferation, and differentiation. In this paper, we investigated whether proteoglycans contribute to the apoptosis induced by cationic liposomes composed of stearylamine (SA-liposome) using RAW264.7 cells.
The effects of proteoglycans on the binding of SA-liposomes to macrophages were investigated by flow cytometry. The liposome-binding to macrophages was clearly inhibited by the addition of chondroitin, chondroitin sulfate, and hyaluronan. These proteoglycans also inhibited the apoptosis induced by SA-liposomes. On the other hand, no inhibition was observed when the cells were treated with SA-liposomes in the presence of heparin and heparan sulfate. Furthermore, the treatment of cells with chondroitinase ABC decreased the binding of SA-liposomes to macrophage. Decrease in DNA content as a result of apoptosis induced by SA-liposomes was also inhibited by the pre-treatment of the cells with the enzyme. We have reported that ROS generation is an initial step on the process of apoptosis triggered by the liposomes. Furthermore p38 MAP kinase activation was required for the apoptosis induced by SA-liposomes. These responses observed by SA-liposome treatment were suppressed, and resulted in the decrease in apoptosis when the cells were pre-treated with chondroitinase ABC. These findings indicate that proteoglycans such as chondroitin sulfate play an important role in the cell apoptosis induced by cationic liposomes.

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Keywords: cationic liposome, proteoglycan, apoptosis, macrophage

Presenting Author: Alexandre Arcaro

A sensitized RNA interference screen identifies a novel role for the PI3K p110gamma isoform in medulloblastoma cell survival and chemoresistance

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Purpose: Medulloblastoma is the most common malignant brain tumour in children and is associated with a poor outcome. We were interested in gaining further insight into the potential of targeting the human kinome as a novel cytostatic approach in medulloblastoma.
Experimental Design: Effects on cell survival in the presence or absence of a low dose of cisplatin were analyzed in medulloblastoma cell lines following down-regulation of the known human protein and lipid kinases (700 genes) using libraries of either short hairpin RNA (shRNA) in plasmids or small interfering RNA (siRNA). The results of the screen were validated by target inhibition with specific pharmacological inhibitors. The expression pattern and functions of the identified targets were investigated in medulloblastoma tumour samples and cell lines.
Results: The analysis of cell survival after RNAi transfection identified new protein and lipid kinases involved in medulloblatoma cell responses. A set of 6 genes comprising ATR, LYK5, MPP2, PIK3CG, PIK4CA and WNK4 were identified as contributing to both cell survival and resistance to cisplatin treatment in the DAOY medulloblastoma cell line. A pharmacological inhibitor of p110gamma (encoded by PIK3CG) impaired cell survival in medulloblastoma cell lines and sensitized the cells to cisplatin treatment. An analysis of the expression of these target genes in primary medulloblastoma tumor samples revealed over-expression of LYK5 and PIK3CG.
Conclusions: Together, our data reveal a novel function of p110gamma in medulloblastoma survival and chemoresistance.

Keywords: medulloblastoma, phosphoinositide 3-kinase, Akt, apoptosis, chemoresistance

Presenting Author: Jane Armstrong

PERK-ATF4 signalling in ER stress-induced apoptosis of neuroectodermal tumour cells

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The endoplasmic reticulum (ER) is the primary organelle responsible for the regulation of intracellular calcium and synthesis of proteins destined for the cell surface or secretion, and as such is a major site controlling cellular homeostasis. Stresses which perturb redox state, energy levels, or calcium homeostasis can trigger the accumulation of unfolded proteins within the ER, resulting in activation of the unfolded protein response (UPR). The UPR is primarily an adaptive response to promote cellular repair and sustained survival; however, if the stress cannot be resolved the UPR initiates apoptosis. In order to exploit this phenomenon as an alternative route to induce death in cancer cells the pathways by which ER stress signalling culminates in apoptosis must move from a phenomenological to a mechanistic understanding.

We have previously demonstrated that the chemotherapeutic agents fenretinide, a synthetic retinoid, and velcade, a 26S proteasome inhibitor, induce apoptosis as a consequence of ER stress in neuroectodermal tumour cells. The aim of this study was to investigate the role of potential apoptotic signalling pathways emanating from the ER during stress in response to fenretinide, velcade, and thapsigargin in neuroblastoma (SH-SY5Y) and melanoma (A375, SK-MEL-28) cells, using a combination of siRNA and small molecule inhibitors. Down-regulation of GADD153, IRE-1α, or inhibition of JNK activity did not abrogate cell death induced by these agents, indicating these factors do not promote stress-induced cell death. However, timecourse studies revealed activation of protein kinase RNA (PKR)-like ER kinase (PERK)-eIF2α-ATF4 signalling, verified by increased eIF2α phosphorylation, ATF4, ATF3 and GADD34 expression, correlated with upregulation of the pro-apoptotic BH3-only protein Noxa. Furthermore, treatment of cells with the eIF2α phosphatase inhibitor salubrinal demonstrated that eIF2α phosphorylation was sufficient to trigger Noxa upreguation. Additional analysis of this pathway revealed that while PERK regulates eIF2α phosphorylation in response to ER stress, overall this protein regulates cell survival pathways as downregulation of PERK either enhanced, or did not inhibit, apoptosis following treatment of cells with ER stress-inducers. However, ATF4 knockdown significantly blocked ER stress-induced Noxa expression and cell death in response to these agents.

These data indicate that PERK signalling regulates both pro-survival and pro-apoptotic machinery, demonstrating a tightly regulated balance between these processes. Furthermore, these data suggest PERK-eIF2α-ATF4 signalling plays an important role in the determination of cell fate in response to ER stress in neuroectodermal tumour cells.

Keywords: ER stress, PERK, ATF4, cancer

Different cellular response of HepG2 cells overexpressing CYP3A4 isoenzyme following treatment with antitumor triazoloacridinone C-1305

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Triazoloacridinones are a group of potent antitumor compounds, synthesized and developed at Gdañsk University of Technology. These compounds exhibited significant cytotoxic activity in vitro and also displayed high antitumor activity against several experimental tumor in mice, especially lekemias and colon carcinoma. Among triazoloacridinones, the most active derivative C-1305 was selected for extended preclinical trials.
Our previous studies have shown that metabolic activation of C-1305 was crucial for biological activity of this drug. Furthermore, studies with rat and human microsomal enzymes revealed that CYP3A4 isoenzyme was suspected to be responsible for this metabolism. It is well known that expression level of enzymes responsible for drug metabolism altered cell cycle progression and cellular response induced by anticancer drug. Therefore, here we elucidate the cellular response to C-1305 in hepatocellular carcinoma cells HepG2 overexpressing CYP3A4 (Hep3A4) compared to empty vector-transfected cells (HepC34). All experiments were performed at biological relevant doses of the drug corresponding to its EC50 value (Hep3A4 = 0,7 mM, HepC34 = 0,5 mM) and for different time periods varying from 3 to 144 h.
Cell cycle analysis revealed that C-1305 induced stable accumulation of both studied hepatoma cell lines in G1 phase of the cell cycle. However, after prolonged incubation with the drug (144 h) this cell cycle effect was associated with gradual increase in cells with a sub-G1 DNA content (about 47% for HepG2 cells overexpressing CYP3A4). In contrast, the population of HepC34 cells with sub-G1 region of DNA did not exceeded 17% of total cell population.
Microscopic survey of cells stained with DAPI showed the presence of small population of cells with multiple micronuclei and occasionally, cells with apoptotic body-like structures. Moreover, after 144 h of C-1305 treatment, most of the Hep3A4 cells demonstrated necrosis-like disruption of intact cellular boundaries, as revealed by Annexin V/PI assay. Surviving hepatocellular carcinoma cells developed features of drug-induced senescence with flattened, enlarged morphology and increased degree of SA-beta-galactosidase staining. The percentage of SA-beta-gal-positive cells increased gradually following prolonged incubation with C-1305. After 144 h of drug treatment the number of SA-beta-gal-positive cells reached about 100% of total population.
The overall results suggest that both hepatocellular carcinoma cells treated with C-1305 underwent senescence-like process, proceeded by the growth inhibition in G1 phase of the cell cycle. Additionally, after prolonged drug incubation significant population of HepG2 cells overexpressing Cyp3A4 isoenzyme died by necrosis.

Keywords: antitumor triazoloacridinone C-1305, HepG2 cells, CYP3A4 isoenzyme, cell cycle, senescence

Clearance of dying autophagic cells induces the inflammasome pathway in human and mouse macrophages

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Autophagy is now recognized as a possible inducer of a distinct cell death mechanism happening under various circumstances (Mizushima N., Nature Reviews, 2008). Clearance of dying autophagic MCF-7 cells but not living or apoptotic ones can lead to pro-inflammatory response in human macrophages (Petrovski et al., Autophagy, 2007). These dying cells could induce activation of caspase-1 (IL-1beta converting enzyme) as early as 1 hour after being co-incubated with human blood-born macrophages as shown by Western blotting. Mouse thioglycollate elicited peritoneal and bone-marrow (BM) derived macrophages could engulf the dying autophagic and apoptotic MCF7 cells with approximately the same capacity as the human ones. Release of IL-1beta from the mouse macrophages upon engulfment of dying MCF7 cells gave so far no consistent response compared to the one with human macrophages. Due to a possible human-to-mouse cross-over inconsistency, we chose to use the mouse Ba/F3 cell line (IL-3 dependent BM derived pro-B cells) as a possible autophagic cell clearance model. Ba/F3 cells have been shown to undergo death under IL-3 depletion with signs of autophagy (Wirawan and Vandenabeele et al., 15th Euroconference, ECDO, 2007). Our present work has shown that dying autophagic Ba/F3 cells can induce the IL-1beta release in mouse peritoneal macrophages in the mouse-to-mouse co-incubation system opening the way of mouse knock out experiments for detailed molecular analysis. Our goal is to test whether knocking down Atg5 and Beclin1 in stably transfected MCF7-GFP LC3 cells could prevent induction of the inflammasome pathway while they are engulfed. Furthermore, studies with THP-1 cells are in progress to deduce the involvement of the members of the inflammasome pathway (caspase-1, ASC, NALP3) as well as exclude the involvement of other inflammatory pathways (Myd88).

Keywords: IL1- beta, inflammasome, phagocytosis, autophagic dying cells

The Prevalence of glutathione S-transferase T1 (GSTT1) null genotype in Iranian liver transplant recipients

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An objective to improve the evolution of transplants is to identify risk biomarkers of morbidity and loss of allograft. In liver transplant (LTX) recipients, an association has been demonstrated between the presence of mismatch for glutathione S-transferase T1 (GSTT1) and the development of de novo immune hepatitis (IH).
The differences in the genotypes of codifying metabolic enzymes between donors and recipients seems to be a prognostic biomarker in order to identify individuals at risk of complication following liver transplantation (LTX). According to previous studies if the patient is null phenotype and the donor is positive (GSTT! Mismatch) then the patient should be long-term monitored for development of atypical autoantibodies and the appearance of de novo Immune hepatitis and graft dysfunction.Atypical autoantibodies were shown to be directed to the enzyme GSTT1, a 29-kDa molecular weight protein, expressed abundantly in the liver and kidney.
The aim of this study was to determine the prevalence of GSTT1 genotype in Iranian population, who had LTX from august 2007 to May 2008 in OrganTransplantation Center affiliated to Shiraz University of Medical sciences.
Determination of GSTT1 in 54 recipients was carried out by an assay based on internal standard controlled polymerase chain reaction (PCR). DNA from the peripheral blood samples of subjects with positive GSTT1 alleles yielded amplification of a 492-bp fragment. As a positive control a fragment of beta- globin was also amplified.
Half of our patients (27/54) have had null genotype. This is a high prevalence. But determination of GSTT1 genotype in both donors and recipients are important because it was previously confirmed that only under one of the four possible genetic combinations (null recipient/positive donor) , an alloimmune response triggered with production of anti-GSTT1 antibodies. Our study is a preliminary report and further research is required in order to elucidate whether in LTX recipients with GSTT1 mismatch, anti-GSTT1 antibodies will be produced and an alloimmune reaction targeted at allograft as posttransplantation IH will developed or not.

Keywords: GSTT1, Iran , Liver, transplant

Presenting Author: Alakananda Basu

Proteolytic cleavage of p70 ribosomal S6 kinase during DNA damage-induced apoptosis

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p70 ribosomal S6 kinase (p70S6K) is a serine/threonine protein kinase, which phosphorylates ribosomal S6 protein and promotes translation of a subset of mRNAs necessary for cell cycle progression. p70S6K has been shown to be constitutively activated or upregulated in several cancers and deregulation in the p70S6K pathway has been associated with cellular transformation. Cisplatin, a DNA damaging agent, is used for the treatment of solid tumors. However its success is often compromised due to relapse and chemoresistance. The objective of the present study was to determine the involvement of p70S6K in cisplatin-induced apoptosis.

The levels of phosphorylated p70S6K were elevated in the small cell lung cancer H69 cells that acquired resistance to cisplatin (H69/CP) compared to parental H69 cells. Cisplatin caused a time-dependent decrease in full-length p70S6K in human small cell lung cancer H69 as well as in non-small cell lung cancer A549 cells. Concomitant to the decrease in level of p70S6K, there was an appearance of a cleaved band in cells treated with cisplatin. The broad specificity caspase inhibitor z-VAD-fmk reversed p70S6K downregulation by cisplatin whereas inhibitors of calpain and the proteasome had no effect. Cell-permeable peptide inhibitors of capsase-3 and -9 inhibited cisplatin-induced proteolytic cleavage of p70S6K. In vitro-translated p70S6K was shown to be cleaved by human recombinant caspase-3. Furthermore, depletion of caspase-3 by siRNA blocked the cleavage of p70S6K. Cisplatin failed to induce cleavage of p70S6K in MCF-7 cells that lack functional caspase-3. Ectopic expression of caspase-3 in MCF-7 cells resulted in cisplatin-induced cleavage of p70S6K. Introduction of the N-terminal cleaved fragment of p70S6K enhanced cisplatin-induced apoptosis. These results suggest that p70S6K is a novel substrate for caspase-3 and the proteolytic cleavage of p70S6K is important for cisplatin-induced apoptosis.

(This work was supported by the grants CA85682 and CA71727 from the National Cancer Institute. Rohini Dhar was supported by the NSF Score grant 0440334.)

Keywords: cancer, DNA damage, apoptosis

ASK1/p38 MAP kinase, c-ABL and c-CBL regulate caspase 8 activation and TNF-α-induced cell death by phosphorylation, ubiquitination and degradation of c-FLIPs

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Introduction: In earlier studies we have demonstrated a role of apoptosis signal regulating kinase 1 (ASK1) in macrophage apoptosis executed by Mycobacterium avium. We now report that at least one mechanism of M. avium-triggered apoptosis is through the release of tumor necrosis factor–alfa (TNF-alfa). We have worked out the detailed mechanism of cell death executed by TNF-alfa in murine macrophages.
Methods: This study has employed the murine macrophage cell line RAW264.7 as well as murine bone marrow-derived macrophages (BMDMs). Cells have been transfected with dominant-negative constructs of ASK1 and p38 MAP kinase in order to elucidate effects attributable to these kinases. Transfection of c-FLIP has been used to decipher the role of c-FLIP in TNF-alfa-induced cell death. RNA interference-based knockdown of the tyrosine kinase c-Abl, ASK1 and c-Cbl has been used to elucidate the effects of these molecules on the cellular signaling pathway triggered by TNF-alfa in macrophages.
Results: Isoforms of c-FLIP have been reported to exert both pro-survival and pro-apoptotic effects. However, c-FLIPs exerts exclusively pro-survival effects. c-FLIPs is the major isoform of FLIP present in RAW264.7 as well as in bone marrow-derived macrophages. We demonstrate that ASK1/p38 MAP kinase activation by TNF-alfa plays a central role in phosphorylation of c-FLIPs on serine-4. At the same time, the tyrosine kinase c-Abl phosphorylates c-FLIPs on tyrosine 211. Phosphorylation on serine 4 and tyrosine 211 results in interaction of c-FLIPs with the E3 ligase c-Cbl, its ubiquitination and degradation. This facilitates activation of caspase 8, one of the proximal steps of TNF-alfa-induced apoptosis.
Conclusions: Our studies bring to light for the first time the novel role of ASK1/p38 MAPK and c-Abl in inducing c-Cbl-dependent ubiquitination of c-FLIPs in response to challenge of macrophages with TNF-alfa. Other studies have shown that the E3 ligase Itch regulates ubiquitination and degradation of FLIPL, and postulated a role of the MAP kinase JNK in this process. We have ruled out any role of Itch or JNK in regulating c-FLIPs degradation in macrophages. This provides new insight into death signaling in immune cells, and suggests that the mechanisms by which the anti-apoptotic effects of FLIP are reversed for the execution of apoptosis, are cell type-specific.

Keywords: Immune signaling, c-FLIP, ASK1, c-Abl, c-Cbl, apoptosis

Investigating the role of the BH3-only proteins Bim and Bmf in mammary gland development and cancer

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Apoptosis is required for normal development and maintenance of tissue homeostasis. Two pro-apoptotic Bcl-2 family members of the BH3-only subgroup, Bim and Bmf, were reported to drive apoptosis upon various stresses including detachment from the extracellular matrix, cytokine deprivation and treatment of cells with chemotherapeutic drugs. Interestingly, both proteins have been recently implicated in developmental cell death during mammary gland formation. Apoptosis plays also a key role during remodelling of the mammary gland after the lactation, a process called involution. Therefore, we started to investigate the expression of BH3-only proteins during these processes. The aim of our study is to investigate the role of Bim and Bmf during involution and malignant transformation of the mammary gland.
We found that both proteins are differentially expressed during mammary gland development and peak in the involution phase. When Bim or Bmf is lost, the involution process appeared delayed, as assessed by histological analysis, supporting an involvement of both proteins to the remodelling of this complex organ. Importantly, Bmf and Bim protein were also found to be highly expressed in MMTV/erbB2-driven tumours, indicating that these proteins may have been induced in response to oncogenic stress. Therefore we started to generate mice overexpressing the MMTV/neu transgene but lacking Bim or Bmf in addition in order to assess their relative tumor suppressor potential in the mammary gland.
The ultimate goal of the proposed study is to deepen the knowledge about breast cancer pathogenesis, possible causes of drug resistance in tumour patients and to identify novel predictors of disease progression and/or drug responsiveness within the Bcl-2 family.

Keywords: BH3, Bim, Bmf, breast cancer, mammary gland

Phenotyping neuronal apoptosis: insult-dependent crosstalk between caspase-dependent/-independent cell death pathways

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Apoptotic-like injury occurs in various neuropathologies and can be triggered by mitochondrial dysfunction as a result of cellular stressors effecting redistribution of apoptogenic proteins. Neurons, like immortalized cell systems, manifest canonical death processes, although the range of stresses that this specialized cell-type experiences does not always invoke “classical” apoptotic death involving the “intrinsic” pathway and caspase activation. Indeed, terms such as aponecrosis and “pathological” apoptosis have been employed to describe the continuum of injury profiles found for neurons. Sequestration of mitochondrial Ca2+ is especially important in various forms of neuronal injury and Ca2+ can induce mPTP opening and activate enzymes, such as calpains, which share specificity for caspase substrates and interact with apoptogenic proteins to induce caspase-independent mechanisms of cell death. We sought to determine whether an interactive model of caspase-dependent and caspase-independent cell death with differential, system-dependent crosstalk applied to neuronal injury induced by stressors implicated in neurologic conditions. Using primary neuronal cultures (excitatory glutamatergic cerebellar granule cells & inhibitory striatal GABAergic neurons) we determined the patterns of activation of proapoptotic mitochondrial signalling and redistribution of apoptogenic proteins induced by various pathologic insults. Progression of injury was correlated with mitochondrial involvement and patterns of protease activation (Beart et al., J Neurochem 103, 2408-2427 (2007)). Concentrations of stressors, including the reference apoptotic inducer staurosporine, were adjusted to produce early, time-dependent Annexin V labeling. Analyses of the kinetics and temporal patterns of redistribution of cytochrome c, Smac/DIABLO, HtrA2/Omi and AIF were undertaken by immunolabelling and confocal microscopy (Lim et al., Exp Cell Res 312, 1174-1184 (2006)) revealing all insults (oxidative stress, excitotoxicity, trophic-factor deprivation, inhibitors of complexes I/II) elicited redistribution of AIF, although there was wide variation in the timing and magnitude of this process. These data confirmed mitochondrial participation in neuronal injury and the hierarchical nature of protein redistribution following permeabilization of the outer mitochondrial membrane. Protease inhibitors failed to block cell death, attenuation was rarely noted, but sometimes progression was slowed - findings which support the existence of alternate death pathways with the establishment of the commitment to die. Recruitment of proapoptotic mitochondrial signalling occurred across a continuum of injury from classical recruitment of the intrinsic pathway (staurosporine & trophic-factor deprivation) to Bax-independent, calpain-dependent (excitotoxicity) injury. Neurologic stressors effecting minimal caspase-3 activation produced a novel form of intrinsic apoptotic-like injury with early involvement of calpain and AIF. Moreover, these patterns of cellular injury appeared to be conserved across different neuronal cell types. By definition of the death machinery we developed models of neuronal cell death reflecting differential insult-dependencies and hierarchies.

Keywords: neurodegeneration, mitochondria, AIF, calpain, caspase-independent, apoptosis

Oxidative stress and chaperone-mediated cancer cell death

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Bcr-Abl expression and its constitutively-activated tyrosine kinase activity are essential for malignant progression in chronic myelogenous leukemia. Here we describe that oxidative stress generated during ascorbate-driven menadione redox cycling (ascorbate/menadione) resulted in Bcr-Abl degradation leading to death of K562 cells (a leukemia cell line expressing Bcr-Abl). Ascorbate/menadione also inhibited tumor growth in K562-bearing Balb/c nude mice. Since hsp90 stabilizes and protects Bcr-Abl from degradation, we looked for putative effects induced by oxidative stress on this chaperone protein. In ascorbate/menadione-treated cells, Western blot analysis against hsp90 showed a second protein band, suggesting hsp90 cleavage. N-acetylcysteine, sodium vanadate and Tyrphostin 46, were all able to suppress hsp90 cleavage and to protect against both Bcr-Abl degradation and cell death. Conversely, neither MG-132, NH4Cl, 3-methyladenine, nor different protease inhibitors were able to suppress hsp90 cleavage. Both hsp90 cleavage and Bcr-Abl degradation were observed by incubating K562 cells with another H2O2-generating system (glucose/glucose oxidase) and by incubating KU812 cells (another leukemia cell line) with ascorbate/menadione. Furthermore, hsp90 cleavage was observed in a large panel of cancer cell lines but not in non-transformed cells. Finally, two additional hsp90 client proteins (Akt and RIP), were also degraded in K562 cells exposed to ascorbate/menadione. Due to the major role of hsp90, these results may have potential clinical applications.

Keywords: cancer, oxidative stress, hsp90, cell death

The role of PIDD in Caspase 2 and NFκB activation

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The p53-induced protein with a death domain (PIDD) is involved in cellular pathways which can induce programmed cell death (apoptosis) or survival via the activation of NF-κB after DNA damage. Depending on the duration and severity of genotoxic stress, PIDD, together with the adapter molecule RAIDD, can aid in the formation of an activating platform for Caspase 2, the so-called “PIDDosome”, or together with RIP1 assist in the activation of NF-κB by promoting sumoylation of NEMO. While the former results in apoptosis, the latter prevents apoptosis and, by enabling the cell to repair the damage, is thought to enhance cell survival. PIDD therefore plays a role in the integration of two opposing signalling pathways. This dual role is based on the capacity of PIDD to autoproteolytic cleavage, which leads to the generation of two different protein fragments, PIDD-C and PIDD-CC, each of which is involved in the induction of one of the aforementioned pathways.
In order to shed further light on these roles and to investigate the in vivo function of PIDD, we have generated a mouse model lacking the pidd gene. Using mouse embryonic fibroblasts (MEFs) derived from those mice as well as from other knockout mice, lacking individual components of the PIDDosome, we performed survival assays and monitored NF-kB activity in response to genotoxic stress.
None of the MEFs lacking a component of the Caspase 2 activating platform showed a significant difference in terms of cell survival, compared to wildtype MEFs in response to different types of DNA damage inducing agents, cytoskeletal disruptors or kinase inhibitors.
To investigate the role of PIDD in NFκB activation after DNA damage, we performed immunofluorescence localisation studies, which showed that the translocation of NFκB transcription factors into the nucleus is not impaired in PIDD deficient cells.
In conclusion, our results suggest that the components of the PIDDosome are not essential for DNA damage induced apoptosis, and that PIDD is not rate-limiting for the translocation of NFκB into the nucleus, pointing towards more selective and redundant functions of this protein that remain to be unraveled.

Keywords: DNA damage, PIDD, Caspase 2, NFkB

Presenting Author: Karina R. Bortoluci

TLR4/MYD88-dependent, LPS-induced synthesis of PGE2 by macrophages or dendritic cells prevents anti-CD3-mediated CD95L upregulation in T cells

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Antigen-presenting cells (APCs) control T cell responses by multiple mechanisms, including expression of co-stimulatory molecules and production of cytokines and other mediators that control T cell proliferation, survival and differentiation. Here, we demonstrate that soluble factor(s) produced by TLR-activated APCs suppress AICD. This effect was observed in non-stimulated APCs, but it was significantly increased after LPS treatment. Using different KO mice, we found that the LPS-induced protective factor is dependent on TLR4/MyD88. We identified the protective factor as prostaglandin E2 (PGE2) and showed that both APC-derived sups and PGE2 prevented CD95L upregulation in T cells in response to TCR/CD3 stimulation, thereby avoiding both AICD and activated T cell killing of target macrophages. The PGE2 receptors, EP2 and EP4, appear to be involved since pharmacological stimulation of these receptors mimics the protective effect on T cells and their respective antagonists interfere with the protection induced by either APCs-derived or synthetic PGE2. Finally, EP2 and EP4 engagement synergistically activate PKA and EPAC pathways to prevent AICD. Taken together, these results indicate that APCs can regulate T cell levels of CD95L by releasing PGE2 in response to LPS via a TLR4/MyD88-dependent pathway, with consequences for both T cell and their own survival.

Supported by FAPESP, CAPES and CNPq.

Keywords: FasL, PGE2, AICD, APC, TLR, apoptosis, immuneregulation

Presenting Author: Vilma Borutaite

Regulation of ischemia-induced heart apoptosis by the redox state of cytochrome c

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In the mitochondrial pathway of apoptosis, the release of cytochrome c from mitochondria is considered to be an irreversible event. However, there is evidence that execution of apoptosis may be regulated even after this step. One possible level of such regulation may be the redox state of cytochrome c which may affect the rate of caspase activation in the apoptosome. In this study, we investigated whether reduction of cytochrome c by TMPD (tetramethyl-phenylenediamine) may prevent either staurosporin-induced caspase activation in cultured HeLa cells or ischemia-induced apoptosis in perfused rat heart. Using cytosols isolated from rat hearts and other mammalian cells we find that oxidation of cytochrome c by added cytochrome oxidase stimulates caspase activation, whereas reduction of cytochrome c by TMPD, ascorbate or yeast lactate dehydrogenase/cytochrome c reductase inhibited caspase activation. Perfusion of the hearts with TMPD strongly inhibited ischaemia/reperfusion-induced activation of caspases and cell death though it did not prevent ischemia-induced release of cytochrome c from mitochondria. We also found that homogenates from healthy cells reduced added cytochrome c, whereas in homogenates from apoptotic cells cytochrome c was rapidly oxidized by endogenous process which was sensitive to azide. This cytochrome c-oxidizing activity was prevented when mitochondria were removed from the homogenate. Mitochondria also posses external NADH-dependent cytochrome c reductase activity which may have an impact on the redox state of cytosolic cytochrome c. This activity is sensitive to DIDS, an inhibitor of voltage-dependent anion channel, and is inhibited during heart ischemia or when isolated mitochondria are exposed to nitrosylating NO-donor nitrosogluthathione but not to NO itself. We conclude that reduction or oxidation of cytosolic cytochrome c by mitochondria or chemical compounds (such as TMPD) can regulate apoptosome-mediated apoptosis in the heart or cultured cells.

Keywords: mitochondria, cytochrome c, caspases, heart ischemia

Presenting Author: Claudio Brancolini

Isopeptidases as anticancer targets for pro-apoptotic therapies

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Isopeptidases comprise a large family of proteolytic enzymes, which deconjugate ubiquitin and ubiquitin-like proteins from target cellular proteins. Isopeptidases through the control of the lifetime, localization, and activity of ubiquitin-modified proteins modulate many biological processes including cell cycle, apoptosis, DNA repair and membrane trafficking. We have recently identified a new non-selective isopeptidase inhibitor, named G5. In cancer cells G5 triggers a peculiar apoptotc response, characterized by Noxa induction, Smac stabilization, caspase-8 activation and apoptosome independence. More recently we have discovered that G5 but not bortezomib, the inhibitor of the catalytic sites of the proteasome, can also elicit a new necrotic response, which in preclinical studies, is relevant for killing glioblastoma cells.
In order to develop new specific isopeptidase inhibitors for a possible clinical use it is important to characterize which, among the hundred of different isopeptidases, are critical in the control of cell death and survival. To answer this question we have silenced 60 different isopeptidases in cells challenged to die with etoposide or bortezomib treatments. With this screening we have discovered that silencing of USP18/UBP43 promotes caspase activation and apoptosis in drug-treated cells. USP18 is an interferon-inducible protein, which removes the ubiquitin-like protein ISG15 from conjugated proteins. We have confirmed that USP18 is an interferon inducible gene. Silencing of USP18 strengthened apoptosis also in cells treated with interferon-α; or in cells co-treated with etoposide and interferon or bortezomib and interferon. We have also analyzed the effect of USP18 downregulation on the expression levels of various Bcl-2 family members and of the regulative caspases 2, 8 and 9. Our data suggest that downregulation of USP18 increases the expression and promotes the activation of caspase-8. Studies are ongoing to define at which level USP18 controls the activation of the extrinsic apoptotic pathway. In conclusion our data indicate that isopeptidases and more specifically USP18 represent important targets for anti-tumor therapies.

Keywords: Cancer, proteasome, caspase-8, interferon, necrosis

Identifying the cellular targets of pro-apoptotic isothiocyanates

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Isothiocyanates are a class of phytochemical with chemopreventive and chemotherapeutic activities including the ability to induce apoptosis in a wide range of cell types and tumour models. We have shown that aromatic isothiocyanates, including phenethyl isothiocyanate (PEITC) can induce apoptosis in Jurkat cells that overexpress the anti-apoptotic proteins Bcl-2 or Bcl-XL [1, 2]. While the biological activities of isothiocyanates are widely recognised their mechanism of action is largely unknown. Isothiocyanates are electrophilic compounds that react with cellular nucleophiles, in particular the sulfhydryl group of cysteine residues in glutathione and thiol proteins, to form dithiocarbamates. We have previously shown that PEITC can oxidize redox-active thiol proteins including mitochondrial peroxiredoxin 3 [3]. In this study we have used an affinity-based target identification approach to capture the direct intracellular binding targets of PEITC. We have synthesised a novel derivative of PEITC that could be conjugated to a solid phase support. Incubation of Jurkat cell lysates with the immobilized PEITC led to the capture of two major cellular thiol proteins. Mass spectrometry is currently being used to identify specific residues targeted by the isothiocyanates. This information will be valuable for exploring the mechanisms by which isothiocyanates trigger apoptosis in cancer cells.

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Keywords: isothiocyanates, apoptosis, thiol proteins

Presenting Author: Pedro Buc Calderon

Murine hepatoma cells are more sensitive than normal hepatocytes to an oxidative stress induced by ascorbate-driven menadione redox cycling.

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It is known that overproduction of reactive oxygen species (ROS) is involved in both initiation and promotion of multistage carcinogenesis. In addition, depending on the nature of the tumour cells, they appear more susceptible to an oxidative stress than the surrounding normal cells. On the other hand, cancer cells readily take up vitamin C in vitro, and studies have demonstrated high vitamin C concentrations in neoplasms compared with the adjacent normal tissue. The mechanism by which cancers accumulate vitamin C, however, is unknown, but most probably it involves the transport of its oxidized form (dehydroascorbate), via facilitative glucose transporters including GLUT1. For instance, in HL60 cells, dehydroascorbate is transported across the cell membrane and accumulated in the reduced form (ascorbate), which is not transportable through the bidirectional GLUTs. We have explored the selective exposure of cancer cells to an oxidative stress generated by ascorbate-driven menadione redox cycling. Since ascorbate is preferentially taken up by cancer cells which appear to be deficient in antioxidant enzymes, we hypothesized that TLT cells (murine hepatoma) will be more sensitive than primary mouse hepatocytes towards an oxidative stress generated by ascorbate-driven menadione redox cycling. The results show that murine hepatomas (TLT cells) have less than 10% of antioxidant enzyme activities as compared to normal hepatocytes. Such a decrease includes the three major antioxidant enzymes, namely catalase, superoxide dismutase and glutathione peroxidase. Catalase is almost not expressed in TLT cells as shown by western blot analysis. Moreover, in TLT cells the content of both GSH and ATP represents less than 25% of normal hepatocytes. Furthermore, the uptake of vitamin C is more important in TLT cells as compared to normal hepatocytes. Finally, the combination of vitamin C (which accumulates in TLT cells) and a quinone undergoing a redox cycling (vitamin K3) leads to an oxidative stress that kills cancer cells in a selective manner. We concluded that the consequences of this differential sensitivity between cancer cells and normal cells may have important clinical applications.

Keywords: oxidative stress, antioxidant enzymes, vitamin C

Metastatic potential changes in human melanoma cell lines after treatment with Alimta

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Previous results of our group have shown that Pemetrexed (Alimta, LY231514) induces cell cycle arrest and apoptosis on human melanoma cell lines. However, this product does not induce cell death on the totally of the cell population, and a subpopulation of the tumoral cells does not respond to the treatment. This fact can lead Alimta to create cells resistant to this treatment.
In the present work we have exposed three human melanoma cell lines A375, HS294T and HT144 to increasing doses of Alimta to generate three cell lines resistant to this drug. The objective of this work was to study metastatic potential changes of these three melanoma cell lines compared to responding cells. The metastatic potential is determined by migration, adhesion and invasion capacity of the tumoral cells. The adhesion to the extracellular matrix is mediated by integrins and results in the actin filaments polymerization, stress fibres assemblement and focal adhesion creation. We have analyzed by flow cytometry the presence of α 4, α 5 and α v in Alimta resistant cells and compared with responding cells resulting in a significant difference between both populations. In the same way, actin filament organization analysis by immunofluorescence assay with phalloidin results in changes in the cytoskeleton organization after treatment with Alimta, in both non-resistant and resistant cells.
The migration capacity has been measured by migration and scratch wound assay, resulting in significant changes in cell migration speed.
Finally, we have studied changes in invasion capacity of human melanoma cells resistant to Alimta with trasnwell chambers.

Keywords: melanoma, metastatic potential, migration, invasion

The PEA-15 Protein Regulates Autophagy in Glioma Cells via Activation of JNK

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PEA-15/PED (phosphoprotein enriched in astrocytoma 15 kDa / phosphoprotein enriched in diabetes) is a death effector domain-containing protein which is known to modulate apoptotic cell death. The mechanism by which PEA-15 inhibits Caspase activation and increases ERK (extra cellular-regulated kinase) activity is well characterized. Here, we demonstrate that PEA-15 is not only pivotal in the activation of the ERK pathway but also modulates JNK (c-jun N-terminal kinase) signaling. Upon over-expression of PEA-15 in U87MG, U251MG, and LN18 glioma cells JNK is potently activated. The PEA-15-induced JNK activation depends on the phosphorylation of PEA-15 at both phosphorylation-sites (serine 104 and serine 116). The activation of JNK is substantially inhibited by siRNA-mediated down-regulation of endogenous PEA-15. Interestingly, JNK activation is abrogated by co-expression of PEA-15 and SEK1/MKK4. MKK4 is a dual-specific kinase, which directly phosphorylates the TPY -motif and coinstantaneously activates JNK. Moreover, we demonstrate that glioma cells stably expressing PEA-15 show increased signs of autophagocytosis in response to classical autophagic stimuli such as gamma irradiation, serum deprivation, or rapamycin -treatment. In conclusion, our data show that PEA-15 is involved in JNK signaling and in the regulation of autophagic processes. We propose a model in which PEA-15-induced autophagy is mediated by the modulation of JNK activity.

Keywords: PEA-15, JNK, autophagy, glioblastoma

Exploring the role of mitochondrial dynamics in T cells: from movement to AICD.

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Mitochondria are highly dynamic organelles that continuously move, divide and fuse in a highly regulated fashion. The balance between the opposing processes of mitochondrial fusion and fission is controlled by a growing family of "mitochondria-shaping" proteins (i.e. OPA1 and DRP1). Evidence is accumulating on the role of these proteins in several physiological functions, from apoptosis to development. We recently unraveled an unexpected role for mitochondrial dynamics in the immune system where it controls migration of T cells. This prompted us to investigate whether mitochondrial shape participates in other crucial processes of T cells. Early in the process of activation-induced cell death (AICD) mitochondria undergo fragmentation and clusterization. This is not caused by changes in the levels of mitochondria shaping proteins, but results from the activation of signaling cascades impinging on the fusion/fission machinery. We will present results on the relationship between mitochondrial fragmentation and AICD.

Keywords: Mitochondria dynamics, AICD, T cells, fragmentation.

Caspase 3 immunohistochemical expression in borderline and invasive serous ovarian tumours

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Aim: To demonstrate the immunohistochemical expression of caspase 3 in borderline and invasive serous ovarian tumours.
Methods: We performed immunohistochemical staining of formalin fixed, paraffin-embedded tissue samples from 40 serous ovarian tumours, using caspase 3 monoclonal antibody, manufactured by R&D Systems (Minneapolis, USA). Apoptotic cells were detected by light microscopy (Olympus BX40). Immunohistochemical results were scored semiquantitatively.
Results: Tissue samples contained apoptotic cells with brown stained cytoplasm that were positive to active form of caspase 3. However considerable number of apoptotic cells did not show any caspase 3 positivity in both borderline and invasive serous ovarian tumours.
Conclusion: Presence of caspase 3 negative apoptotic cells imply the possibility of activation of different apoptotic pathways in ovarian tumour cells. This implicates that in cancer therapy we should consider targeting divers molecules at the same time for the therapy to be successful.

Keywords: serous ovarian tumours; caspase 3; apoptosis

Induction of apoptosis in embryonal tumors by targeting the oncogene Myc

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Embryonal tumors (ET) such as neuroblastoma (NB), medulloblastoma (MB), Ewing sarcoma family of tumors (ESFT), Wilm's tumor (WT), retinoblastoma (RB), and malignant rhabdoid tumor (MRT), all together account for about 30% of childhood malignancies. Although these diseases are very different in terms of etiology, genetics, and progression, changes in the expression of the Myc family of oncogenes and/or mutations along Myc-related cellular pathways are important contributors to the malignant phenotype of ET. Among different strategies of targeting Myc, we have considered two approaches aimed at destabilizing Myc either at the mRNA or at the protein level, by using small interference RNA (siRNA) or a small molecule inhibitor (quassinoid NBT-272), respectively. In particular, we investigated the induction of apoptosis that can be related to Myc expression in ET-derived cell lines, upon repression of Myc by either strategy. The apoptotic response triggered by the Myc-inhibitor NBT-272 varied considerably in different ET entities, therefore suggesting that apoptosis alone cannot explain the very potent effect of the compound on cell viability in vitro. However, concentrations as small as 10 nM were able to trigger cell death in MB, ESFT, and MRT cell lines, as assessed by quantifying caspase activation by different approaches. The expression analysis of the two major forms of Myc in different ET cell lines led us to speculate that over-expression of c-Myc, rather than MYCN, can lead to a more pronounced sensitivity to NBT-272-induced apoptosis. This observation was also confirmed by using c-Myc-deficient cells that were considerably more resistant to apoptosis induced by NBT-272. A complementary study using gene expression profiling (by cDNA microarrays) allowed us to correlate Myc expression and the activation status of apoptosis-related genes in a MB-derived cell line (DAOY), where c-Myc was either up-regulated or silenced. Consistent with the role of Myc as an activator of cell proliferation, many cell cycle-promoting genes (e.g. cyclins of the D family) became repressed, whereas pro-apoptotic genes were up-regulated, upon c-Myc knock-down by siRNA. Among this last group of genes triggering apoptosis, we could find different caspases, Ras-related proteins, as well as many members of the tumor necrosis factor (TNF) receptor superfamily and TNF ligands. Some of these genes could be direct targets of Myc and may open the way to alternative strategies for selective therapeutic intervention. In conclusion, investigating Myc-dependent apoptotic pathways is not only instrumental for increasing our knowledge in the biology of ET, but will also possibly lead to the identification of novel tumor markers and drug targets for the treatment of ET over-expressing Myc family oncogenes.

Keywords: Embryonal tumors, tumor targeting, Myc

Presenting Author: Claudia Cerella

Extrusion of Ca2+-loaded mitochondria as a survival strategy in anoxia/reperfusion stress

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After ischemic injuries, reperfusion is probably the major cause of cell loss, mostly due to a paroxysm of cytosolic Ca2+ increase. Tumor cells develop resistance programmes to hypoxia/anoxia during tumor progression, providing exemplar systems where to study cell survival strategies to ischemia/reperfusion. We investigated the mechanisms of cell survival of in vitro anoxia/reoxygenation of two tumor lymphoma cells, U937 and E2r. Both survive reoxygenation, but with different strategies: U937 limit cytosolic Ca2+ rise, whereas E2r survive in spite of substantial and prolonged Ca2+ increase. In both cells, mitochondria act as Ca2+ stores during reoxygenation, since prevention of mitochondrial Ca2+ uptake with the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) greatly increases intracellular Ca2+ concentration([Ca2+]i); this does not affect U937 survival, because [Ca2+]i remains below a toxic threshold; instead, CCCP turns reoxygenation into a cell killing treatment in E2r, implying that Ca2+ stored into mitochondria of reoxygenating E2r is a potential killer. To understand how E2r extinguish this toxic potential, we followed mitochondria behaviour during reoxygenation, and witnessed a peculiar phenomenon: at ~70, E2r rapidly extrude actin-lined cytoplasmic vesicles highly enriched of charged mitochondria, losing about half of the population; this has no toxic implications but rather, E2r dispose of dangerous Ca2+ loaded mitochondria as a pro-survival strategy, rapidly recovering regular mitochondria number.

Keywords: calcium, anoxia, reperfusion, mitochondria, survival

Presenting Author: Boris Chernyak

The small fraction of mitochondria can determine the fate of the cell under oxidative stress. The study of protective effect of the mitochondria-targeted antioxidants.

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Plasoquinone conjugated with triphenyl phosphonium cation (SkQ1) is a powerful antioxidant in bilayer lipid membranes and in isolated mitochondria. Plastoquinone conjugated with positively charged fluorophore rhodamine-19 (SkQR1) selectively accumulates in mitochondria of HeLa cells and human fibroblasts. Accumulation was completed in 2h. Uncoupler FCCP suppressed the accumulation and stimulated the release of SkQR1. Incubation with nmolar SkQ1 or SkQR1 for 2h prevented oxidation of glutathione and fragmentation of mitochondria caused by H2O2. However, 2h incubation with antioxidants did not protect HeLa cells and human fibroblasts against apoptosis induced by H2O2 or other prooxidants (menadione, paraquat). The protective effect was observed only after 24h incubation. After prolonged (6-7 d) incubation the effect of SkQ1 or SkQR1 became pronounced at 0.01-1 nM. The rate of SkQR1 release from the cells measured after prolonged incubation is much slower then after 2h incubation. We have shown that the mitochondria-targeted antioxidants promoted slow structural and functional fusion of mitochondria increasing the size of electrically-united mitochondrial network. Incubation of fibroblasts with SkQR1 for 2h revealed significant heterogeneity of mitochondria while after prolonged incubation mitochondria became uniformly stained. Probably the small fraction of mitochondria with low membrane potential accumulated mitochondria-targeted antioxidants slowly and remained unprotected in short-term experiments. Prolonged incubation with antioxidants improved the quality of these mitochondria probably due to fusion with “good” mitochondria in the cell. This could explain slow development of the protective effect of the mitochondria-targeted antioxidants. It is suggested that small fraction of “bad” mitochondria could determine the fate of the cell under various stresses.

(Sponsored by O. V. Deripaska)

Keywords: mitochondria, oxidative stress, mitochondria-targeted antioxidants.

Presenting Author: Young-Eun Cho

mHGTD-P mediates hypoxic neuronal cell death via the caspase-independent pathway

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HGTD-P is a hypoxia-responsive pro-apoptotic protein that transmits hypoxic signals directly to mitochondria. It localizes to mitochondria and facilitated apoptotic cell death via typical mitochondrial apoptotic cascades, including permeability transition, cytochrome c release, and caspase 9 activation. However, much is unknown about post-transcriptional modification and signaling networks of HGTD-P in association with cell death-regulating proteins. We performed this work to elucidate the effects of the pro-apoptotic protein HGTD-P on neuronal cell death induced by hypoxia and to investigate the cell death mechanisms activated during this process. In this study, we show that mouse HGTD-P(mHGTD-P) is transcriptionally increased by hypoxia and that its overexpression triggers neuronal cell death with affected cells displaying shrunken cytoplasm neuronal cells from hypoxic injury. Taken together, our data show that mHGTD-P induces the mitochondrial release of apoptosis-inducing factor into the cytoplasm. Finally, we suggest that mHGTD-P participates in caspase-independent hypoxic neuronal cell death.

Keywords: HGTD-P, Neuron, Hypoxia, Apoptosis, Apoptosis-inducing factor

JNK is activated but does not mediate hippocampal neuronal apoptosis in experimental neonatal pneumococcal meningitis

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Pneumococcal meningitis is associated with caspase 3-dependent apoptosis of recently post-mitotic immature neurons in the dentate gyrus of the hippocampus. The death of these cells is implicated in the learning and memory deficits in patients surviving the disease. The stress-activated protein kinase c-Jun N-terminal kinase (JNK) has been shown to be an important mediator of caspase 3-dependent neuronal apoptosis. However, whether JNK is involved in hippocampal apoptosis caused by pneumococcal meningitis has not been investigated so far. Here we show in a neonatal rat model of pneumococcal meningitis that JNK3 but not JNK1 or JNK2 is activated in the hippocampus during the acute phase of infection. At the cellular level, JNK3 activation was accompanied in the dentate gyrus by markedly increased phosphorylation of its major down-stream target c-Jun in early immature (Hu-positive) neurons, but not in migrating (doublecortin-positive) neurons, the cells that do undergo apoptosis. These findings suggested that JNK may not be involved in pneumococcal meningitis-induced hippocampal apoptosis. Indeed, although intracerebroventricular administration of D-JNKI-1 or AS601245 (two highly specific JNK inhibitors) inhibited c-Jun phosphorylation and protein expression in the hippocampus, hippocampal apoptosis was unaffected. Collectively, these results demonstrate that JNK does not mediate hippocampal apoptosis in pneumococcal meningitis, and that JNK may be involved in processes unrelated to apoptosis in this disease.

Keywords: c-Jun N-terminal kinase, neuronal apoptosis, brain development

Contribution of autophagy to drug-induced death of malignant glioma cells

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Autophagy is a cellular process in which portions of cytoplasm and intracellular organelles are sequestered within double-membrane vesicles known as autophagosomes before delivery to lysosomes for degradation. It has been proposed that autophagy is involved in cell survival or cell death. In many conditions apoptosis and autophagy can both contribute to cell elimination. The relationship between autophagy and apoptosis is especially complex as both are induced by similar stimuli and impact on each other's functions. The mammalian target of rapamycin (mTOR) is involved in the control of cancer cell metabolism, growth and proliferation but also can control programmed cell death type I (apoptosis) or type II (autophagy).

We have previously demonstrated that cyclosporin A (CsA), an immunophilin ligand and calcineurin inhibitor, induces growth arrest or cell death of cultured glioblastoma cells and inhibits tumor growth in vivo. CsA-induced cell death in rat C6 glioma cells was accompanied by caspase 3 activation followed by PARP cleavage, DNA fragmentation, although these hallmarks of apoptosis were not so strong as after UVC irradiation or adriamycin treatment. Moreover, CsA triggers programmed cell death with the appearance of cytoplasmic vacuoles in human malignant glioma cells. Therefore, in this study we investigated whether CsA induces nonapoptotic autophagic cell death in malignant glioma cells.

We found that CsA-induced cell death was companied by several features characteristic of autophagy: development of the acidic vesicular organelles, autophagosome membrane association of microtubule-associated protein light-chain 3 (LC3), and a marked increase in expression of levels of LC3-II in some malignant glioma cells. We found that CsA treatment affetcts mTOR/p70S6 kinase pathway decreasing p70S6K phosphorylation. Further, the inhibitory effect of CsA on the phosphorylation of 4EB-P1 (eukaryotic initiation factor 4E-binding protein 1) and S6 ribosomal protein (two downstream target molecules of p70S6K) was observed. The presented results demonstrate that CsA induces autophagic cell death in malignant glioma cells via inhibition of mTOR/p70S6K pathway and show possibility to induce nonapoptotic autophagic cell death in malignant gliomas bearing numerous defects in apoptotic pathway.

Keywords: autophagy, glioma, cyclosporin A

Presenting Author: Saskia Cillessen

A subset of chemotherapy-refractory diffuse large B-cell lymphomas is characterized by constitutive activation of the intrinsic apoptosis pathway

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Inhibition of the apoptosis cascade is an important cause of therapy resistance in diffuse large B-cell lymphomas (DLBCL). In this study, we investigated the functionality of the intrinsic apoptosis pathway in lymphoma cells of thirty DLBCL biopsies. Two DLBCL groups were identified using RT-Multiplex Ligation dependent Probe Amplification (RT- MLPA) analysis; one with low expression levels of both pro- and anti-apoptotic genes and one group with high expression levels of these genes. DLBCL with high expression levels of pro- and anti-apoptotic genes frequently appeared to be refractory to clinical chemotherapy. Functional analysis in these latter DLBCL samples and DLBCL cell lines with comparable expression profiles revealed high levels of spontaneous caspase 9 activity, mitochondrial membrane depolarization and release of cytochrome c in the cytosol, without induction of apoptosis, indicating disruption of the apoptosis pathway downstream of caspase 9 activation. Furthermore, high levels of p53 expression were found in most of these DLBCL patient samples and DLBCL cell lines. Upstream inhibition of the intrinsic pathway with a p53-inhibitor resulted in a decrease in caspase 9 activity in DLBCL cell lines.
We conclude that the intrinsic caspase 9-mediated apoptosis pathway may be constitutively activated with concomitant downstream inhibition of the convergence apoptosis pathway in chemotherapy-refractory DLBCL. Constitutive caspase 9 activation might be caused by stabilization of p53 expression.

Keywords: lymphoma, intrinsic apoptosis pathway, chemotherapy-resistance

ML-10 - A novel fluorinated small-molecule probe for apoptosis

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Apoptosis has an important role in numerous medical disorders, and respectively, targeting of apoptotic cells may substantially advance patient care. Novel, low molecular weight fluorinated detectors of apoptosis may be beneficial as potential positron emission tomography (PET) probes for clinical imaging of apoptosis in vivo. In the development of such probes, we focused on the unusual amino acid gama-carboxyglutamic-acid (Gla), which plays a vital role in the binding of clotting factors to negatively-charged phospholipid surfaces, as encountered in apoptotic cells. Based on the alkyl-malonate motif of Gla, we have developed and now present ML-10 [2-(5-fluoro-pentyl)-2-methyl-malonic-acid, MW=206 Da], the prototypical member of a novel family of small-molecule detectors of apoptosis. ML-10 is responsive to the apoptotic hallmarks of irreversible membrane depolarization and cytosolic acidification, occurring early in apoptosis, while membrane integrity is still preserved. Accordingly, ML-10 crosses the membrane of the apoptotic cell from the early stages of the death process, and accumulates within the cytoplasm. ML-10 was found to be specific for apoptotic cells, while being excluded from either viable or necrotic cells. ML-10 uptake was also found to correlate with caspase activation, Annexin-V binding and disruption of mitochondrial membrane potential. A crucial role for the malonate moiety in ML-10 function as an apoptosis detector was also observed. ML-10 is therefore the most compact apoptosis probe known to date. Being a fluorinated compound, ML-10 is amenable for radio-labeling with the 18F isotope, towards future use for clinical PET imaging of apoptosis.

Keywords: apoptosis probe, molecular imaging, membrane potential depolarization, intracellular acidification, Gla domain

Presenting Author: Sébastien Conus

Caspase-8 is activated by cathepsin D initiating neutrophil apoptosis during the resolution of inflammation

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In the resolution of inflammatory responses, neutrophils rapidly undergo apoptosis. We describe a new proapoptotic pathway in which cathepsin D directly activates caspase-8. Cathepsin D is released from azurophilic granules in neutrophils in a caspase-independent but reactive oxygen species-dependent manner. Under inflammatory conditions, 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 that is in addition to their role in bacterial defense mechanisms: to regulate the life span of neutrophils and, therefore, the duration of innate immune responses through the release of cathepsin D.

Keywords: apoptosis, azurophilic granules, caspase-8, cathepsin D, inflammation, neutrophils, reactive oxygen species, septic shock

Presenting Author: Carolina Costa-Ramos

Studies on phagocyte apoptosis induced by AIP56, a novel virulence factor of Photobacterium damsalae subsp. piscicida

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Apoptosis is an evolutionary conserved, genetically controlled process of cell death, which plays an important role in several physiological processes but which can also be modulated by pathogens to subvert the host immune defences. This is the case in the sea bass; one of the most economically important marine fish species, where apoptosis of both professional phagocytes, macrophages and neutrophils, is induced by AIP56, an exotoxin of 56 kDa secreted by the pathogen Photobacterium damselae ssp. piscicida (Phdp). Using an ex-vivo model, this apoptotic process has been characterized and found to involve both the extrinsic pathway, with activation of caspase-8 and the intrinsic pathway with activation of caspase-9, changes in mitochondrial membrane potential, cytochrome c translocation from mitochondria and also an increased production of ROS. A better understanding of this pathogenicity mechanism, in which bacteria use exotoxins to destroy the phagocytic cells of the host by inducing an apoptotic cell death, will lead to a greater knowledge on specific host-pathogen interactions which will allow for more effective design of preventive vaccines.

Keywords: Bacterial toxins, phagocytes, apoptosis

Presenting Author: Andrew Cox

Apoptosis induced by the thioredoxin reductase inhibitor auranofin is regulated by the Bcl-2 family: Role of peroxiredoxin 3 oxidation and mitochondrial stress

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The peroxiredoxins (Prxs) are a family of abundant thiol peroxidases that decompose H2O2. Catalytic activity involves the formation of disulfide-linked dimers that are reduced by the thioredoxin network. Due to their abundance and high reactivity with H2O2 [1], Prxs are increasingly being recognised as crucial players in redox signalling, including the regulation of events leading to apoptosis. We have previously demonstrated that mitochondrial Prx3 becomes oxidised during the initiation of receptor-mediated apoptosis [2]. In the current study we assessed the impact that inhibiting the thioredoxin network has on mitochondrial Prx3 and the induction of apoptosis. We assessed the redox state of the Prxs in Jurkat T-lymphoma cells treated with auranofin, a gold (I) compound that potently inhibits thioredoxin reductase and triggers apoptosis by an as yet undefined mechanism. Mitochondrial Prx3 was considerably more sensitive to oxidation than the cytosolic Prx1 and 2, indicating selective mitochondrial stress. Prx3 oxidation was detected at apoptotic doses of auranofin in several cell types, and occurred before other mitochondrial events including cytochrome c release and mitochondrial depolarisation. Auranofin was also able to sensitise U937 cells to TNF-α mediated apoptosis. Auranofin-induced apoptosis was effectively blocked by the overexpression of Bcl-2, and Bax/Bak deficient mouse embryonic fibroblasts were resistant to apoptosis, indicating a central role for the pro-apoptotic proteins of this family in auranofin-triggered apoptosis.

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Keywords: peroxiredoxin, apoptosis, auranofin

Influence of BCR-ABL expression on cell death mechanisms in haematopoietic cells.

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Chronic Myeloid Leukaemia (CML) is a biphasic disease. The initial chronic phase is often controlled with drugs such as Imatinib/Glivec. The disease can then progress to a more aggressive blast crisis that is highly resistant to both Imatinib and DNA targeting drugs. A major feature of both initial Imatinib resistance and disease progression is elevated expression of the BCR-ABL oncogene. Bcr-Abl has been reported to be anti-apoptotic. However, the effect of Bcr-Abl on other cell death mechanisms remains unknown, and needs to be established in order to fully understand the molecular basis for drug resistance.

We are investigating the influence of Bcr-Abl expression on cell death mechanisms. We have employed two independent cell line models with variable expression of Bcr-Abl. The 32D mouse myeloid cell line was transfected with BCR-ABL and two clones were established that express low (Clone 2) and high levels of BCR-ABL (Clone 4). The second cell line is the TonB.210 cells which express a tetracycline (Tet) inducible BCR-ABL gene. We can also induce this cell line to produce low or higher expression levels of Bcr-Abl. We have used these models evaluate the influence of Bcr-Abl expression on cell death induced with a DNA damaging drug VP16/etoposide, over 24 hours. In addition, we have examined the long term (48- 96 hours) ability of drug treated cultures to recover.

In cells without Bcr-Abl expression (32D & uninduced TonB.210) two morphologies are evident in drug treated cultures. Apoptosis is clearly visible and caspase activity is detectable (using active caspase 3 antibody staining). The second morphology consists of highly vacuolated cells, with intact nuclei, and resembles the morphology described for autophagy. We are currently evaluating typical markers for this process (LC3 cleavage /MDC staining). Following drug removal, these cultures recover from drug treatment. During recovery, cells are swollen, highly vacuolated and display abnormal nuclei. The majority of cells however return to their original morphology after a further 96hrs.

When cells expressing low levels of Bcr-Abl (C2 & TonB0.2ug Tet) are treated with VP16, apoptosis is pre-dominant, and caspase activity is also evident. These cultures cannot recover, and have lost viability within 48hr of drug removal. In cells with elevated levels of Bcr-Abl (C4 and 2.0ug Tet) there is an absence of apoptotic morphology, consistent with low levels of caspase activity. The cells display a marked elevation in the number of vesicles. Previous work from this group has shown that C4 cells are resistant to apoptosis and initially preserve a more intact plasma membrane (as shown by propidium iodide staining). We now show that these cells can recover from drug treatment.

Effective chemotherapy requires cancer cells to be more sensitive to drugs than normal cells. Our data indicates that cells expressing a low level of Bcr-Abl (which resembles chronic phase) are more susceptible to drug treatment than normal cells – this is associated with the induction of primarily an apoptotic phenotype. However when cells express elevated Bcr-Abl, the predominance of non-apoptotic phenotype severely limits cell death induction and permits recovery of the leukaemic cells. This process may play a major role in the ineffectiveness of treatment in patients expressing elevated Bcr-Abl.

Keywords: Cancer, Chronic Myeloid Leukaemia, Bcr-Abl, apoptosis

Presenting Author: Carsten Culmsee

Bid induces caspase-independent neuronal cell death by the rapid release of apoptosis inducing factor (AIF) from mitochondria to the nucleus

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Mitochondrial dysfunction and release of pro-apoptotic factors such as cytochrome c or apoptosis inducing factor (AIF) from mitochondria are key features of delayed neuronal cell death after acute brain injury and in neurodegenerative diseases. The precise mechanisms regulating mitochondrial demise, the release of pro-apoptotic mitochondrial proteins and their particular role in neuronal cell death signaling, however, are largely unknown.
Here, we demonstrate that 8-10 hours after induction of glutamate toxicity AIF translocates from mitochondria to the nucleus in primary neurons and HT-22 neuronal cells. Using an AIF-GFP construct in video microscopy studies we demonstrate that AIF translocation occurs in a rapid manner and induces nuclear fragmentation and cell death within only a few minutes in HT-22 neurons. This markedly fast translocation of AIF to the nucleus is preceded by increasing translocation of the pro-apoptotic bcl-2 family member BH3-interacting domain death agonist (Bid) to mitochondria, perinuclear accumulation of Bid-loaded mitochondria, and loss of mitochondrial membrane integrity. Bid siRNA or a small molecule Bid inhibitor preserved mitochondrial membrane potential, prevented nuclear translocation of AIF, and abrogated glutamate-induced neuronal cell death. In HT-22 cells and primary neurons, AIF siRNA attenuated glutamate induced cell death. In contrast, activation of caspase-8 or caspase-3 was not detectable or occurred very late at 18 h after glutamate exposure, respectively. Moreover, inhibitors of caspase-2, -3, -or -8, calpain inhibitors, and cathepsin inhibitors failed to prevent glutamate toxicity. Notably, cell death induced by tBid was inhibited by AIF siRNA indicating that caspase-independent AIF signaling is the main pathway through which Bid mediates cell death. It is important to note that Bid inhibition and AIF siRNA failed to prevent HT-22 cell death after staurosporine treatment, an experimental paradigm of programmed cell death that involves early caspase activation but not mitochondrial AIF release to the nucleus.
In conclusion, Bid-mediated mitochondrial release of AIF followed by rapid nuclear translocation is a major mechanism of glutamate-induced neuronal death. These data expose Bid and AIF as key factors in caspase-independent neuronal cell death that may play a major role in prominent neurological diseases such as ischemic stroke and brain trauma.

Keywords: mitochondria, Bid, AIF, neuronal death, glutamate

Presenting Author: Nicolas Dejeans

Ascorbate-driven menadione-redox cycling induces an oxidative stress that affects signal transduction pathways and leads to MCF7 cell death.

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Several new approaches are designed to target specifically the members of the ERK MAPK pathway which is associated with tumour proliferation and bad prognosis. Here we describe that an oxidative stress generated by ascorbate-driven menadione redox cycling (ascorbate/menadione) leads to the formation of reactive oxygen species (ROS), among them hydrogen peroxide (H2O2). This oxidative stress provokes ERK and RAF deactivation, does not modify JNK but activates p38, prior to induce cell death in MCF7 breast cancer cells. In addition, ROS induce DNA damage within MCF7 cells as shown by the increasing amounts of poly-ADP-ribosylated proteins and the appearance of ƒ×-H2AX during the first hours of incubation. The oxidative stress by ascorbate/menadione also inhibits the glycolysis pathway leading to a strong decrease in the rate of lactate formation and to a depletion of both NAD and ATP levels. Reinforcing a major role of oxidative stress in the cytotoxicity by ascorbate/menadione, the addition of N-acetylcysteine into cell cultures suppresses MCF7 cell death, while the addition of 3-aminotriazole (a well-known catalase inhibitor) results in an increase of cell death. Since ascorbate is preferentially taken up by cancer cells (rendering favourable an in situ ascorbate-driven menadione redox cycling) and these cells are deficient in antioxidant enzymes, we conclude that oxidative stress should affect cancer cells rather than normal cells. The consequences of this sensitivity may have important clinical applications.

Keywords: Ascorbate, menadione, oxidative stress, cell death, cancer

Presenting Author: Araceli Diez-Fraile

The extracellular calcium-sensing receptor in avian granulosa cells: a key to survival during folliculogenesis

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Introduction: Calcium influx through plasma membrane channels or release from the endoplasmic reticulum plays a central role in the initial stimulus for apoptosis in different cell systems (Berrigde et al., 2003). However, the role of intracellular calcium in the development of apoptosis is ambiguous. While elevation of intracellular calcium may act as a trigger for cell death in rat thymocytes (McConkey et al., 1989), it was found to be a defense mechanism against apoptosis in cerebellar granule cells (Galli et al., 1995). In our hands, addition of calcium into the medium was able to elicit antiapoptotic as well as proapoptotic effects when acting as first or second messenger, respectively, in quail granulosa explants (Mussche et al., 2000). Interestingly, increasing concentrations of extracellular calcium were able to attenuate granulosa cell (GC) apoptosis in a dose-dependent manner upon 24 h of culture, suggesting a receptor-mediated effect (Mussche et al., 2000).
Objective: The current study aimed at investigating the possible involvement of the calcium-sensing receptor (CaR) in quail GC survival signalling.
Methods: The presence of the CaR at the GC surface of quail (Coturnix coturnix japonica) follicles was assessed by western blot and immunohistochemistry. The effect of the polyvalent cations calcium and magnesium, the polycationic molecule spermine, and the positive allosteric modulator R-568 on apoptosis was evaluated in serum-deprived F1 granulosa quail explants after addition of C8 ceramide or LY294002 for 24 h at 38ºC. Fluorescence microscopy was used to quantify the percentage of apoptotic cells upon nuclear staining with DAPI.
Results: The CaR was identified as a 130 to 140 kDa protein expressed in quail follicles selected for ovulation and in cultured granulosa explants. No immunoreactive signal was evidenced at the cohort of previtellogenic or small vitellogenic follicles. Addition of the different CaR agonists caused inhibition of apoptosis elicited by gonadotropin withdrawal or C8 ceramide. Incubation in the presence of LY294002 elicited GC apoptosis, indicating that the PI3-K pathway is involved in GC survival. However, LY294002–induced apoptosis could be attenuated by incubation with CaR agonists suggesting that the PI3-K pathway is not the major survival system activated through the CaR.
Conclusions: This report shows the first direct evidence of the presence of CaR in preovulatory granulosa explants and suggests a pivotal regulatory role of the CaR in follicle selection.

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Keywords: Calcium-sensing receptor, survival signalling, apoptosis, granulosa explants, avian

The dialoge between apototic cells and phagocytes

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The phagocytosis of apoptotic cells is mediated by specific epitopes, eat-me signals, that are exposed on the surface of the death cells, allowing their removal. Differently from the phagocytosis of necrotic cells, the phagocytosis of apoptotic cells is peculiar not allowing inflammatory and autoimmune responses. On the other hand defective or delayed clearance of apoptotic cells gave rise to pathologies like Lupus eritematotus, fibrosis cystic, obstructive pulmonary disease etc. The exposure of eat-me signals only on the surface of apoptotic cells could not, however, be sufficient for their fast and safe removal. Find-me signals secreted by the apoptotic cells allow phagocytes to come to apoptotic cells. Then, eat-me signals allow to recognize them and, finally, the intracellular signaling elicited by the ligand-receptor binding allow the engulfment and digestion of corpses. Thus, the machinery for apoptotic cell clearance is comprised by phagocyte receptors and engulfing signaling on one side and by secreted and exposed molecules from dying cells on the other.
Aim of our work has been the identification of the find-me signals released from apoptotic cells and the evaluation of their effect(s) on the phagocytes. To these purposes in vitro cultures of macrophagic cell lines at different stage of maturation, i.e. pro-monocytic U937 cells, monocytic THP-1 cells and Raw 264.7 macrophages were challenged with apoptotic cells in phagocytosis assays. Raw 264,7 macrophages were able to internalize apoptotic cells. U937 and THP-1 cells cannot engulf apoptotic cells, unless they were differentiated with phorbol ester (TPA for three days). Different cell types (U937, THP1, lymphocytes etc.) were induced to apoptosis with CHX 10 mM for 18 h followed by 4 h incubation in fresh medium. At the end of recovery the culture medium was analyzed for identification of released signal molecules as well the medium of the phagocytosis assays. NMR analysis showed that apoptotic cells released many molecules of diverse nature into the culture medium during the 4h of phagocytosis assay and recovery time. Many aminoacids were found in the medium; particularly high was the concentration of phenylalanine. Surprisingly, incubation of U937 or THP-1 cells with apoptotic cells or with conditioned medium induced time-dependent cell death in 50% of U937 cells and 25% of differentiation. While in the literature has been reported a pro-apoptotic effect of high phenylalanine concentration, it is the first time that the ability to induce macrophage differentiation of the molecules released by apoptotic cells is reported. The release of find-me signals from apoptotic cells has been investigated in phagocytosis assays in which a porous membrane separated an upper chamber containing Raw 264.7 macrophages by the below chamber containing apoptotic cells. Macrophages moved toward apoptotic cells by passing trough the porous of the membrane to reach and phagocyte the dead cells.
All together our data indicate a complex crosstalk between apoptotic cells and phagocytes, whose significance is not yet fully deciphered.

Keywords: phagocytosis, find-me signals, chemotaxis

GSH content in the vulnerable brain regions of Wistar rats intrastiratally poisoned with paraquat and diquat

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Paraquat (PQ) and diquat (DQ), widely used contact herbicides of bipyridinium structure, exert their toxicity through free radical production. During their aerobic metabolism produced superoxide anion radical initiates chain radical reactions which contribute oxidative stress (OS) development. Recently, PQ neurotoxicity attracts attention of researches, because after inhalation route of PQ administration (the most frequent one) target organs are lungs and kidneys, while convulsions observed in PQ poisoning are of less toxicological importance. Target organs for DQ poisoning are liver and kidneys and there is almost nothing in literature about its neurotoxicity.
The aim of the study was to investigate if depletion of glutathione (GSH), the most important endogenous antioxidants occurred after acute intrastriatal (i.s.) poisoning with PQ and DQ.
The experiment was conducted on Wistar rats (both sexes, 11 weeks old) divided in three groups: control group (5 animals) – intact untreated group (0 time); PQ group (24 animals) - i.s. poisoned with PQ (50 mg/kg) and DQ group (24 animals) - i.s. poisoned with DQ (50 mg/kg).
GSH was spectrophotometrically determined in mitochondrial crude fraction of ipsi- and contra- lateral side of vulnerable brain regions (VBR) (striatum, hippocampus, cortex), 30 min, 24 h and 7 days after the treatment (for each time point 8 animals were sacrificed).
Measured control values of GSH (nmol/mg prot) in examined brain structures are very closed: cortex - 16.84 ± 4.927; striatum - 17.3 ± 4.416 and hippocampus 15.47 ± 3.519.
Significant GSH depletion occurred 24 hours after PQ poisoning (~ 25 % of controls) in both sides of all examined VBR, unlike elevated GSH values were observed at 7 day (~ 135 % of controls). Within the first 30 minutes in all examined VBR of rats poisoned with DQ, GSH decreased without significance, while in striatum, 24 hours after the poisoning GSH were significantly low (~ 80 % of controls). Later on, measured GSH levels were closed to controls. Mortality of the rats poisoned with DQ was distinguished (20 % of poisoned animals) immediately after the poisoning, unlike PQ, where reversible Parkinson like symptoms were observed in the same time.
Determined GSH values do not differ between VBR within certain measurement time points.
Different dynamics of GSH changes in VBR of Wistar rats obtained after poisoning with PQ and DQ emphasise diverse neurotoxic mechanisms, although both of them exert their toxicity through free radical production. More severe symptoms were achieved immediately after the DQ poisoning, while remarkable OS followed by significant GSH depletion were observed 24 hour after PQ poisoning, what are in accordance with literature. Obviously, GSH cycle is more involved in antioxidative defence from PQ free radical mediated toxicity.

Keywords: paraquat, diquat, neurotoxicity, glutathione, oxidative stress

Antioxidant SkQ1 leads to morphology differentiation of various transformed cells and protects mitochondria from H2O2 induced fragmentation

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We investigated new mitochondria-targeted antioxidant SkQ1 that scavenges ROS in mitochondria of various cells at nanomolar concentrations. We used 10/3 and 10/3-ras mouse fibroblast cell lines, SiHa human cervical carcinoma, MRC5 human lung fibroblasts and their SV-40 -transformed counterparts MRC5-V2, and human subcutaneous fibriblasts. Morphometry method, confocal microscope, immunofluorescence staining were used to investigate cell morphology, cytoskeleton and mitochondrial system. Western analysis was used for the protein quantification. It was shown that under protection of SkQ1 transformed epithelial SiHa cells showed deceleration of proliferation rate and the normalization of morphological state: actin bundles were present as a circumferential rings around cells and E-cadherin- and beta-cathenin-positive cell-cell contacts characterized differentiated epithelial phenotype. SkQ1 treatment of Ras- and SV-40-transformed fibroblast cell lines caused restoration of normal fibroblastic morphology: re-appearance of stress fibers and focal adhesion contacts, and enlargement of cell area. The changes of actin system in SkQ1-treated cells were connected with increase in the portion of TGF-beta and phosphorylated cofilin. SkQ1 treatment protected the mitochondria from H2O2-induced fragmentation. Antioxidant protection of mitochondria was critical for normalization of transformed cells. Possible mechanisms of observed alterations will be discussed.

Keywords: antioxidant, transformation, cytoskeleton, mitochondria,cell culture

Phagocytosis substrate specificity of macrophages

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Macrophages are professional phagocytes. They are able to engulf a wide variety of substrates. They remove foreign substances and thus fight infection. They also participate in the efficient clearance of dying cells. The regulation of their phagocytic activity is thus important for physiological processes ranging from immunity to the the maintance of tissue homeostasis.
It is well established that macrophage is a heterogeneous cell type; different substrates are often phagocytosed by only a subset of these cells. However, little is known about the molecular mechanisms that allow the macrophage to decide whether a recognized particle is taken up or not.
We have labeled murine apoptotic cells, a variety of microorganisms and latex beads with various fluorescent molecules. We have added these labeled phagocytosis substrates in various concentrations to both murine macrophages differentiated ex vivo from bone-marrow cells and to thioglycolate elicited peritoneal macrophages; and measured the percentage of cells engulfing the labeled substrate using flow cytometry and fluorescent microscopy. We also added the various substrate categories in combination at non-saturating concentrations, and determined whether the uptake of one macrophages phagocyte one or more class of substrates at the same time. We proved that heterogeneity of macrophages for different substrates exists. Investigation on the molecular explanation of this phenomenon is still in progress.

Keywords: phagocytosis, macrophages heterogenity

Presenting Author: Zsuzsanna Dunai

Pharmacological characterization of necrosis-like cell death forms in U937 human lymphoma cell line.

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Until recently necrotic cell death was considered as a consequence of cell injuries that resulted in an irreversible energetic failure of the cell. Accumulating evidences indicate that necrotic cell death can be a genetically regulated event and can be classified as programmed cell death in line with apoptosis. A bunch of studies support the suggestion that distinct necrosis-like cell death types can be dissected on the basis of activation of divergent (convergent?) biochemical pathways.
Most recently, after PARP-mediated or autophagy-promoted necrotic processes, necroptosis was described as death receptor-initiated, RIP1 protein kinase-mediated necrosis-like cell death form. Simultaneously, a potent new pharmacon, necrostatin-1 was discovered that was suggested to halt necroptosis by inhibiting RIP1 kinase activity. Necrostatin partially protected neuronal and cardiac cells from ischemia-reperfusion-induced damage additively with caspase inhibitors.
In our study we dissected the necrotic mechanisms in U937 human lymphoma cells exposed to h. r. TRAIL –50 ng/ml- for death receptor while and staurosporine -1 µM- for mitochondria mediated death pathways. Specific chemical inhibitors were applied to reveal the (necrosis relevant) attendant molecules activated by these stimuli: caspase inhibitor (Z-VD-fmk, 5 µM), cathepsin inhibitor (CA-074-Me, 10 µM), autophagy inhibitor (3-methyladenine, 10 mM), PARP inhibitor (PJ-34, 1 µM) and RIP1 kinase inhibitor (necrostatin-1, 10 µM). Necrosis (plasma membrane damage) was detected by propidium iodide staining and flow cytometry. In our U937 cell model we found that
1. Both TRAIL and staurosporine activated apoptotic processes (caspase activation, DNA fragmentation and condensed cell morphology) followed by secondary necrosis. PARP inhibitor reduced secondary necrosis for both stimuli without reducing apoptosis. Neither necrostatin-1 nor CA-074 was effective on, while 3-methyladenine enhanced both processes.
2. Caspase inhibitor halted apoptotic processes but allowed necrosis to proceed. In caspase inhibited U937 cells PARP inhibitor did not reduced necrosis for TRAIL or staurosporine.
3. TRAIL induced pure necroptosis in caspase-compromized cells as necrostatin-1 protected necrosis for up to 20 hours. CA-074 had the similar effect at concentration that was much higher than required for cathepsin B inhibition in these cells.
4. Staurosporine induced more divergent necrotic processes in caspase-compromized cells: necrostatin-1 fully reduced early necrosis at 8 hours but only partially at later (50% at 20 hours). Autophagy inhibitor also reduced necrosis at 20 hours and was additive with necrostatin-1. CA-074 fully protected cells from necrosis and mitochondrial breakdown as well detected by DiOC6(3) staining.
In conclusion, our results indicate that in U937 cells i) the signaling pathway leading to secondary necrosis is different from necrotic pathway initiated the same stimuli in caspase-compromised cells, ii) staurosporine can induce several distinct pathways of necrosis-like cell death in parallel; one of them is possibly mediated by autocrine death receptor signaling eliminated by necrostatin-1. iii) Necroptotic and autophagic necrosis pathways have a common, cathepsin-like, CA-074 sensitive mediator that is likely not cathepsin B.
This work was supported by OTKA T049008

Keywords: necrosis, necroptosis, programmed cell death, cancer, flow cytometry

Construction of a new oncolytic adenoviral vector with doxycycline-inducible expression of CD95L: Efficient combination of selective oncolysis and apoptosis induction in melanoma cells

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No suitable therapy is available for metastasized melanoma, thus characterized by high mortality rates. Apoptosis deficiency is considered as a critical factor for therapy resistance. In previous work, we have shown that TNF-related death ligands (CD95L/FasL and TRAIL) can induce apoptosis and enhance chemosensitivity of cultured melanoma cells, and CD95L could prevent melanoma growth in a nude mouse model. For preparing gene therapeutic strategies, we constructed a conditional replication-competent, oncolytic adenoviral vector (Ad5-FFE02), in which E1A and thus selective adenoviral replication is driven by a melanoma-specific tyrosinase promoter, and CD95L expression is triggered by a doxycycline-inducible promoter. As a further step addressing a safe application, a mutated variant of E1A was used, which lacks the pRB binding site and thus allows replication preferentially in tumor cells.
The efficiency and selectivity of gene expression as well as induction of apoptosis and oncolysis by this construct was analysed in a representative panel of melanoma cell lines and compared to several non-melanoma cell lines. Replication-competent adenovectors without CD95L and replication-deficient adenoviral vectors encoding for luciferase were used as controls.
Applying Ad5-FFE02, after infection and induction with doxycycline, efficient induction of apoptosis was the result in tyrosinase-positive and CD95L-sensitive melanoma cell lines, but not in tyrosinase-negative or CD95L-resistant cells, and particularly there was no effect in non-melanoma cell lines. Protein analyses revealed highly selective expression of E1A and selective induction of CD95L in melanoma cells. Four days after infection, substantial oncolysis was found in tyrosinase-positive melanoma cells, whereas non-melanoma cell lines showed no response. These data prove the high selectivity of tyrosinase promoter-driven adenoviral vectors in melanoma cells as well as the high efficacy of the encoded CD95L. Selective replication-competent adenoviral vectors offer novel therapeutic strategies, which may enable the use of proapoptotic genes in therapeutic approaches for melanoma.

Keywords: Cancer, gene therapy, CD95L, oncolytic adenovirus

The Novel Viral Homologue Ov9 Induces Apoptosis in COS-7 Cells

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The ovine herpesvirus 2 (OvHV-2) belongs to the family Herpesviridae (subfamily Gammaherpesvirinae) and is known as the causative agent of the sheep-associated malignant catarrhal fever (SA-MCF), a generally fatal lymphoproliferative disease affecting animals such as pig, cattle, bison and deer (Ackermann, 2005).
In a previous study, the OvHV-2 open reading frame (ORF) Ov9 was partially characterized (Laimbacher, 2006).The prediction based on the amino acid sequence of Ov9 revealed a Bcl-2 homology, consisting of the Bcl-2 homology domain BH1 and the conserved C-terminal transmembrane domain. The Ov9 ORF was cloned in frame with a fluorescent marker (EYFP) to be expressed from a herpes simplex virus-1 (HSV-1) based amplicon vector. Using confocal laser scanning microscopy, Ov9 was shown to localize to mitochondria.
In order to test whether Ov9 plays a role in the regulation of the mitochondrial apoptotic pathway, a flow cytometry based assay for apoptosis with Annexin V-Cy3 and Propidium Iodide (PI) was established. COS-7 cells were transduced with HSV-1 amplicon vectors containing the Ov9-EYFP and, as control, EYFP without Ov9. Cells were tested at 15h to 30h post-transduction. Interestingly, Ov9 did induce apoptosis in transduced COS-7 cells. Moreover, those dying COS-7 cells displayed fragmented and swelled mitochondria in time lapse experiments using live confocal laser scanning microscopy. These changes are typical signs of outer mitochondrial membrane permeabilisation, a crucial event of the mitochondrial apoptotic pathway.
In conclusion, Ov9 represents a novel variant of viral Bcl-2 homologues, which possesses only the BH1 domain and induces apoptosis without having a BH3 domain. The further characterisation of Ov9 could be a useful tool to gain insights into novel mechanisms of the intrinsic apoptotic pathway. SA-MCF is associated with untimely and uncontrolled multiplication of OvHV-2-infected lymphocytes, which simultaneously overexpress Bcl-2. Since Ov9 expression has not been detected in those cells, it may be hypothesized that Ov9 counteracts the function of Bcl-2 in animals which do not develop the disease and, hence, the absence of Ov9 could explain the uncontrolled multiplication of lymphocytes during MCF.

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Keywords: viral Bcl-2 homologue, apoptosis, Ov9

Presenting Author: Sylvie Eigeldinger-Berthou

Rasagiline reduces apoptotic photoreceptor cell death in the Prph2/rds mice

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Purpose: The Prph2/rds mouse, a well characterized animal model for retinitis pigmentosa (RP), was used to evaluate the neuroprotective potential of rasagiline, a novel second-generation of propargylamine compounds, which exerts a dose-dependent anti-apoptotic effect in neurons. Rasagiline acts principally by modifying activation of caspase-3 and expression of Bcl-2 family members. To assess the effect of rasagiline on the pathogenic mechanisms causing retinal degeneration in the Prph2/rds mouse, we examined morphologically and biochemically the time course of photoreceptor cell (prc) death from post-natal day 10 until post-natal day 56.
Methods: Animals were treated orally with various doses of rasagiline (2, 5, 25, 50 mg/kg/day) starting at post-natal day 1 (P1) till euthanasia at post-natal day 10, 14, 18, 28 and 56 (P56). Morphological approaches consisted in H&E staining and morphometric analysis of the outer nuclear layer (ONL) thickness of the neuroretina. Apoptotic cells were identified during retinal development and degeneration by TUNEL-assay, immunoblotting and immunohistochemistry.
Results: Differences in apoptotic events between Prph2/rds and wild type mice were already apparent at P14. Between post-natal day 14 and 18, cleaved caspase-3 was detected in the ONL of Prph2/rds mice, but was absent in control mice. Oral treatment with rasagiline was well tolerated even in newborn pups, and results at P56 in an inverse dose dependent prc rescue measurable by an increase of the ONL thickness, which was highest (~18%) and highly significant at the concentration of 2 mg/kg/day ($p < 0.01$, Kruskal test) in the Prph2/rds mouse. Procaspase-3, -6 and -7 expressions were reduced in the Prph2/rds mouse. In wild type animals, rasagiline drastically reduces caspase activation and increases the expression level of Bcl-2 and Bcl-XL.
Conclusions: Apoptosis is a normal physiological mechanism in the development of the retina. In the Prph2/rds mice, aberrant expression and activation of caspases leads to retinal degeneration. Rasagiline exerts its neuroprotective effect mainly by decreasing the expression level of caspase precursors in the Prph2/rds mice. It also interferes in the normal remodeling of the neuroretina by blocking caspase activation, as seen in the wild type mouse. Rasagiline is neuroprotective in the Prph2/rds degeneration and may in future have a putative role for therapeutic strategies of human retinal diseases, such as retinitis pigmentosa.

Keywords: rasagiline, caspase-3, photoreceptor, apoptosis, rds, neuroprotection

Altered Sub-cellular Localization of Anti-apoptotic Bcl-xL in Murine and Human Cell Models of Chronic Myeloid Leukaemia

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Chronic Myeloid Leukaemia (CML) is a disease of haematopoietic stem cells characterized by the Philadelphia Chromosome that harbours the chimeric gene BCR-ABL. The resultant Bcr-Abl fusion protein is a tyrosine kinase that constitutively activates several signalling pathways. The oncogenic Bcr-Abl tyrosine kinase has been shown to induce expression of the anti-apoptotic protein Bcl-xL, which is considered to be an important factor in resistance to drug treatment of CML.
In this study we demonstrate that, in addition to inducing protein expression of Bcl-xL, Bcr-Abl tyrosine kinase also alters the intracellular distribution of Bcl-xL.
We have developed a murine model of CML in haematopoietic 32D cells. Clones expressing low (C2 cell line) or high (C4 cell line) expression of Bcr-Abl were employed for analysis of Bcl-xL. In addition, we examined the human Bcr-Abl-expressing cell line K562 and compared it to the non-Bcr-Abl-expressing leukemic HL-60 cell line. Firstly, increased expression of Bcl-xL was confirmed in all Bcr-Abl-expressing cell lines (C2, C4 and K562) compared to control cell lines (32D and HL-60, respectively). Secondly, all cell lines (control and Bcr-Abl-expressing cell lines) were subjected to sub-cellular fractionation by sucrose density ultra-centrifugation. The sub-cellular fractions were resolved by SDS PAGE and immunoblot analysis. Antibodies against specific markers for intracellular organelles were used to detect distribution of organelles in the gradients.
Sub-cellular fractionation analysis of the above mentioned cell lines showed that in control cell lines Bcl-xL was detected in cytosolic and mitochondrial fractions, as expected. In contrast, in both murine and human Bcr-Abl-expressing cell lines Bcl-xL was also found to be associated with endoplasmic reticulum (ER) and plasma membrane fractions. To further establish that this altered distribution of Bcl-xL is linked to the oncogenic activity of Bcr-Abl, the effect of Bcr-Abl inhibition on Bcl-xL sub-cellular distribution is currently being investigated.
Indirect but increasing evidence suggests the presence of Bcl-xL at the ER membrane. Our study demonstrates that in control haematopoietic cell lines, Bcl-xL mainly resides in the cytosol and mitochondria. However, in Bcr-Abl-positive cell lines Bcl-xL was found to be associated with the ER and plasma membrane. Placing Bcl-xL at the ER membrane may imply a new function for this anti-apoptotic protein in modulating ER signalling pathways and may provide a novel therapeutic target for treatment of chronic myeloid leukaemia.

Keywords: Bcl-2 family, Bcl-xL, cancer, leukeamia, sub-cellular fractionation, Bcr-Abl, endoplasmic reticulum

Different types of cell elimination in urothelial remodelling during early postnatal bladder development

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The structure of early postnatal urothelium is of a short-term and »transitional« character so cell elimination is involved in tissue remodelling, which finally leads to the terminally differentiated urothelium. In order to find out how urothelial architecture is destroyed, we investigated the urothelium by scanning and transmission electron microscopy, by TUNEL method and by immunodetection of active caspase-3 as a key effector caspase and Beclin-1 as a marker of autophagy. Apoptotic indices were scored on the base of data of quantitative analysis, obtained by results of TUNEL reaction.
C57BL/6JOlaHsd adult male mice older than 8 weeks and mouse offspring of postnatal ages from the day of birth to postnatal day 14 were sacrifised.
During the early postnatal period, three types of urothelial cell elimination were observed: apoptosis, autophagic cell death and desquamation. Apoptosis was detected during all fourteen postnatal days, massive cell desquamation was observed between postnatal days 6 and 9, while occasional superficial cells underwent autophagic cell death accidentally throughout the first fourteen days.
Apoptotic indices revealed that apoptosis was present in the urothelium already on the day of birth and it increased to day 6, where the most intense apoptotic activity was detected during the first fourteen days after birth. After day 6, apoptosis decreased to day 14, whereas in terminally differentiated urothelium of adult mice no apoptotic activity was observed. We detected extruded apoptotic bodies on the urothelial lumenal surface or phagocytosed by cells of different urothelial layers.
Between postnatal days 6 and 9, superficial and intermediate urothelial cells desquamate individually or in groups. Cells, which underwent release from the urothelium, showed no morphological signs of apoptosis, although the majority of them were caspase-3 positive. We assume that cells with activated caspase-3 started to split cell-cell contacts and detach, which precedes the termination of the initial apoptotic pathway. Results of TUNEL method revealed that some desquamated cells also were TUNEL positive, confirming our hypothesis that desquamation is initiated by apoptotic program.
Autophagic cell death of superficial cells was confirmed by intense positive reaction against Beclin-1 and by ultrastructural features like non-apoptotic centrally condensed chromatin, well developed Golgi complex and heavily vacuolized cytoplasm with many autophagic vacuoles.
Our results demonstrate that intense cell death is present during urothelial early postnatal remodelling. Cells mostly die in apoptotic manner, but when fast and intense cell elimination is needed, massive desquamation starts, probably induced by the activation of caspases. The presence of autophagic cell death of individual superficial cells remains to be elucidated in further studies.

Keywords: apoptosis, desquamation, autophagic cell death, urothelium

Identification of apoptotic factors and charaterization of their molecular mechanisms in human cancer cells: new strategies for prevention and treatment of cancer

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Apoptosis is an efficient method for preventing malignant transformation. Dysregulation of apoptosis cause signifecant human diseases and promote cancer development. Fucosing on identifying of apoptotic factors in human cancer cells and understanding their molecular mechanisms may inhance our understanding on the oncology and on the biology of human cancer. Moreover, this may offer new therapeutic stratrgies to exploit agents that inactivate oncogens or activate tumor suppressor genes which could lead to apoptosis in cancer cells.
Human cancer cell lines of colon, breast and prostate were exposed to different dietary indole derivatives (indol-3-carbinol or to 3-3¡¦ diindolylmethane) or natural antioxidents (NAOs) extracted from the leaves of Spinach (S), Inulla viscosae (IV) and Citrullus Colocynyhis (CC). Our results indicated that these factors inhibit proliferation and DNA synthesis of the cancer cells in vitro. Moreover, these factors induced cell cycle arrest and apoptosis. Gene expression analysis indicated that the induction of apoptosis was p53-independent and it was through the mitochondrial pathway by releasing cytochrom C and induction of capase 9 followed by activation of caspase 3 and PARP. In vivo studies indicated that treatment of the animals with these facors three times a week for five weeks, caused a significant deceleration in the volumes and weights of tumors which were induced in C57BL/6 mice, by transplanting the TRAMP-C2 prostate cell line subcunaneously. This effest was found to be mediated by inducing apoptosis as it was detected by morphological and staining studies. Moreover, pre-treatment of animals with pre-apoptotic facors for five weeks before transplanting the TRAMP-C2 cells, significantly reduced tumor development as compared to controls. Tumors were developed in 78% of control and 20-40% of treated animals. The tumors developed in treated animals were significantly (pf¬0.01) smaller than that developed in controls. In addition, the results indicated that natural antioxidants have no effect on animal weight and liver or kidney functions. These results indicated that these agents are not toxic and may prevent tumor development. Thus, it appears that natural derivatives induced apoptosis in human cancer cells and it may offer an effective and non-toxic natural anti-tumorigenic compounds in humans.

Keywords: natural antioxidents, apoptosis, prostate cancer, breast cancer, colon cancer

Presenting Author: Stephanie Felscher

Identifying novel Bax binding partners by a combination of gel filtration and blue native PAGE followed by mass spectrometry

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Organ development and tissue turnover depend on the finely tuned regulation of programmed cell death. The members of the Bcl 2 family are central regulators of the intrinsic pathway of apoptosis by integrating survival and death signals. The pro-apoptotic protein Bax primarily localizes to the cytosol of healthy cells, where it has to be strictly controlled to prevent activation and translocation to mitochondria, an event that occurs upon apoptosis induction. A variety of proteins have been identified to interact with Bax in the cytosol by yeast two hybrid screens, interaction cloning, affinity columns or over-expressing various Bax forms. Most of these methods were performed in the presence of detergent and/or relied on artificially high Bax expression. We wanted to identify proteins, which bind to endogenous cytosolic Bax under healthy conditions. For that purpose, we separated the cytosol of factor dependent mouse monocytes (FDMs) cells by AEKTA gel filtration chromatography. Fractions containing Bax (identified by Western blotting) were pooled and applied to blue native polyacrylamide gel electrophoresis (BN-PAGE) followed by 2nd dimension SDS-PAGE. After silver staining and localizing Bax on the SDS-PAGE by Western blotting, gel slices in the area of Bax and above (where interaction partners should reside) were cut, digested with trypsin and subjected to LC-MS/MS mass spectrometry. To exclude proteins which were not in a complex with Bax, but migrated similarly on SDS-PAGE, we ran fractionated cytosol from Bax-/- FDMs on the same gel. We obtained some candidate proteins, which will now be tested for their interaction with endogenous Bax in healthy cells.

Keywords: Apoptosis, Bax, binding partners, gelfiltration, blue native PAGE, mass spectrometry

NFAT signalling in malignant melanoma.

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Malignant melanoma is the most aggressive form of skin cancer and metastatic melanoma is notoriously unresponsive to current therapies, leaving an acute need for novel chemotherapeutic targets. NFAT is a calcium-regulated transcription factor reported to play a pro-carcinogenic role in many cancers, partly via induction of cyclooxygenase-2 (COX-2). Since increased expression of COX-2 contributes to melanoma growth, invasion, and apoptotic resistance, NFAT may therefore represent a potential target for therapeutic intervention. The aim of the present study was to investigate NFAT signalling in metastatic melanoma cells and determine the relative contribution of this pathway to COX-2 regulation.

Of 4 known NFAT proteins (NFAT 1-4), NFAT 2 and 4 are expressed in human metastatic melanoma cell lines at both the mRNA and protein level. Furthermore, NFAT transcriptional activity was induced by the classical NFAT activators TPA/ionomycin and blocked by the calcineurin inhibitor cyclosporin A (CsA). Interestingly, both basal and inducible NFAT transcriptional activity was higher in metastatic melanoma cells bearing activating mutations of the B-RAF protein kinase. B-RAF mutations are present in up to 70% of melanomas and result in increased activation of oncogenic RAS-RAF-ERK signalling. Treatment of B-RAF mutated melanoma cells with the downstream MEK inhibitor PD98059, resulted in reduced levels of activated (phosphorylated) ERK (pERK) as well as reduced NFAT transcriptional activity. Conversely, over-expression of mutated BRAFV600E protein resulted in both increased levels of pERK and increased NFAT transcriptional activity.

Western blotting confirmed COX-2 over-expression in melanoma cells compared to normal melanocytes, induction of which was increased by activating NFAT using TPA/ionomycin and blocked by CsA and PD98059. COX-2 promoter-driven luciferase reporter vectors containing mutated NFAT binding sites, were induced to a lesser extent than vectors containing wild-type NFAT binding sites, indicating that NFAT regulates COX-2 promoter activity and COX-2 protein induction in metastatic melanoma cell lines.

Take together, our results suggest that NFAT activity in melanoma cells is increased by mutant B-RAF signalling and that the pro-carcinogenic functions of COX-2 may be mediated, in-part, by upstream NFAT signalling. Targeting NFAT may therefore represent a novel therapeutic strategy for the treatment of metastatic melanoma.

Keywords: melanoma, NFAT, COX-2

Uncovering the quantitative role of the c-FLIP isoforms in CD95 signaling

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CD95 is a member of the TNF receptor family, which can trigger apoptosis upon binding to its ligand but can also induce survival pathways if cells are stimulated with a low amount of CD95 (Lavrik et al, 2007). Activation of the receptor leads to the formation of the death-inducing signalling complex (DISC) which consists of CD95, FADD, procaspase-8/10 and c-FLIP. There are two short and one long isoform of c-FLIP known. The short isoforms (c-FLIPR, c-FLIPS) compete with procaspase-8 for binding to the DISC and thereby inhibit the signalling. The long isoform (c-FLIPL) can promote procaspase-8 processing and activation at low concentrations but can also inhibit procaspase-8 binding to the DISC if expressed in high amounts. Cleavage and activation of procaspase-8 at the DISC leads to the initiation of apoptosis. CD95 stimulation can also lead to the induction of survival pathways: It induces Erk, IkBα and JNK phosphorylation. Therefore the composition of the DISC, e.g. the amount of c-FLIPS/R, c-FLIPL, procaspase-8, CD95 and FADD is crucial for the life-death decision.
In this study the different isoforms of c-FLIP will be investigated by constructing a mathematical model of CD95 signal transduction. We have established several HeLa cell lines which express different amounts of the c-FLIP isoforms. The response of these cell lines upon CD95 stimulation will be measured with quantitative immunoblots and bioplex. Further, the total amount of the main molecules involved in CD95 signal transduction will be measured with mass spectrometry. This data will then be used to build a mathematical model of CD95 signalling which will allow a better understanding of this pathway.

Keywords: CD95, DISC

Presenting Author: Vlasta Furstova

Different effect of saturated and unsaturated fatty acids on cell death induction in pancreatic beta cells

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Chronically elevated concentrations of fatty acids (FAs) in blood contribute to pancreatic beta-cell loss in type 2 diabetes, mainly due to apoptosis. However, precise molecular mechanisms of their cell death inducing effect are unclear.
Employing human pancreatic beta-cell line NES2Y and defined culture media, we compared the effect of six FAs differing in their carbon chain length, degree of saturation and spatial conformation of their double bond: palmitic acid (16 carbons, saturated), stearic acid (18 carbons, saturated), palmitoleic acid (16 carbons, unsaturated with cis double bond), oleic acid (18 carbons, unsaturated with cis double bond), elaidic acid (18 carbons, unsaturated with trans double bond) and linoleic acid (18 carbons, unsaturated two double bonds).
Saturated FAs showed effects on cell proliferation in a dose dependent manner. After 2 days, almost all cells incubated with of 1mM of palmitic or stearic acid were dead. In contrast, 1 mM and 3 mM cis unsaturated FAs caused only a decrease in proliferation activity of the cells after 4 days of incubation. Elaidic acid appeared more effective than its cis counterpart oleic acid. Its inhibitory effect on the proliferation was already detectable at 0.1 mM concentration in contrast to 1 mM in the case of oleic acid. Cell death induced by saturated FAs was not accompanied by a significant increase in caspase-3 activity. However, we detected a significant (2-5x) increase in caspase-2 activity after palmitic and stearic acid treatment. Unsaturated FAs did not activate caspase-3 as well as caspase-2.
Taken together, we can conclude that saturated fatty acids, in contrast to unsaturated fatty acids, induce cell death in human beta cells NES2Y at physiologically relevant concentrations. Our data indicate that the effect of FAs is influenced by spatial conformation of the double bond because a trans unsaturated fatty acid is significantly more effective in inhibiting beta cell proliferation than its counterpart cis unsaturated fatty acid. Furthermore, we newly demonstrated that beta cell death induced by saturated fatty acids is related to caspase-2 activation but not to caspase-3 activation.

This work was supported by research program MSM 0021620814 of the Third Faculty of Medicine, Charles University Prague.

Keywords: saturated fatty acids, unsaturated fatty acids, trans fatty acids, cell death induction, caspase-2, pancreatic beta cells

Presenting Author: Guzel Gafijatullina

Circulatory maintenance of embryonic neurotransplant of a brain cortex at rats

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Circulatory maintenance of embryonic neurotransplant of a brain cortex at rats
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Parallel with a problem of immunologic compatibilities as bases of the transplanted tissue growing, special interest represent questions of functional implants' integration. Neurotransplantation of embryonic tissue can be used as an alternative method of treatment for indemnification of some proceeding in cells of a brain pathological processes, correction of the broken functions of nervous system. Efficiency of embryonic neurotransplant (ENT) integration depends on reorganization of a microvascular channel components in a adult animal brain and a level of circulatory-metabolic ENT maintenance. The factors causing apoptosis in an adult brain, can induce the process growing of ENT neurons (Snyder E., 2004), so special interest represent questions of functional implants' integration.

In this connection the aim of research was studying local cerebral blood flow (LCBF), ðÎ2 and character of vascular reactions in ENT 8 months after its homotopical allotransplantations into the barrelfield of somatosensory recipient's brain cortex of the rat. Donor tissue received from 17-18-old day embryos of Wistar line rats at which from corresponding of somatosensory cortex zone, took the fragment of a tissue used such as ENT.

Received embryonic tissue carried out transplantation to 6-month's rats of Wistar line. As the control rats with intact somatosensory cortex served. In a zone of a projection Ñ1 whisker in ENT intensity of LCBF has made 47,0±2,5ml/100g/min. Also it has been reduced (ð<0,05) in comparison with the control. The greatest LCBF values are registered in ENT on depth of 0,5-0,7 mm. The ðÎ2 tissue level has made in ENT 23,8±2,96 mm Hg. The item also did not differ from the control. At the activation caused by Ñ1 whisker's stimulation, reactions of increase in LCBF and ðÎ2, reflecting formation local functional hyperemia, developing by means of local vascular reactions are revealed. The level of LCBF intensity grew with the latent period 6,8±0,76 s, the amplitude post stimulated increases of a blood flow in ENT has been reduced (ð<0,01) in comparison with the control: 12,7±1,34 % and 21,8±2,26 %, accordingly, that testified to reduction of vessels' dilatation efficiency. The received data specify insufficiency of ENT blood supply, but simultaneously with it testify to ability of microvascular network ENT to realization of the active vascular reactions directed on regulation of a blood flow in a nervous tissue.

Keywords: neurotransplantation, embryonic tissue,circulatory maintenance

C. elegans SIR-2.1 translocation linked to a proapoptotic pathway parallel to cep-1/p53-like during DNA damage induced apoptosis

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We use DNA damage induced germ cell apoptosis in C. elegans as a model system to study apoptosis. Worm apoptosis generally requires or core apoptosis pathway that includes the BH3 only domain EGL-1, Bcl-2 like CED-9, CED4/Apaf-1 and the CED-3 caspase. We found that in the germ line that anti-apoptotic CED-9 and CED-4 do not colocalize. This is in contrast to apoptosis occurring during worm development where CED-4 pro-apoptotic activity is thought to be kept at bay by its direct interaction with mitochondrial CED-9. Thus C. elegans germ cell apoptosis might be more similar to mammalian apoptosis as previously assumed. Our results raise the question of how mitochondrial CED-9 functionally interacts with cytoplasmic and perinuclear CED-4.
We found that that C. elegans sir-2.1 might be one such factor. Sir-2 was previously known to have a role in aging and stress responses, is essential for DNA damage induced apoptosis. S. cerevisiae Sir2p is an NAD+ dependent protein deacetylase implicated in transcriptional repression, ageing and stress responses. In mammals, the homolog SIRT1 plays important roles in diverse processes such as angiogenesis, glucose, cholesterol, lipid homeostasis, stress responses and apoptosis.
Genetic analysis shows that sir-2.1 acts in the DNA damage pathway downstream or parallel to the worm p53 like gene cep-1. sir-2.1 does not interfere with the execution of apoptosis downstream of ced-4, since other forms of apoptosis, such as developmental apoptosis and physiological germ cell apoptosis are unaffected by deleting sir-2.1. It is therefore likely that sir-2.1 acts at the level of egl-1/ced-9/ced-4 and in parallel to cep-1.
Immunostaining of irradiated germ lines shows that during apoptosis SIR-2.1 translocates from the nucleus to the cytoplasm of dying cells. SIR-2.1 translocation is independent of cep-1 and ced-3, as it also occurs in cep-1 and ced-3 loss of function mutants. Even though we have so far been unable to find a direct interaction between SIR-2.1 and CED-4 we found that CED-4 and SIR-2.1 transiently colocalize at the nuclear periphery. Furthermore, the perinuclear hyper-accumulation of CED-4, a hallmark of apoptosis progression, is abrogated in sir-2.1 and cep-1 mutants. Therefore our cytological observations confirm our genetic data and indicate that SIR-2.1 is part of a pathway that acts in parallel to CEP-1 to regulate CED-4 activity.

Keywords: C. elegans, germ cell apoptosis SIR2, CEP-1/p53 CED-4

Presenting Author: Marine Gasparyan

The new DR-5 selective mutant of TRAIL

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TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is a cytokine that preferentially induces apoptosis in tumor cells compared with normal cells through two receptors (DR4 and DR5). Many cancer cell lines express both DR4 and DR5 but DR5-induced apoptosis appeared more effective. Recently DR5 specific TRAIL variant bearing 6 substitutions (DR5-8) was selected using phage display [1]. This mutant TRAIL did not induce apoptosis in DR4-responsive cell lines. Another mutant TRAIL (D269H) was found to be more efficient in DR5-responsive cell lines [2]. Based on these results we have generated new DR5-selective mutant variants of TRAIL, which contains all six substitutions plus D269H (DR5-B). Wild type and all mutant variants of TRAIL were expressed in E.coli as fusion proteins with thioredoxin. Purified fusion Trx\TRAIL was cleaved by recombinant human enteropeptidase light chain enteropeptidase and TRAIL was separated from thioredoxin on Ni-NTA agarose [3]. High yields of wild type TRAIL and mutant variants without N-terminal methionine and tags were obtained. Analytical sedimentation confirmed that 98% of all preparations formed trimers. The mutants of TRAIL were tested on 3 cell lines: Hela (express both DR4 and DR5 at comparable levels), K562 (DR4-responsive) and Jurkat (DR5-responsive).
It was shown that in HeLa cancer cell DR5-B proapoptotic activity was similar to wild type and D269H mutant but significantly higher then activity of DR5-8. In K562 cells (DR4-responsive) D269H mutant was as effective as a wild type while DR5-B and DR-8 efficiency was much lower. Finally in Jurkat (DR5-responsive) all the mutants were more effective then the wild type TRAIL. Taken together, these results indicate that the new mutant TRAIL (DR5-B) has increased affinity for DR5 being ineffective in DR4-resposive cells. It can serve as a good tool in studies of TRAIL-induced apoptosis in cancer or normal cells which express the both death receptors.

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Keywords: TRAIL, DR5-selective mutant, death receptors

PI3Ks play a role in TNFα-induced neutrophil cell death

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Innate immunity is essential as the first line of defence against invading organisms. Key effector cells of the innate immune system are neutrophils. Upon cellular stimulation, neutrophils migrate to sites of inflammation where they phagocytose infectious agents and release toxic mediators. Tight regulation of granulocyte function is critical for the removal of pathogens, and strict control of cell death is important for an efficient resolution of inflammation.
TNF receptor engagement in neutrophils results in either survival or cell death depending on TNFα concentrations and co-stimulatory signals. In order to gain insight into the signalling mechanisms leading to apoptosis downstream of TNF receptor, we treated neutrophils with high TNFα concentrations in combination with small-molecule inhibitors against JAK/STAT, MAPK and PI3K pathways.
When PI3K activity was blocked by broad-spectrum and class IA isoform-selective PI3K inhibitors, neutrophil viability was increased upon TNF receptor ligation. These results were surprising since PI3K inhibition induces death in most cell types. Increased neutrophil viability under these conditions was not due to the inhibition of caspases, whose processing pattern remained comparable to neutrophils treated with TNFα alone. However, inhibition of PI3Ks completely abolished the immediate generation of reactive oxygen species (ROS) upon TNFα stimulation. The reduction in ROS levels following PI3K inhibition resulted in increased mitochondrial transmembrane potential, and in enhanced neutrophil viability. In line with these findings, neutrophils from CGD patients (who lack a functional NADPH oxidase) did not produce ROS and where therefore long-lived when treated with TNFα.
In summary, class IA PI3Ks control ROS production and thus play a role in the regulation of neutrophil cell death induced by TNFα.

Keywords: phosphoinositide 3-kinase, reactive oxygen species, TNFα, neutrophil

FKHRL1/FOXO3 promotes re-expression of caspase 8 in neuroblastoma cells

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Background: Neuroblastoma, a pediatric malignancy of neural crest origin is the most common solid tumor in children and accounts for approximately 10% of all childhood cancers. Deregulated neurotrophin signaling and lack of caspase 8 are frequently observed in aggressive neuroblastoma tumors. We previously reported that FKHRL1 triggers apoptosis via the mitochondria in human SH-EP and STA-NB15 neuroblastoma cells. Due to the fact that the majority of aggressive neuroblastoma do not express caspase 8, we further investigated the potential function of FKHRL1 on the extrinsic death pathway.
Methods: A 4OH-tamoxifen (4OHT) inducible, phosphorylation-independent FKHRL1(A3)ERtm allele was retrovirally introduced into two low passage, caspase 8-deficient neuroblastoma cells. Expression of transgenic FKHRL1, caspase 8, CREB, pCREB and TRAIL was analyzed by immunoblot and quantitative RT-PCR. The effect of FKHRL1 and transgenic caspase 8 on cell survival was assessed by flow cytometry.
Results: Transgenic FKHRL1 transiently induced the death ligand TRAIL and spontaneous cell death in one out of two neuroblastoma cell lines. Retroviral expression of caspase 8 restored or accelerated apoptosis induction by FKHRL1, indicating that the extrinsic apoptosis pathway is critical for cell death decision in these cells. Interestingly, transgenic FKHRL1 per se caused re-expression of caspase 8 in the caspase-8-deficient cell lines. The re-expression of caspase 8 was preceded by the phosphorylation of CREB at Ser133, which has been reported to bind to an intronic region of the caspase 8 gene, thereby inducing the expression of caspase 8.
Conclusions: The combined data suggest that FKHRL1-induced cell death in neuroblastoma is critically modulated by the presence of caspase 8 and that FKHRL1 may regulate the re-expression of caspase 8 via the phosphorylation of CREB.

Keywords: FOXO3/FKHRL1, neuroblastoma, caspase 8

Presenting Author: Doron Ginsberg

E2F1 regulates autophagy and the transcription of autophagy genes.

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The RB pathway is often inactivated in human tumors resulting in deregulated E2F activity that can induce both proliferation and cell death. While the role of E2F in apoptosis is well characterized, little is known regarding its putative participation in other cell death pathways.
We show here that activation of E2F1 up-regulates the expression of four autophagy genes - LC3, ATG1, ATG5 and DRAM. E2F1-mediated induction of LC3, ATG1 and DRAM is direct and indeed, endogenous E2F1 can be found bound to regions encompassing the promoters of these genes. Regulation of ATG5 by E2F1 is indirect.
Importantly, we demonstrate that E2F1 activation enhances autophagy and conversely, reducing endogenous E2F1 expression inhibits DNA damage-induced autophagy. Moreover, inhibition of autophagy increases significantly DNA damage-induced cell death, suggesting that E2F1-mediated autophagy contributes to cell survival after DNA damage.
Altogether, this study identifies E2F1 as a transcriptional regulator of autophagy, and for the first time establishes a role for E2F1 in DNA damage-induced autophagy.

Reference: Polager S., Matan Ofir M. and Ginsberg D. E2F1 regulates autophagy and the transcription of autophagy genes. Oncogene. 2008. In Press.

Keywords: autophagy, E2F, transcription

Mitochondria as a therapeutic target for killing tumor cells

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The past decade has revealed a new role for the mitochondria in cell metabolism – regulation of cell death pathways. Targeting the mitochondria appears to be a promising strategy for the induction of cell death in tumor cells. A redox-silent analogue of vitamin E, α-tocopheryl succinate (TOS), was shown to kill selectively malignant cells at concentrations non-toxic to normal cells. Treatment with TOS led to mobilization of pro-apoptotic factors, such as cytochrome c and Smac/Diablo; however, the mechanism of the outer mitochondrial membrane (OMM) permeabilization, responsible for their release, remained obscure. We have shown that TOS caused suppression of Complex I of the mitochondrial respiratory chain and mitochondrial uncoupling in a variety of malignant cells. In addition, TOS stimulated mitochondrial production of reactive oxygen species. Analysis of cell death induced by TOS in Tet21N neuroblastoma cells, characterized by amplification of MycN oncogene, revealed that the effect of the drug was markedly stronger when MycN amplification was blocked. Turning MycN off caused inhibition of respiration, a decline in calcium buffering capacity, and activation of caspase-3. It is believed that mitochondrial pathway in apoptosis on advanced stages of neuroblastomas is suppressed due to an imbalance between anti-apoptotic and pro-apoptotic Bcl-2 family proteins in favor of former. Destabilization of mitochondrial respiration together with ROS-mediated oxidation of critical thiols facilitated induction of mitochondrial permeability transition (MPT), with subsequent mitochondrial swelling, rupture of the OMM and cytochrome c release. Stimulation of the OMM permeabilization via induction of MPT seems to be a powerful tool in overcoming the protective effects of Bcl-2 and Bcl-XL. Thus, combined alteration of MycN status and mitochondrial targeting by TOS can be viewed as a promising tool facilitating neuroblastoma cell death.

Keywords: mitochondria, cell death, reactive oxygen species

SLS- a novel stress induced cell death pathway in Trypanosoma brucei

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Trypanosomes are protozoan parasites and the causative agent of major parasitic diseases affecting millions of people mostly in developing countries. Trypanosomes are ancient eukaryotes that diverged early from the eukaryotic lineage. We recently discovered a novel stress-induced mechanism that leads to programmed cell death. This PCD-like process was revealed in RNAi silenced cells for the signal recognition particle receptor, and was elicited by shutting-off the transcription of the most essential small RNA in the cell, the SL RNA. This process was termed SLS for SL RNA Silencing (SLS) (EMBO reports 8, 4, 408–413, 2007). SLS blocks mRNA production since all trypanosome mRNAs utilize SL RNA for their processing by trans-splicing. Transcription shut-off of SL RNA is correlated with the failure of the SL RNA specific transcription factor, tSNAP42 to bind to its cognate promoter. We currently do not know how is the signal that triggers SLS is transmitted to the nucleus.

Here we show that SLS can be induced under variety of stresses including perturbations in protein sorting by silencing of SEC63 and SEC61 that function in translocation of proteins across the ER membrane. Moreover, SLS is induced by triggers of the unfolded protein response (UPR) in other eukaryotes such as reducing and oxidative agents, as well as glycosylation competitors. These treatments first result in classical UPR response, diagnosed by induction of the ER chaperone BiP. However, upon prolonged exposure to UPR conditions, SLS was induced. We find that SLS induction is not reversible and leads to cell death, suggesting that SLS may represent a programmed cell death pathway which is activated upon prolonged ER stress. We also show that during this PCD process reactive oxygen species (ROS) are produced, and the mitochondrial membrane potential is reduced. In addition, SLS cells were AnnexinV positive, and DNA fragmentation was observed using TUNEL, propidium-iodide staining and DNA laddering assays. SLS may represent a unique parasite specific cell death pathway that stops cell metabolism in a single step. SLS can potentially serve as a novel drug target for parasite eradication.

Keywords: ER stress, Trypanosoma brucei, SL RNA Silencing

B10, a new betulinic acid derivative, induces a mixed phenotype of cell death in glioblastomas.

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Since the discovery of it's cytostatic and cytotoxic properties, betulinic acid has stimulated the developement of a number of studies aiming at evaluating it's therapeutic potential. Although promising results show that betulinic acid induces cell death in various cancer cell lines without affecting healthy cells, poor hydrosolubility, and low efficiency restrict the promotion of betulinic acid as a therapeutical agent. In order to offer an alternative to these limitations, we present here the study of the mode of action of B10, a synthetic glycosylated compound designed on betulinic acid molecular scaffold. In addition to an increased solubility in aqueous medium, and the potential to be uptaken through the blood-brain barrier, B10 also embed an increased in vitro cytotoxic activity when compared to the natural betulinic acid. Interestingly B10 treatment of glioblastomas results into morphological, and biochemical changes that argue for a mixed phenotype of cell deaths. Both apoptotic, necrotic and autophagic features can actually be detected in cells upon B10 treatment. Although the origin of these changes, their interplay and their sequence in time are still investigated, it appears that conversely to betulinic acid, mitochondria are not the main target of B10. Instead, the lysosomal compartment is primarily affected, and displays significant alterations along the time course of the cell death process induced by B10. These results suggest that B10 may act as a lysomotropic drug via lysosomal permeabilization. As a consequence of this property, B10 may preferentially affect cells presenting an increased lysosomal activity, and thus provide a specific therapeutic window against cancer cells.

Keywords: betulinic acid, lysosomes, apoptosis, necrosis, autophagy

Presenting Author: Francesca Grespi

Investigating isoform-specific functions of the BH3-only protein Bmf

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Bmf is a proapoptotic BH3-only protein of the Bcl-2 family. It is widely expressed in the hematopoietic system and during mammary gland development. Bmf protein expression can be induced by histone deacetylase inhibitors (HDACi) and its function is regulated post-translationally by binding to the cytoskeleton via dynein light chain-mediated interaction with the myosin V actin motor complex or JNK-mediated phosphorylation. At least two isoforms can be detected by Western blot analysis, suggesting that alternative promoter usage and/or alternative splicing may provide additional means of regulating its pro-apoptotic function. Interestingly, the longest isoform (BmfCUG) arises from the use of an unconventional CUG START codon that is highly conserved between mammals. We aim to understand the physiological functions of the two isoforms, by comparing their binding pattern to the antiapoptotic members of the Bcl-2 family, their relative killing efficacy, their sub-cellular localization as well as relative changes in expression levels during cell death initiation.
We demonstrate by co-IP that BmfCUG has the same binding affinity to pro-survival molecules as Bmfs, when overexpressed. To evaluate possible differences in the potency of cell-killing, we generated IL-3 dependent BAF3 cells that express antiapoptotic proteins alone or together with Bmfs or BmfCUG. Cells were deprived of IL-3 and survival was assessed thereafter, but no differences in apoptosis-induction were observed between the two isoforms. Cell death induced by Bmf isoforms can occur in a Bax or Bak dependent manner as evaluated by retroviral transduction of wt, bax-/-, bak-/- and double deficient MEF. We also noticed that both isoforms colocalize with mitochondria in NIH3T3 and HEK293T cells when overexpressed.
Finally, we investigated whether the additional base pairs in BmfCUG and its 5´UTR could mediate IRES-dependent translation of Bmfs under conditions when CAP-dependent translation is blocked. Preliminary experiments with HEK293T cells overexpressing a ECFP-EYFP-fusion protein, separated by these additional base pairs, support this hypothesis and luciferase assays excluded residual promoter activity in this region. To induce conditions where CAP-independent translation is preferred, we treated NIH3T3 cells and HC11 cells with LY-294002, a PI3-K inhibitor, and with EGI-1, a drug mimicking the action of 4EBP1. Surprisingly, immunoblotting revealed an increase of both isoforms over time, suggesting that both isoforms could be translated from this putative IRES.

Keywords: BH3-only proteins, apoptosis, IRES-mediated translation

Collagen VI deficiency drives muscular cells to apoptosis in myopathic diseases

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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 three muscular diseases: Bethlem myopathy, Ullrich congenital muscular dystrophy (UCMD) and myosclerosis myopathy.
ColVI knockout (Col6a1–/–) mice display a myopathic phenotype affecting diaphragm and other muscles. Col6a1–/– muscle fibers have a loss of contractile strength associated with ultrastructural alterations of mitochondria and sarcoplasmic reticulum, mitochondrial dysfunction and spontaneous apoptosis. We established that patients affected by UCMD have mitochondrial swelling and an increased rate of apoptosis in muscle biopsies and in myoblast/fibroblast cultures derived from them, thus resembling the murine model. These changes are matched by a latent mitochondrial dysfunction that can be revealed by addition of the F1F0 ATPase inhibitor oligomycin, which caused PTP-dependent mitochondrial depolarization in cultures from Col6a1–/– mice and UCMD patients. The defects are reversible and could be completely reverted by plating cells onto ColVI or after treatment with Cyclosporin A (CsA), a drug that desensitizes PTP opening. Remarkably, the structural alterations and apoptotic defects of Col6a1–/– muscles can be normalized by in vivo treatment with CsA. Based on these findings, we recently carried out a pilot trial with CsA in five patients affected by ColVI myopathies. Prior to treatment, all patients displayed mitochondrial dysfunction and increased apoptosis, as determined in muscle biopsies. Both these pathological signs were largely normalized after 1 month of oral CsA administration, which also increased muscle regeneration. These findings demonstrate that ColVI myopathies can be effectively treated with drugs acting on the pathogenic mechanism downstream of the genetic lesion.
Our studies indicate that extracellular ColVI must relay a set of trophic signals which regulate cell survival in skeletal muscle. We are currently performing studies aimed at investigating ColVI-driven signal transduction and dissecting the molecular pathways that are altered in Col6a1-/- muscles. In unpublished studies, we found that Col6a1-/- myoblasts display marked changes in the activation status of some signal transducers playing a key role in the control of cell survival. These changes are reversible, as they are fully normalized by plating cells onto ColVI, but not other extracellular matrix substrates. The altered signaling pathways of Col6a1-/- mice and UCMD patients are currently under active investigation.

Keywords: collagen VI, mitochondria, apoptosis

Presenting Author: Jennifer Guerriero

Chemotherapy induces tumor clearance independent of apoptosis

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It is widely accepted that the impairment of apoptosis is a crucial step in tumorogensis. Despite this, DNA alkylating agents remain an effective means of treating cancer in patients, indicating non-apoptotic cell death pathways may be responsible for tumor regression. Here, we utilize a xenograft mouse tumor system generated from genetically defined mouse embryonic fibroblasts (MEFs) deficient in key apoptotic proteins Bax and Bak. We show that bax-/-bak-/- tumors treated with a commonly prescribed DNA alkylating agent, cyclophosphamide (CP), regress, similar to wild-type tumors. Immunohistochemistry, transmission electron microscopy, and immunoblotting analysis show that apoptosis occurs in CP-treated wild-type tumors, but not in CP-treated bax-/-bak-/- tumors. Rather, sporadic necrosis is observed in both apoptosis-competent and deficient tumors evident by tumor cell morphology, extracellular release of the high mobility protein B1 (HMGB1), and activation of innate immune cells. Our findings indicate that necrosis may play a fundamental role in tumor clearance by stimulating the innate immune response.

Keywords: chemotherapy, apoptosis, necrosis, Bcl-2, innate immunity

CD44 variant isoforms are expressed in MS lesions, and their genetic deletion ameliorates active and passive EAE by increased apoptotic activity

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CD44 variant isoforms (CD44v) are transmembrane molecules, which play an important role in the development of various autoimmune disorders, including colitis and arthritis. The role of CD44v has been explored to only a limited extent in multiple sclerosis (MS).
Therefore, we determined the functional role of selected CD44v isoforms in MS and experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. Among various isoforms tested, CD44v3, CD44v7 and CD44v10 isoforms were expressed on glial cells and on perivascular infiltrating inflammatory cells in MS lesions. In the EAE model, brain infiltrating CD4+CD44v10+ T cells preceded EAE onset and paralleled disease severity scores. Moreover, antigen specific restimulations of draining lymph node cells in vitro resulted in a striking upregulation of CD44v10 on resting T cells from 30 to almost 100%. This upregulation of CD44v10 was paralleled by a strong increase in their encephalitogenic potential.
Independent genetic deletion of the CD44v7 and the CD44v10 isoforms significantly reduced clinical EAE burden, as well as the number of inflammatory infiltrates in the brain and the spinal cord. Furthermore, adoptive transfer experiments showed that CD44v7 and CD44v10 expression on both, effector T cells and antigen presenting cells, participated in the development of EAE. Both CD44v7 and CD44v10 expression contributed to the development of EAE by increasing the longevity of autoantigen specific CD4+ T cells. Vice versa, CD44v10 and CD44v7 deficiencies led to increased apoptosis in the infiltrates, resulting in marked reduction of disease activity.
In conclusion, we show that the selected CD44v isoforms, CD44v7 and CD44v10, are critically involved in the pathogenesis of EAE and probably of MS. Targeting these CD44v isoforms might therefore reduce inflammatory processes and clinical symptoms in MS.

Keywords: MS, EAE, CD44v isoforms,

Presenting Author: Günther Haase

Amino Acids in Apoptosis, Proliferation and Activation of p53

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528 different tumorcases 1985-2007
All had undergone several operations and chemos and developed consequently relapses.The immunsystem showed severe damages (CD3, CD4,CD8,CD19) besides WBC,RBC and platelets.Some got already transfusions.We devided the patients in 3 groups:1.NoneImmunresponders(NIR),2.Medium-Immunresponders(MIR),3.Good Immunresponders(GIR).
1.NIR,225-42%,survival 2-15 weeks(8.5 weeks),no immunresponses.
2.MIR,160-31%,survival 5-23 months(14 months),shivering and fever 38-38.5℃ over 4-6 hours.3.GIR,143-27%,survival 2,5-15 years(8.6) Red and swollen spots at the injectionsite,39-40℃,8-10 hrs.sometimes 1-2 days.In GIR are still 15 patients alive without any tu- mors.These patients get now every 4-5 weeks 1 treatment to keep the immunsystem on a high standard to block new tumordevelopment We use specific amino-and nucleic acids,embryonic peptides and radical scavenger chinons!Natalia Chalisova gave the scientific prove:APOPTOSIS in immature cells and PROLIFERATION in mature cells of braincortex,liver and spleen and the production of p53.Besides Valery Radchenko showed the clinical prove in Hepati-tis C virusinfected cells.Destruction of the cells,regrowth of new livercells and normalisation of the bad bloodsamples!These are the proves,that my method is working antiviral and antitumoral.This was already stated,that some of my substances are acti- ve in this way by the Encyclopedia of Virology of the Academic Press 1995!"CORRECT IMMUNOREGULATION GIVES THESE FINE RESULTS"
More in lecture- or posterform! N.Chalisova,G.Haase,V.Radchenko

Keywords: amino acids,apoptosis,p53

Presenting Author: Sabine Haecker

Restoring caspase-8 expression by histone deacetylase inhibitors sensitizes neuroectodermal tumors with epigenetic inactivation of caspase-8 for TRAIL-induced apoptosis

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Resistance of human cancers to current therapies is often due to defects in apoptotic programs. In medulloblastoma caspase-8, a key mediator of death receptor-induced apoptosis, is frequently inactivated by epigenetic silencing and loss of caspase-8 is associated with unfavourable survival outcome of medulloblastoma patients. In search for novel strategies to overcome apoptosis resistance of cancer cells, we investigated the role of chromatin remodeling in restoring caspase-8 expression. Here, we identify aberrant histone deacetylation as novel mechanism of epigenetic inactivation of caspase-8. Histone deacetylase inhibitors (HDACI), e.g. valproic acid (VA), SAHA or MS-275, trigger re-expression of caspase-8 in a variety of medulloblastoma cell lines lacking caspase-8 with VA being the most potent one. Interestingly, combined treatment with VA and interferon-gamma (IFN-gamma) cooperates to upregulate caspase-8 expression. VA and IFN-gamma treatment increases acetylation of histone H4, leading to enhanced caspase-8 promoter activity and subsequently to upregulation of caspase-8 mRNA levels. Importantly, re-expression of caspase-8 by VA/IFN-gamma results in enhanced recruitment of caspase-8 into the death-inducing signalling complex (DISC) upon addition of TNF-related apoptosis-inducing ligand (TRAIL) compared to single agent treatment, resulting in enhanced caspase-8 activation. Furthermore, combination treatment promotes TRAIL-induced activation of proapoptotic Bax, which in turn triggers loss of mitochondrial membrane potential, release of cytochrome c into the cytosol and finally activation of effector caspase-3, further promoting TRAIL-induced apoptosis. Intriguingly, inhibition of caspase-8 upregulation by a vector-based RNA interference system completely abolished the sensitizing effect of VA/IFN-gamma for TRAIL-induced apoptosis. Similar results were obtained using the caspase-8 inhibitor zIETD.fmk, demonstrating the importance of caspase-8 upregulation for the sensitization effect of VA/IFN-gamma to TRAIL. Importantly, a similar synergistic effect of VA and IFN-gamma in restoring caspase-8 expression and thereby sensitivity for TRAIL-induced apoptosis is observed in additional cancers lacking caspase-8, e.g. neuroblastoma and Ewing's sarcoma, as well as in primary medulloblastoma cells freshly isolated from patient material. By demonstrating that HDACI, especially in combination with IFN-gamma, restore caspase-8 expression and thereby sensitize resistant tumor cells for TRAIL-induced apoptosis, our findings have important clinical implications for novel strategies targeting defective apoptosis pathways in neuroectodermal tumors.

Keywords: Histone deacetylase inhibitors, caspase-8, medulloblastoma

Presenting Author: Leonhard Heinz

Molecular Characterization of Unc5CL/ZUD, a potent activator of NF-κB

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ZU5 and death domain (DD) containing proteins form a small family of molecules implicated in a variety of biological processes including apoptosis, axon guidance and cell scaffolding. Prominent members include PIDD, Unc5 receptors and ankyrins. The recently identified family member Unc5CL/ZUD contains an N-terminal ZU5 domain and a C-terminal DD and has been described as a negative regulator of the transcription factor NF-κB in response to a broad range of upstream signals. The aim of this study was to get further insights into Unc5CL/ZUD protein topology and function:
RT-PCR experiments on human tissue cDNAs reveal expression in human pancreas and kidney as well to a lesser extent in small intestine and thymus. The protein contains an N-terminal transmembrane domain which mediates membrane association. Proteinase K protection assays show that its C-terminus is exposed to the cytosol permitting signalling in this compartment. In addition, we show that Unc5CL/ZUD can also act as a potent inducer of the NF-κB signalling pathway. This activation of NF-κB involves phosphorylation of IκB α and can be blocked by a dominant negative IκB kinase beta or a stabilized form of IκB α. Furthermore activation of NF-κB requires the DD as mutants that lack or have a destabilizing point mutation within this domain are rendered non-functional.

This work is supported by the Marie Curie Research Training Network “ApopTrain” funded by the 6th Framework Programme of the European Union.

Keywords: NF-κB, death domain

S-Adenosylhomocysteine hydrolase overexpression is associated with adenosine-induced apoptosis in HEK-293 cells

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S-Adenosylhomocysteine hydrolase (AdoHcyase) catalyzes the reversible hydrolysis of S-adenosylhomocysteine (AdoHcy) to adenosine and homocysteine. Previous studies have shown, that induction of AdoHcyase expression precedes Epstein-Barr virus lytic gene activation in ganglioside-stimulated lymphoma cells. However, the cellular consequences of high or elevated AdoHcyase activity have not been investigated in detail.
Since adenosine is formed by AdoHcy hydrolysis and since AdoHcyase is an adenosine-binding protein, we hypothesized that elevated AdoHcyase expression increases intracellular adenosine levels, which might lead to the induction of apoptosis.
To investigate the effect of elevated AdoHcyase activity on adenosine metabolism and cell viability, we generated HEK-293 cells stably overexpressing AdoHcyase and selected five clones (cd20 1, cd20 3-6 cells), which showed an increase in AdoHcyase activity between 2-fold (cd20 5) and 16-fold (cd20 6 cells) compared to sham-transfected control cells.
In fact, AdoHcyase overexpression increased intracellular adenosine levels from 0.05±0.006 (sham-transfected HEK cells) to 0.81±0.07 nmol/10^7 cells (cd20 6 cell line) whereby, we found a correlation between AdoHcyase activity and adenosine levels ($r=0.89$, $p<0.05$).
Moderate elevation of AdoHcyase activity (2-5-fold in cd20 4 and cd20 5 cell lines) did not decrease cell viability whereas, a 7-16 fold increased AdoHcyase activity in cd20 1, cd20 3, and cd20 6 cells was associated with significantly reduced cell viability. Caspase-activity assays and DNA fragmentation analysis revealed that the cell death was due to apoptosis. The observation that administration of adenosine induced apoptotic features in sham-transfected HEK cells is consistent with our hypothesis that elevated intracellular adenosine levels play a crucial role in inducing apoptosis in AdoHcyase overexpressing cells. Transient expression studies also revealed that 6-fold increased AdoHcyase activity is associated with reduced cell viability.
Taken together, our data suggest that up to 5-fold increases in AdoHcyase activity are compatible with physiological cell function, while greatly enhanced AdoHcyase activity results in adenosine-induced apoptosis.

Keywords: S-adenosylhomocysteine hydrolase, overexpression, adenosine-induced apoptosis

Inhibition of B-RAF signalling down regulates IAP expression resulting in increased ER stress-induced apoptosis

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Malignant melanoma is one of the most difficult cancers to treat, with an increasing incidence which has risen faster than any other malignancy over the past 40 years. Arguably the most important breakthrough in melanoma research has been the discovery that mutations in the B-RAF protein kinase occurring in approximately 70% of all melanomas correlates with disease progression and resistance to apoptosis. The mechanism by which B-RAF renders melanoma cells resistant to apoptosis however, remains unclear but is likely caused by activation of mitogen-activated protein kinase (MAPK) pathways resulting in enhanced expression of members of the inhibitor of apoptosis protein family (IAP).
Resistant to apoptosis induced by conventional mechanisms, we have shown that metastatic melanoma cells undergo apoptosis as result of increased ER stress (Lovat et al 2008 in press), although cells harbouring a B-RAF mutation are inherently more resistant to the novel agents fenretinide and velcade than B-RAF wild type cells. Western blot analysis shows the expression of IAP's survivin and XIAP is also increased in B-RAF mutated A375 and WM266-4 cells.
To test the hypothesis that inhibition of B-RAF-induced MAPK down regulates XIAP and survivin expression resulting in increased ER stress-induced apoptosis, B-RAF signalling was inhibited either by RNAi mediated knockdown of B-RAF V600E or using down stream MEK specific inhibitors. Results demonstrated inhibition of B-RAF signalling down regulated both XIAP and survivin expression as well as increasing the sensitivity of cells to fenretinide-induced apoptosis and inhibition of cell viability. These data suggest that inhibition of B-RAF-induced MAPK or in combination with inhibiting the IAP family member most crucial for melanoma survival may define a more effective therapeutic strategy for metastatic melanoma increasing the clinical efficacy of ER stress-induced apoptosis.

Keywords: Melanoma, BRAF, ER stress, inhibitor of apoptosis proteins, apoptosis

Apoptotic brain damage in Bacterial Meningitis: Evaluation of Regenerative Strategies using Stem Cells

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Background: Apoptosis in the dentate gyrus of the hippocampus correlates with the extent of learning and memory deficits as a sequel of experimental bacterial meningitis. This form of brain damage is a likely histomorphological correlate for neurofunctional disabilities observed in up to 50 % of the children surviving the diseases. In experimental bacterial meningitis hippocampal injury is characterized by apoptotic cell death of neuronal stem cells and/or their progeny in the subgranular zone of the dentate gyrus, a brain region where life-long neurogenesis occurs, and therefore potentially well equipped for brain repair. Multipotency and the capacity for continuous self-renewal make stem cells from various sources attractive candidates for cell-replacement studies. We are systematically developing an in vitro system to evaluate the regenerative potential of proliferative cells and to study brain regeneration in bacterial meningitis.
Method: Here we used a co-culture model of long-term hippocampal slice cultures from postnatal rats (P5) and embryonic stem/progenitor cells from the subventricular zone (E16) to evaluate the potential of transplanted cells to survive and to integrate into organotypic hippocampal slice cultures for potential therapeutic approaches. To this end, we grafted chemically labelled embryonic-derived stem/progenitor cells into organotypic hippocampal slices in the region of the dentate gyrus. Cells were allowed to grow in such co-culture conditions with the addition of epidermal (EGF) and basic fibroblast growth factor (bFGF). The survival and integration of grafted cells was examined on cryosections of organotypic slice cultures by using immunohistochemistry.
Results: Sections showed movement and neurite outgrowth of transplanted subventricular cells into the dentate gyrus at day 7 after engraftment. In the presence of bFGF and EGF embryonic derived stem/progenitor cells were able to differentiate and to mature into neurons.
Conclusion: Here we demonstrate that embryonic stem/progenitor cells are well suited for subsequent migration, proliferation, differentiation and integration into organotypic slice cultures in vitro and may thus hold promise for regenerative therapies aimed at repair of apoptotic brain damage in patients with bacterial meningitis.

Keywords: bacterial meningitis, apoptosis, stem cells, regeneration

Presenting Author: Jiann Hong

Dynamics of Positive and negative control systems on RGNNV-induced host necrotic cell death in fish cells

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The betanodavirus is the causative agent of viral nervous necrosis (VNN), an infectious neuropathological condition characterized by necrosis of the central nervous system including the brain and retina, and which is accompanied by clinical signs such as abnormal swimming behavior and darkening of the fish.
Betanodavirus induces cell apoptosis or secondary necrosis by an ill-understood process. Presently, RGNNV how to exert the negative-off and positive-on cell death systems on control host necrotic cell death in fish cells will be address. In early replication stage, B1 non-structural protein was expressed at 12 h post-infection (p.t.) time, which may reduce host cell death through regulate the viral gene expression. In this stage, B1 may play a necrotic cell death blocker for enhance its viral expression. In contrast, the necrotic cell death inducers were expressed at middle replication cycle, which include B2 (non-structural protein, siRNA silencing suppressor) and capsid protein (protein á) also exist a necrotic death function for triggering necrotic cell death and spreads virus. Interesting, this caspase-independent cell death was also correlated to induce progressive loss of mitochondrial membrane potential (MMP), which type cell death also blocked by Bcl-2 family member zfBcl-xL.
We conclude that RGNNV induces apoptosis followed by secondary necrotic cell death through a mitochondria-mediated death pathway, which controlled by using negative-off and positive-on cell death systems in fish cells.

Keywords: nervous necrosis virus, anti-necrotic gene B1, pro-necrotic gene B2, mitochondria, fish cell

Presenting Author: Catherine Hor Hong Huan

AMIGO is expressed in multiple brain cell types and may regulate dendritic growth and neuronal survival

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In this study, we report the potential roles of a brain-enriched transmembrane immunoglobulin (Ig) superfamily protein, AMIGO (amphoterin-induced gene and ORF) in neurite outgrowth and neuronal survival. AMIGO consists of six leucine-rich repeats (LRRs), an immunoglobulin (Ig) domain close to the transmembrane region, a transmembrane domain and a cytosolic domain. Full-length AMIGO (AMIGO-FL) and its mutants, which are AMIGO-LRR (consist of ectodomain with LRRs only) and AMIGO-Ig (consist of ectodomain with Ig only) were found to exhibit different neurite localization in morphologically mature primary neurons. Transfected AMIGO-FL and AMIGO-LRR are predominantly localized to dendrites while AMIGO-Ig, contrasting with AMIGO-FL and AMIGO-LRR, is predominantly axonal. In line with AMIGO's dendritic localization, siRNA-mediated silencing of AMIGO showed retarded dendritic outgrowth. Overexpressed AMIGO also exerts neuroprotective effects from staurosporine and hydrogen peroxide induced cell death in SH-SY5Y human neuroblastoma cells stably transfected with AMIGO-FL.AMIGO could therefore transducer signals associated with dendritic outgrowth as well as neuronal survival.

Keywords: AMIGO, Ig superfamily, leucine rich repeats, neuron

An Acidic Microenvironment Confers in vitro Selection of Resistance to Diverse Apoptotic-Inducing Stimuli by Increased Expression of Bcl-2

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Background: Apoptosis, or programmed cell death, helps regulate the kinetic balance between cell proliferation and cell death. Metastatic cancer cells exhibit an increased resistance to apoptosis thus gaining a selective growth advantage over normal cells. Previous research has shown an acidic microenvironment to be a stressor that promotes resistance to apoptosis. The anti-apoptotic protein, Bcl-2, and the pro-apoptotic protein, Bax, interact in many cancers to regulate apoptosis.
Purpose: To determine how cells maintained in an acidic environment will respond to diverse pro-apoptotic stimuli including: serum deprivation,γ-irradiation, hyperthermia and heat shock. A further objective was to evaluate Bcl-2 and Bax expression following these stimuli.
Methods: We utilized the well-characterized, poorly metastatic B16-F1 murine melanoma cell line throughout. Briefly, B16-F1 cells previously conditioned in an acidic media (pH of 6.8 for 20 weeks) were subjected to various pro-apoptotic stimuli. The percentage of cell death was assessed at 2, 6 and 12 hours for serum deprivation (1% FCS) and hyperthermia (430C). Heat shock (400C) was measured at 2 hours followed by hyperthermia at 2 and 4 hours. Dosages of 20, 30 and 40 Gy were administered in the γ-irradiation study. Viability was assessed by trypan blue exclusion and apoptotic death was demonstrated by DNA fragmentation on gel electrophoresis. For each condition each experiment was carried out in triplicate. Expression of Bcl-2 and Bax were measured by Western blot analysis. Results: The data show less apoptotic cell death in acidic conditioned, acidic-resistant B16-F1 cells compared with non-acidic conditioned B16-F1 cells. Percentages of cell death in both serum deprivation and hyperthermic conditions were significantly lower at all time points for cells at pH 6.8 versus pH 7.4. The percentage of cell death was lower for heat shock and subsequent hyperthermic conditions in pH 6.8 cells vs pH 7.4. Percentage of cell death after γ-irradiation was lower in cells at pH 6.8 at all radiation dosages vs pH 7.4. An absolute increase in the expression of Bcl-2 and an increase in the ratio of Bcl-2 to Bax were both demonstrated.
Conclusions: Prolonged culture in an acidic media selects for acidic resistant cells that develop resistance to diverse apoptotic inducing stimuli including: serum-deprivation, γ-irradiation, hyperthermia and heat-shock. Furthermore, this resistance is associated with both increased expression of Bcl-2 and an increased ratio of Bcl-2 to Bax.

Apoptotic Inducer	Media pH	Time (hours)/ % Cell Death	Time (hours)/ % Cell Death	Time(hours)/ % Cell Death
Serum Deprivation	6.8	2/31.70	6/48.74	12/82.61
	7.4	2/43.45	6/63.54	12/97.32
Hyperthermia	6.8	2/34.82	6/46.91	12/83.73
	7.4	2/44.63	6/64.18	12/94.88
Heat Shock	6.8	2 (400C)/22.83	2 (430C)/35.10	6(430C)/46.64
	7.4	2 (400C)/34.21	2 (430C)/43.80	6(430C)/59.99

	Dose (Gy)/ % Cell Death	Dose (Gy)/ % Cell Death	Dose (Gy)/ % Cell Death
gamma irradiation	6.8 20/29.99	30/42.13	40/48.54
	7.4 20/39.10	30/52.61	40/63.43

Keywords: apoptosis, cancer, Bcl-2

Presenting Author: Michelle Hughes

RNAi targeting of XIAP sensitizes tumor cells to TRAIL-induced apoptosis and reveals an additional regulatory role for XIAP upstream of mitochondria

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X-linked inhibitor of apoptosis (XIAP) is a member of the Inhibitor of Apoptosis (IAP) family that has been implicated in the suppression of apoptosis. XIAP is proposed to inhibit apoptosis by virtue of its ability to suppress caspase-9/-3 and -7 activity but also via targeting pro-apoptotic molecules for ubquitination and subsequent proteasomal degradation. Here, we further investigate the role of XIAP in regulating apoptosis by examining the effect of small interfering (si) RNA-mediated silencing of the XIAP gene in breast tumor cells. In MCF-7 cells stably expressing vector-borne shRNAs, silencing of XIAP sensitized both wild-type MCF-7 cells (caspase-3-null) and MCF-7 cells over expressing caspase-3 (MCF-7-casp-3) to different apoptotic stimuli, including TRAIL and etoposide. Sensitization to TRAIL was associated with increased mitochondrial release of the pro-apoptogenic factors, cytochrome c and Smac. Intriguingly, increased cytochrome c release was coupled with increased caspase-8-mediated Bid cleavage, and enhanced Bax translocation in RNAi-targeted cells suggesting a role for XIAP upstream of the mitochondria. We show that this, in part, is mediated via a caspase-6-dependent feedback-amplification loop, which in turn results in increased processing of procaspase-8. By contrast, analysis of the TRAIL death-inducing signalling-complex (DISC) in XIAP depleted cells did not reveal any significant increase in caspase-8 processing within the DISC itself. Taken together, these data reveal an additional, as yet unidentified, regulatory role for XIAP upstream of mitochondria, but downstream of the DISC, in death-receptor-mediated apoptosis. Thus, we establish that XIAP suppresses apoptosis by targeting multiple steps in the apoptotic pathway and that modulation of XIAP represents an attractive therapeutic target in breast tumor cells, irrespective of their caspase-3 status.

Keywords: Caspase-8; DISC; MCF-7; RNA interference; TRAIL; XIAP

Presenting Author: Ingram Iaccarino

Identification of Praf2 as a novel Bcl-xL-interacting protein

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Protein-protein interactions among members of the Bcl-2 protein family is an essential control point in apoptosis, and has been shown to be a promising level of intervention for cancer therapy. To gain insights into the biochemical implications of Bcl-xL overexpression in tumour development, we performed an extensive screen for membrane proteins interacting with Bcl-xL using the Tandem Affinity Purification (TAP) technology. This strategy led to the identification of several novel Bcl-xL-interacting proteins. They can be divided in 4 functional groups: pro-apoptotic members of the Bcl-2 protein family; proteins with a known or predicted role in general metabolism; proteins with a known or predicted role in cellular trafficking; proteins with unknown function. We focused our effort on the characterization of Praf2, a protein belonging to the PRA1 (Prenylated Rab Acceptor) protein family. The ability of Praf2 to interact with Bcl-xL was confirmed by co-immunoprecipitation. Deletion analysis showed that the C-terminal TM domain of Bcl-xL mediates the interaction with Praf2. We found a general ability of members of the Bcl-2 protein family (Bcl-xL, Bcl-2, Bak and Bax) to interact with members of the PRA family (Praf2 and Praf3). Transient transfection of Praf2 results in a time dependent induction of apoptotic cell death, which is prevented by the co-transfection with Bcl-xL or treatment with the caspase inhibitor zVAD. Futhermore, knock-down of Praf2 expression by RNA interference decreases the sensitivity of HeLa cells to etoposide-induced cell death. We have evidences that Praf2 has the ability to modulate protein secretion. We found that Praf2 knocked down RPE cells have an increased secretion of Urokinase (uPA) in the cell culture medium. At the same time, overexpression of Praf2 in 293T cells completely blocks secretion of co-transfected uPA, and concomitant expression of Bcl-xL is, at least in part, able to rescue uPA expression. Finally we observe a reduced targeting of multi membrane-spanning receptors to the plasma membrane following Praf2 expression. We propose that Praf2 could influence cellular viability by modulating the autocrine secretion of growth/survival factors and the amount of membrane receptors presented on the surface.

Keywords: cancer, cell survival, protein secretion

Presenting Author: Maili Jakobson

N-Bak is regulated post-transcriptionally and causes apoptotic changes in the sympathetic neurons

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mRNA for N-Bak, a BH3-only splice variant of Bak is expressed only in neurons, whereas Bak mRNA is expressed in all tissues but not in the neurons. Despite the normal levels of N-Bak mRNA, endogeneous N-Bak protein is difficult to demonstrate, suggesting a post-transcriptional regulation. Although N-Bak mRNA meets the formal criteria for nonsense-mediated decay, our preliminary results show that it is not the case in the primary neurons.
When overexpressed in the cultured sympathetic neurons deprived of nerve growth factor (NGF), N-Bak retards their apoptotic death. In the NGF-maintained neurons, overexpressed N-Bak localized to Golgi in a C-tail-dependent manner. It caused Golgi dispersal and apoptotic morphology of the mitochondria The death of the neurons was still retarded, probably by blockage of caspase activation.

Keywords: BH3-only protein, neuron

Presenting Author: Irmela Jeremias

Anti-apoptotic signaling of pro-apoptotic cytotoxic drugs on tumor cells

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Both vinca-alkaloids and anthracyclines are cytotoxic drugs in current clinical use and induce apoptosis in target tumor cells.
Here we show that anthracyclines inhibited vinca-alkaloids-induced apoptosis by more than halve in certain tumor cells. Counteraction was found in 10/16 (63 %) tumor cell lines, 4/4 (100 %) anthracycline-resistant cell lines and 4/26 (15 %) primary tumor samples from children with acute leukemia. The anthracycline doxorubicin inhibited vinca-alkaloid vincristin-induced phosphorylation of Bcl-2 family members, mitochondrial depolarization, activation of caspases and cell death, while phosphorylation of JNK was still intact. The inhibitory function of doxorubicin was dependent on ATM and G2-arrest, but independent from p53 as shown by knockdown experiments.
Our data describe for the first time that pro-apoptotic anthracyclines exert anti-apoptotic functions on tumor cells which should be avoided during anti-cancer therapy.

Keywords: cancer, cytotoxic drugs, intracellular signaling

Presenting Author: Tarja Joensuu

Gene expression profiling in cerebellum of Cstb-deficient mouse model for Unverricht-Lundborg disease

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Progressive myoclonus epilepsy of Unverricht-Lundborg type (EPM1, OMIM 254800) is a recessively inherited neurodegenerative disorder characterized by stimulus-sensitive progressive myoclonus, tonic-clonic epileptic seizures and ataxia with an onset at age of 6-15 years. Thus far, ten mutations on the cystatin B gene (CSTB, OMIM 601145) encoding a cysteine protease (cathepsin) inhibitor have been reported to contribute to the disease. Of these an unstable minisatellite expansion of over 30 copies located on the promoter region accounts for 90% of the disease alleles worldwide. The pathological consequence of majority of EPM1 mutations is reduced CSTB gene and protein expression resulting in increased cathepsin activity. The physiological function of CSTB and the molecular pathogenesis of EPM1 are unknown.
A mouse model for EPM1 has been generated by targeted disruption of the mouse Cstb gene. The clinical features, progressive ataxia and myoclonic seizures, of Cstb-deficient mice are similar to symptoms seen in human disease. Mice also show severe loss of cerebellar granule cells due to apoptosis and widespread gliosis, which suggests that CSTB has a role in preventing apoptotic death of specific nerve cells.
Our research aim is to understand and characterize the disease mechanisms and disturbed metabolic pathways of EPM1 starting from microarray-based monitoring of genome-wide gene expression in cerebellum and cultured cerebellar granular neurons of Cstb-deficient mice. The analysis was done with Affymetrix GeneChip Mouse Genome 430 2.0 chips and GeneSpring (v.7.3) program. The pathway analyses were performed using public programs WebGestalt and GeneTools. In the cerebellum, the expression profiling revealed changes in genes related to myelinization, ion transport, and immune response, as well as in genes encoding lysosomal and cytoskeletal proteins. In addition, genes encoding various proteins involved in cellular stress response and in signal transduction were upregulated indicating microglial activation in response to neuronal death. In cultured neuronal cells, genes involved in cell cycle and cell division were upregulated indicating cell cycle re-entry of neurons. Some of the down-regulated genes encode proteins involved in heparan sulfate (HS) biosynthesis or that bind heparan sulfate proteoglycans (HSPGs). These proteins play important roles in various neurodevelopmental processes forming complexes with various extracellular matrix proteins mediating cellular proliferation, differentiation, and migration, as well as in protecting the neuronal cells from apoptosis by activation of cell survival pathways. We have confirmed several of the findings by real time quantitative PCR and have initiated functional analyses of some of the pathways involved.
Our data give insight into the early disease processes of EPM1 and provide a basis for further detailed studies on the pathophysiology of this devastating disease.

Keywords: Cystatin B, progressive myoclonus epilepsy, neurodegeneration, micro-array

Presenting Author: Sarvenaz Karimpour

Investigation of nucleotide binding properties of the NOD domain of NAIP by fluorescence spectroscopy

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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. 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. Fluorescence spectroscopy was used to investigate the interaction of various nucleotides with the NOD domain. In order to do so, the intrinsic fluorescence of the protein was measured at increasing concentrations of various nucleotides either in the presence or absence of magnesium chloride. Thus, the samples were excited at 280 and 295 nm and the emission spectra were obtained. Upon interaction, the fluorescence intensity was increased considerably. The results revealed that presence of magnesium was very important for effective interaction of the nucleotides with the protein.

Keywords: NAIP, NOD domain, nucleotide binding, fluorescence spectroscopy

NF-κB at the crossroads of DNA damage / repair and apoptosis in glioblastoma – a new face of a multi-faceted protein

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Glioblastoma is the most common primary brain tumour and highly resistant to current treatment approaches. Since anticancer therapy primarily acts by inducing DNA damage and subsequently apoptosis in cancer cells, defects in the apoptotic program can provoke resistance. Although the transcription factor NF-κB has predominantly been implicated in protecting cells by inducing the expression of anti-apoptotic genes, there is growing evidence that it can also mediate cell death depending on the cellular context.
Here, we investigated the role of NF-κB in DNA damage and repair processes and apoptosis resistance in glioblastoma by engineering cell lines that stably express a mutant form of IκBα (IκBα superrepressor). It cannot be phosphorylated and consequently sequesters NF-κB permanently in the cytosol. Activation of NF-κB upon treatment with DNA-damaging drugs such as Doxorubicin was completely blocked by overexpression of IκBα superrepressor.
Interestingly, we found that the level of drug-induced DNA damage upon treatment with Doxorubicin was significantly reduced in U87MG cells if NF-κB activation was blocked. Concomitantly, levels of apoptosis were decreased when NF-κB was inhibited by overexpression of IκBα superrepressor. Similarly, blockage of NF-κB activation reduced DNA damage and apoptosis following incubation with Daunorubicin and Mitoxantron. Knockdown of p53 by vector-based RNA interference in U87MG cells, which are p53 wildtype, increased DNA damage levels in cells expressing IκBα superrepressor, pointing to an inhibitory role of NF-κB on p53-mediated DNA repair. Western blot analysis revealed that p53 similarly accumulated upon treatment with DNA-damaging agents in the presence or absence of NFκB, showing that NF-κB did not interfere with p53 accumulation.
Moreover, apoptosis triggered by the death-receptor ligand TRAIL was profoundly inhibited when NF-κB was blocked, indicating that NF-κB promotes TRAIL-induced apoptosis.
These findings explicitely point to an involvement of NF-κB in the regulation of DNA damage and repair processes. Furthermore, NF-κB exerts a pro-apoptotic role in glioblastoma cell lines, which might have important implications for the use of NF-κB inhibitors in the treatment of glioblastoma.

Keywords: NF-κB, DNA damage, glioblastoma

SPIKE – A Cellular Signaling Pathways Resource for the DNA Damage and Apoptosis communities

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Signaling networks that govern cellular physiology form an intricate web of tightly regulated interlocking processes. The DNA damage response (DDR), which encompasses various DNA repair mechanisms, cell cycle check-points, apoptotic and non-apoptotic forms of programmed cell death, as well as general stress responses, exemplifies this overwhelming complexity. Data on these regulatory networks are accumulating at an unprecedented pace, and hence, their assimilation, visualization and interpretation have become a major challenge in biological research.
To cope with this challenge, we are developing the SPIKE knowledge-base of signaling pathways. SPIKE contains three main software components: 1) A database (DB) of biological signaling pathways. Carefully curated information from the literature and data from large public sources constitute distinct tiers of the DB. 2) A visualization package that allows interactive graphic representations of regulatory interactions stored in the DB and superposition of functional genomic and proteomic data on the maps. 3) An algorithmic inference engine that analyzes the networks for novel functional interplays between network components.
SPIKE is designed and implemented as a community tool and therefore provides a user-friendly interface that allows users to upload and share signaling data with other research labs. SPIKE's database contains extensive, up-to-date, and highly curated data on pathways induced by DNA damage, such as cell-cycle regulation, DNA repair, apoptosis, and other stress responses.
Our vision is that data and network maps will be created and shared in a distributed and highly collaborative effort undertaken by multiple groups in the research community, where each group contributes data in its field of expertise. Expanding the collaborative effort will provide the research community with an integrated, comprehensive and constantly up-to-date KB of the ever-growing DDR networks.
SPIKE is available at: http://www.cs.tau.ac.il/~spike/

Keywords: Signaling networks, apoptosis

AIF Deficiency in the Adult Forebrain leads to Neurodegeneration

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Loss of apoptosis-inducing factor (AIF) has been shown to result in impaired mitochondrial respiration followed by rapid cell death. To evaluate the requirement of AIF in the mature brain, mice carrying a floxed AIF allele were interbred with those carrying CamKIICre, to generate mice lacking AIF in the forebrain. As previously reported, western blot analysis revealed a reduction in the 39 kDa subunit of complex I, which was evident as early as one month of age. Cell loss, however, was only evident by after 3 months. Surprisingly, these animals survive up to 3 months of age despite the metabolic defect resulting from impaired oxidative phosphorylation. Cell counts revealed significant neuronal cell loss within the cortex and CA1 region of the hippocampus. Immunohistochemical analysis revealed the upregulatoin of autophagy markers including Beclin-1 and Atg-7. Relevant to the induction of autophagy, we also examined total and phosphorylated AMPK, an energy sensing kinase. Total AMPK levels did not differ between wild type and knockout animals at any time point. However, phosphorylated AMPK was reduced in knockout animals at three months of age. Taken together, these findings demonstrate that AIF-deficiency in the adult brain results does not induce rapid cell death but instead results in cell loss by a mechanism reminiscent of autophagy resulting in a gradual neurodegeneration.

Keywords: Autophagy, neurodegeneration

Evidence of BNIP3 involvement in TNF-á induced cell death pathway

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BNip3 is a pro-apoptotic molecule that belongs to BH3-only Bcl-2 family proteins. When overexpressed, BNip3 induces apoptotic, necrotic, or autophagic cell death, depending on types of cells or injuries. Adenoviral E1B19K protein, a homologue of mammalian Bcl-2 protein, specifically interacts with BNip3 and inhibits its pro-apoptotic activity. Given that the suppression of apoptotic cell death by viral protein is an early and essential step for efficient viral replication, we have hypothesized that BNip3 may function as a cell death machinery that has to be blocked by early viral proteins. On this hypothesis, we speculated the involvement of BNip3 in cell death signals provoked by cellular defense mechanisms against viral infection. In this study, we show that BNip3 inserted into the mitochondrial membrane, which is the requisite for BNip3-induced cell death, when cell death is triggered by TNF-α. These observations suggest that caspase-8-mediated release of cathepsin B from lysosomes enhances BNIP3 activation but C-Jun NH2-terminal kinase (JNK)-independent pathway in TNF-α-treated. Furthermore, our results show that BNip3-induced necrotic cell death is an alternative route selected when main Bid pathway was blocked. In conclusion, our data suggest that BNip3 is an effecter molecule responsible for TNF-α-induced caaspase-8/cathepsin B-associated mitochondrial damage.

Keywords: Apoptosis, BNIP3-mediated Necrotic cell death, TNF-α, Cathepsin B

Presenting Author: Yong-Jun Kim

The putative tumor suppressor GLTSCR2 induces apoptotic cell death and its expression is decreased in glioblastomas

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Glioma tumor suppressor candidate region gene 2 (GLTSCR2/PICT-1) is localized within the well-known 1.4-Mb tumor suppressive region of chromosome 19q, which is frequently altered in various human tumors including diffuse gliomas. Aside from its chromosomal localization, several lines of evidence, including PTEN phosphorylating and cell-killing activities, suggests that GLTSCR2 participates in the suppression of tumor growth and development. However, little is known about the biological functions and molecular mechanisms of GLTSCR2 as a tumor suppressor gene. We investigated that GLTSCR2 can induce cell death through the cell cycle arrest and inhibition of cell division, resulting in cellular catastrophe with several apoptotic features. Also we found that GLTSCR2 expression is downregulated in glioblastomas, suggesting that GLTSCR2 is the tumor suppressor gene. In addition, direct sequencing analysis and fluorescence in situ hybridization clearly demonstrates the presence of genetic alterations, such as a nonsense mutation and deletion, in the GLTSCR2 gene in glioblastomas. Finally, our immunohistochemical study on glial tumors demonstrates that GLTSCR2 is sequentially downregulated according to the histological malignant progression of the astrocytic glial tumor. Taken together, our results suggest that GLTSCR2 is cell death gene and involved in astrocytic glioma progression.

Keywords: GLTSCR2, tumor suppressor gene, cell death gene, glioblastoma

Presenting Author: Krisztina Koroskenyi

Adenosine A2A receptors mediate partially the anti-inflammatory phenotype of macrophages exposed to apoptotic cells

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Phagocytosis of a variety of targets normally triggers a battery of proinflammatory responses in macrophages, including the generation of reactive oxygen-derived intermediates, the release of proteolytic enzymes and the production of numerous inflammatory cytokines. In sharp contrast, ingestion of apoptotic cells by macrophages induces an anti-inflammatory phenotype. Apoptotic cells do not simply fail to provide proinflammatory signals, but they actively interfere with the inflammatory program, as preincubation with apoptotic cells strongly suppresses the lipopolichacharide (LPS)-induced inflammatory response of macrophages. Adenosine has been known for a long time to mediate anti-inflammatory effects on macrophages via its adenosine A2A receptors. Since previous studies have demonstrated the release of adenosine from macrophages digesting apoptotic cells, in the present study the possible involvement of adenosine A2A receptors in the development of anti-inflammatory phenotype in macrophages exposed to apoptotic cells was investigated. Using cytokine array we show, that the lack of adenosine A2A receptors in adenosine A2A null macrophages does not affect the proinflammatory cytokine production of macrophages exposed to apoptotic cells. Neither did it affect the ability apoptotic cells to suppress the proinflammatory cytokine production of LPS-induced macrophages. However, the loss of adenosine A2A receptors, resulted in a higher LPS-induced proinflammatory cytokine production. In addition, macrophages exposed to apoptotic cells produced sufficient amount of adenosine to inhibit LPS-induced proinflammatory cytokine production of macrophages, which themselves were not exposed to apoptotic cells. Our data report for the first time that one of the mediators of the anti-inflammatory phenotype induced by apoptotic cells is the macrophage derived adenosine.

Supported by OTKA 049445 and ETT 115/2006.

Keywords: adenosine, apoptotic cell uptake, inflammation

Measurement of NK-mediated target cell cytolysis by flow cytometric detection of caspase 6 activity

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NK-mediated cytolysis of target cells constitutes a form of apoptosis, and is accompanied by many of the same events associated with receptor-mediated apoptosis, including caspase activation. With fluorogenic substrate (OncoImmunin, Inc., USA) incorporated into a target cell line the caspase 6 activation in target cells can be measured following coincubation with cytolytic cells. This method engages prior tagging of the target cell line with a tracking dye followed by substrate loading and plating with the cytolytic cell of interest. The cells are then analyzed by single-laser flow cytometry, gating for tagging dye-positive cells. Cytotoxicity of NK cells and NK-like cell lines toward standard target K562 cells was analyzed with the caspase 6 detection method. A portion of K562 cells contained high level of activated caspase 6 augmented corresponding the increase of effector:target ratio and amounted to 90% after 1 hour incubation with NK cells. This approach was then employed for assessment of NK-cell-mediated cytolysis of MICA-positive cells. Human NK cells were isolated from peripheral blood mononuclear cells using the Miltenyi Biotec negative selection magnetic bead system and then were activated with IL-2 in 5 days that caused several-fold increase of NKG2D expression on NK cell surface. It is known that interaction of receptor NKG2D with stress-induced protein MICA expressed on a target cell results in NK cell activation and stimulation of cytolytic function. By caspase 6 fluorescence detection, we demonstrated three-fold difference of cytotoxic lysis of C1R cells and C1R-MICA cells that constitutively expressed MICA on their surface. Blocking with MICA- or NKG2D-specific antibodies resulted in decrease of C1R-MICA cell cytolysis and loss of the difference.

Keywords: NK cells, cytotoxicity, caspase 6

Presenting Author: Jan Kovar

Mechanisms of cell death induced by classical taxane paclitaxel and novel taxane SB-T-1216 in sensitive and resistant breast cancer cells

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Taxanes are mitotic poisons that are widely used in cancer therapy. However, molecular mechanisms of taxane-induced cell death as well as molecular mechanisms causing resistance of cancer cells to taxanes remain unclear.
Employing taxane-sensitive cell line MDA-MB-435 and taxane-resistant cell line NCI/ADR-RES, we compared the effect of classical taxane paclitaxel with the effect of novel taxane SB-T-1216 on cell cycle progression, the activation of caspases and the formation of interphase microtubule bundles and aberrant mitotic spindles.
Novel taxane SB-T-1216 was more effective than paclitaxel, particularly in resistant cells. The C50 of the novel taxane SB-T-1216 (concentration resulting in 50% of living cells in comparison with the control) in sensitive cells was approximately 0.5 nM versus approximately 1 nM for paclitaxel. However, the C50 of SB-T-1216 in resistant cells, was approximately 3 nM versus approximately 300 nM for paclitaxel. Cell death induced by paclitaxel in sensitive as well as in resistant cells by the lethal concentrations (30 nM for sensitive and 3000 nM for resistant cells) was associated with the accumulation of cells in the G2/M phase. Cell death induced by lethal concentrations (10 nM for sensitive and 100 nM for resistant cells) of SB-T-1216 seemed to be rather associated with decreased number of the G1 cells and the accumulation of hypodiploid cells. Cell death induced by both paclitaxel and SB-T-1216 was accompanied with activation of caspase-3, caspase-9, caspase-2 and caspase-8 in sensitive as well as resistant cells. Both SB-T-1216 and paclitaxel at a concentration of 100 nM induced the formation of interphase microtubule bundles and mitotic multipolar spindles in resistant cells. SB-T-1216 at this concentration induced death in nearly all resistant cells during 96-hour incubation in contrast to 100 nM paclitaxel which was completely ineffective in cell death induction.
We conclude that cell death induced by taxanes in breast cancer cells is not necessarily related to the formation of interphase microtubule bundles and mitotic multipolar spindles and to the accumulation of cells in G2/M phase. It seems that novel taxane SB-T-1216 can induce cell death via a pathway involving the accumulation of hypodiploid cells.

This work was supported by grant NR9426-3/2007 from IGA, Ministry of Health of the Czech Republic.

Keywords: cell death, novel taxane SB-T-1216, paclitaxel, cell cycle, microtubule bundles, caspases, breast cancer cells

Emotional stress activates anti-apoptotic signaling in prostate cancer

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The potential connection between emotional stress and cancer has been a subject of a long-standing debate. Some epidemiological studies supported the notion that emotional stress increases incidence of cancer and aggravate the course of existing cancer. Other studies came to the opposite conclusion.
This controversy is due in part to the lack of information on the mechanisms that connect emotional stress and cancer. Indeed, no direct effects of stress hormones on cancer cells have been documented.
In the course of analyzing anti-apoptotic network in prostate cancer, we observed that epinephrine, in physiologic concentrations, comparable to those observed after emotional stress, can protect prostate cancer cells from apoptosis. The anti-apoptotic mechanism of epinephrine primarily involved activation of beta2-adrenergic receptors that lead to BAD phosphorylation by PKA. Expression of BAD with mutated PKA phosphorylation site S112 significantly reduced anti-apoptotic effect of epinephrine.
Activation of epinephrine/PKA axis was not limited to tissue culture conditions. Thus, subjecting mice to emotional stress (exposure to the fox scent) or injection with epinephrine activated PKA in xenograft tumors. Activation of PKA/BAD pathway was observed until 12 hours after stress/epinephrine administration. Therefore single stressful event may result in prolong activation of anti-apoptotic signaling mechanism in tumors.
Furthermore, stress or epinephrine injection increased resistance of prostate cancer xenografts to apoptosis induced by PI3K inhibitor LY294002. Thus, increased stress/epinephrine interfere with anti-cancer therapies in mouse model of prostate cancer.
Recent epidemiological studies support the possibility that emotional stress/epinephrine may contribute to prostate cancer. Indeed, increased levels of mood disturbances have been reported among prostate cancer patients; at the same time 15% reduction of prostate cancer incidence was reported in patients who regularly take beta-blockers.
In light of these evidences it might be valuable to identify patients with increased levels of stress hormones. Reducing stress/epinephrine levels in such patients is expected to improve their responses to anti-cancer therapies.
To our knowledge, this is the first report that demonstrates activation of an anti-apoptotic mechanism by emotional stress in cancer cells in tissue culture and in vivo.

Keywords: emotional stress, epinephrine, prostate cancer, PKA, BAD

Vaccinia virus anti-apoptotic F1L is a novel Bcl-2-like domain swapped dimer that binds a highly selective subset of BH3 containing death ligands

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Programmed cell death (apoptosis) is a critically important mechanism that enables multicellular organisms to eliminate damaged, infected or unwanted cells. The Bcl-2 family of proteins, which contains both pro- and antiapoptotic members, plays a central role in regulating apoptosis. The two pro-apoptotic members Bax and Bak are activated in response to apoptotic stimuli and play a pivotal role by triggering the release of pro-death factors by a series of unknown conformational events that result in mitochondrial membrane permeabilization (MMP). In healthy cells, Bax and Bak are held in check by anti-apoptotic family members such as Bcl-2, Bcl-xL and Mcl-1. Apoptotic stimuli result in the release of pro-apoptotic BH-3 only proteins that neutralize anti-apoptotic Bcl-2, thus freeing Bak and Bax to cause MMP.
Apoptosis is recognised as a key innate immunity defence mechanism, and viruses have developed different strategies to ensure their survival in the face of host immune responses. Some viruses express proteins homologous in sequence and function to mammalian pro-survival Bcl-2 proteins. Anti-apoptotic F1L expressed by vaccinia virus is essential for survival of infected cells, but it bears no discernable sequence homology to proteins other than its immediate orthologs in related pox viruses. The crystal structure of F1L reveals a Bcl-2-like fold with an unusual N-terminal extension. The protein forms a novel domain-swapped dimer in which the α;1 helix is the exchanged domain. Binding studies reveal an atypical BH3 binding profile, with sub-micromolar affinity only for the BH3 peptide of pro-apoptotic Bim and low micromolar affinity for the BH3 peptides of Bak and Bax. This binding interaction is sensitive to F1L mutations within the predicted canonical BH3-binding groove, suggesting parallels between how vaccinia virus F1L and myxoma virus M11L bind BH3 domains. Structural comparison of F1L with other Bcl-2 family members reveals a novel sequence signature that redefines the BH4 domain as a structural motif present in both pro- and anti-apoptotic Bcl-2 members, including viral Bcl-2-like proteins.

Keywords: Vaccinia virus, Bcl-2, mitochondria, X-ray crystallography

BH3 mimetics re-activate autophagic cell death in anoxia-resistant malignant glioma cells

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Here, we investigated the specific roles of Bcl-2 family members in anoxia tolerance of malignant glioma. Flow cytometry analysis of cell death in 17 glioma cell lines revealed drastic differences in their sensitivity to oxygen withdrawal (< 0.1 % O2). Cell death correlated with mitochondrial depolarisation, cytochrome C release and translocation of GFP-tagged LC3 to autophagosomes, but occurred in the absence of caspase activation or phosphatidylserine exposure. In both sensitive and tolerant glioma cell lines, anoxia caused a significant upregulation of BH3-only proteins previously implicated in mediating anoxic cell death in other cell types (BNIP3, NIX, PUMA and Noxa). In contrast, we detected a strong correlation between anoxia resistance and high expression levels of anti-apoptotic Bcl-2 family proteins Bcl-xL, Bcl-2 and Mcl-1 which function to neutralise the pro-apoptotic activity of BH3-only proteins. Importantly, inhibition of both Bcl-2 and Bcl-xL with the small-molecule BH3 mimetics HA14-1 and BH3I-2´ and by RNA interference reactivated anoxia-induced, autophagic cell death in previously resistant glioma cells. Our data suggest that endogenous BH3-only protein induction may not be able to compensate for the high expression of anti-apoptotic Bcl-2 family proteins in anoxia-resistant astrocytomas. They also support the conjecture that BH3 mimetics may represent an exciting new approach for the treatment of malignant glioma.

Keywords: Bcl-2 family, hypoxia, cell death, autophagy, Bnip3

PUMA mediated apoptosis can be essential for tumor formation

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DNA-damage induced apoptosis in lymphocytes is mainly mediated by the pro-apoptotic Bcl2-family member PUMA. It is well established that PUMA is upregulated in different cell types in a p53-dependent manner upon ã-irradiation and its absence renders thymocytes and mature lymphocytes highly resistant to ã-irradiation induced apoptosis. Since it is widely believed that apoptosis triggered in response to DNA-damage serves to suppress tumorigenesis, we explored the role of PUMA in the formation of radiation-induced thymic lymphomas.
Surprisingly, whereas loss of one or both alleles of p53 drastically accelerated tumor development, loss of puma, but not loss of a related BH3-only protein, bad, rescued these mice from lymphomagenesis. The observed inhibitory effect on tumor development required functional p53, since mice lacking p53 and puma succumbed to disease as quickly as p53-deficient mice. Based on previous findings, we speculated that tumor formation in this cancer model may be caused by neoplastic transformation of damaged hematopoietic stem cells that are forced to proliferate to re-populate the thymus and secondary lymphatic organs. We found that both thymocytes as well as bone marrow derived hematopoietic progenitors deficient for PUMA are highly resistant to irradiation-induced apoptosis. Noteworthy, mice over- expressing a BclxL-transgene exclusively in thymocytes, but not in hematopoietic progenitors, developed thymic lymphomas as readily as wild type mice. Therefore, we propose that in wild type mice, ã-irradiation introduced DNA-damage reduces the number of hematopoietic progenitors that carry damaged genomes and need to repopulate the hematopoietic system of the host culminating in massive asymmetric cell proliferation. This might generate a pool of pre-malignant tumorigenic stem cells that can acquire subsequent oncogenic lesions which then receive the final fully transforming hit with highest frequency in the thymus, due to its rapid cellular turnover rate and TCR-rearrangement activity.
In summary, our data suggest that the acute DNA-damage response is dispensable for tumor suppression and can even foster tumorigenesis.

Keywords: PUMA, cancer, DNA damage, stem cells

Presenting Author: Thomas Landes

A new OPA1 partner links mitochondrial morphology and apoptosis

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The morphology of the mitochondrial network depends on the equilibrium between antagonistic forces acting on the fission and the fusion of mitochondrial membranes. The state of the mitochondrial outer membrane is determined both by fission driven by Dnm1p/Drp1, and fusion controlled by Fzo1p/Mfn1-2. The dynamics of the inner membrane, and the mechanisms involved in the modeling of the cristae, are not well characterized. The dynamin Mgm1p/Msp1p/Opa1 is believed to control these processes and as a consequence to regulate apoptosis. Since mutations in Opa1 are associated with autosomal dominant optic atrophy, a cause of inherited blindness, a better understanding of the functions of this protein is crucial to gain further insight into the pathological processes associated with this disease.
Using 2H strategy, we identified a proapoptotic member of the Bcl2 family as an Opa1 partner. The interaction was confirmed by GST pull-down and co-immunoprecipitation experiments. Overexpression of this Opa1 interactor induced mitochondrial fragmentation by loss of fusion and also triggers apoptosis, both effects being alleviated by Opa1 overexpression.
This finding constitutes the first evidence of a direct link between Opa1 and the apoptotic machinery, shedding a new light on the relationships between mitochondrial morphology and apoptosis.

Keywords: apoptosis, mitochondrial morphology, Opa1

Presenting Author: Maike Laussmann

Intracellular signalling kinetics during bortezomib-induced apoptosis.

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The proteasome inhibitor bortezomib is a therapeutically relevant apoptosis-inducing drug. Proteasome inhibition leads to complex alterations of the intracellular protein composition resulting from impaired protein degradation and stress-induced transcriptional responses.
In HeLa cervical cancer cells, we analyzed bortezomib-induced cell death signalling by both biochemical bulk analysis and single cell imaging. We found that bortezomib induces upregulation of Bim, mitochondrial outer membrane permeabilisation (MOMP) and subsequent sequential activation of caspases-9 followed by -3 and -8. Cells died individually with cell death peaking between 24 h and 48 h. Fluorescence resonance energy transfer (FRET)-based time-lapse microscopy was used in single living cells for the detection of effector caspase activation. Cleavage of the FRET probe at the caspase cleavage site DEVD was detected approximately 2 min after MOMP.
Pathologically high expression of anti-apoptotic Bcl-2 family members is a frequent cause of tumour resistance to therapy. Even though Bcl-2 over-expression inhibited MOMP and the release of cyt-c and Smac into the cytosol, we still detected a slow increase in processed caspase-3 and -8, as well as caspase-3-processed caspase-9 subunits. Furthermore, we detected submaximal caspase activity resulting in mild cleavage of the DEVD-FRET probe in absence of MOMP in individual living cells.
Our preliminary results thus suggest an alternative, MOMP-independent mechanism of caspase-3 activation that can manifest in response to proteasome inhibition by bortezomib.

Keywords: bortezomib, time-lapse microscopy, caspase activation

Presenting Author: Jacqueline Lessig

Contribution of components of inflammatory processes to spermatozoa quality

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Chronically proceeding processes in the male genital tract frequently occur as silent inflammations without clinical symptoms. Although the existence of silent inflammations is often underestimated they are one reason for unwanted childlessness.
Inflammatory tissue is invaded by polymorphonuclear leukocytes participating in the regulation of inflammations. Polymorphonuclear leukocytes are associated with the release of a certain amount of immune mediators. Immune mediators (e.g. from the azurophilic granules secreted proteins as myeloperoxidase and elastase) affect both pathogen killing and tissue destruction. The elastase-release emphasizes the transition of apoptotic to necrotic cells associated with an increasing generation of pro-inflammatory cytokines and subsequent prolonged inflammation.
In this study we focussed our attention on the influence of elastase and pro-inflammatory cytokines responsible for chronic inflammations on spermatozoal properties.
Concentration- and time-dependent spermatozoa treatment with elastase provokes a significant increase in annexin V-binding at phosphatidylserine epitopes on cell surfaces as well as a vitality loss. These investigations were performed by flow cytometry.
Contrary, pro-inflammatory cytokines (TNFα, IL6, IL11) only cause a slight increase of the apoptotic pathway whereas TNFα and IL6 additionally alter the lipid composition of spermatozoal membranes characterized by an increase of lysophosphatidylcholine formation. This lysophosphatidylcholine formation was measured with matrix-assisted laser desorption & ionization time-of-flight mass spectrometry and can be inhibited to one third by seminal plasma addition. 50 pg/ml IL11 lead to a significant impairment of sperm vitality, whereas 200 pg/ml cause an absolute vitality loss.
Although, our experiments were performed with mature germ cells, it can be assumed, that the factors serving as markers for silent chronic inflammations cause an induction of necrosis in germ cell precursor cells. Necrosis is accompanied by macerated cell membranes. Elastase secreted from necrotic cells causes vitality loss with membrane damage. Pro-inflammatory cytokines as TNFα and IL6 responsible for the formation of lytic lysophosphatidylcholines further supply macerated spermatozoal membranes.
Subsequently, inflammatory mediators may prolong inflammatory processes by induction of necrosis. This effect might become chronic with impairment of sperm quality.

Keywords: Chronic Inflammation, Cytokines, Elastase, Flowcytometry, Lysophosphatidylcholine, MALDI-TOF MS, Spermatozoa

Small molecule XIAP inhibitors enhance drug- and death receptor-mediated apoptosis in acute and chronic leukemia

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Resistance towards chemotherapy in leukemia can be due to defects in apoptosis pathways, e.g. upregulation of antiapoptotic proteins like "Inhibitor of Apoptosis Proteins" (IAPs). Therefore, inhibition of these proteins seems to be a promising approach to restore defective apoptotic programs in leukemia. Among the IAPs, XIAP is the best characterized and displays the highest potency in inhibiting caspases. In this study, we used small molecule inhibitors to target XIAP in acute and chronic leukemia. XIAP inhibitors sensitized precursor B-cell acute lymphocytic leukemia (pre-B-ALL) cell lines Reh and Nalm-6 for apoptosis induced by various anticancer drugs, for example Cyclophosphamide, Taxol and importantly also AraC, which is a first-line drug in the therapy of acute leukemia. In addition, clonogenic survival of Reh cells was markedly decreased following double treatment with XIAP antagonists and AraC compared to single agent treatment, indicating that the combination treatment also has an impact on long-term survival. Cell death induced by XIAP inhibitors together with AraC was caspase-dependent, as it could be blocked by the broad-range caspase inhibitor zVAD.fmk. Western Blot analysis revealed strong increase of AraC-induced cleavage of caspase-8 and caspase-3 by XIAP inhibitors in Reh cells. Furthermore, XIAP inhibitors enhanced mitochondrial perturbations like loss of mitochondrial membrane potential and release of cytochrome c in AraC-treated Reh cells. Importantly, also primary blasts from a child with acute lymphocytic leukemia were sensitized by XIAP inhibitors for AraC-induced apoptosis. Moreover, small molecule XIAP inhibitors enhanced death receptor-mediated apoptosis. XIAP inhibitors sensitized T-cell acute lymphocytic leukemia (T-ALL) cell lines Jurkat, CEM and Molt-4, but not a variety of pre-B-ALL cell lines to apoptosis induced by a soluble hexameric CD95 ligand, suggesting a preferential benefit of the use of XIAP antagonists together with CD95 ligand in T-ALL compared to pre-B-ALL. Furthermore, XIAP antagonists increased apoptosis in apoptosis resistant chronic lymphocytic leukemia cell lines following treatment with TRAIL or agonistic anti-CD95 antibody. These findings suggest that inhibition of XIAP by small molecule antagonists together with apoptotic stimuli such as chemotherapeutic drugs or death receptor ligands is a promising approach to overcome apoptosis resistance in acute and chronic leukemia.

Keywords: apoptosis, leukemia, XIAP

Presenting Author: Muhammad Mansha

"Bimbam", a novel glucocorticoid-regulated BH-3 containing transcript from the BCL2L11/Bim Locus

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Proapoptotic Bcl-2 family members provoke cell death by neutralizing their anti-apoptotic relatives, which in turn maintain cell viability by regulating the activation of the cell death effectors, the caspases. In this study we investigate a potential novel splice variant of human BCL2L11/Bim, termed "Bimbam". First evidence for Bimbam was obtained by Affymetrix-based whole genome comparative expression profiling, where a corresponding probe set was found to be induced in 7 of the 13 ALL children and 1 adult during systemic glucocorticoid (GC) monotherapy (Schmidt et al., Blood 107: 2061, 2006). The postulated Bimbam mRNA consists of the 5' portion of Bim (up to and including the BH3-containing exon 8) and the 3' portion of Bam, a cDNA originally discovered in a multiple myeloma cDNA library (Claudio et al, 2002). Thus, it encodes a putative protein consisting of the N-terminal region of Bim (including its BH3 domain) and 40 C-terminal amino acids derived from Bam and not present in any known Bim transcript. RT-PCR revealed that the postulated Bimbam mRNA is indeed present in ALL cell lines. Bimbam and Bim proteins have similar molecular masses and could not be distinguished by conventional Western technology. However, the two isoforms were readily discriminated employing 2D gel approach. Regarding subcellular localization of Bimbam, preliminary data suggest that it localizes in mitochondria like Bim, even though it has an entirely different C-terminus. To investigate its function and potential role in GC-induced apoptosis, recombinant Bimbam was cloned into an HIV-derived lentiviral vector for conditional gene expression (pHRtet-CMV-Bimbam). The construct was transfected into 293T packaging cells and virus particles were used to infect human T-ALL cells (CEM-C7H2-2C8) that constitutively express a tetracycline responsive transactivator (rtTA). Stable transfected 2C8 subclones were generated and analyzed by real time RT-PCR, Western blotting and FACS analyses. These analyses revealed that recombinant BimBam appears to be a killer protein with potency similar to that of Bim. Thus this novel splice variant may contribute to the anti-leukemic effects of GCs and perhaps other apoptotic responses.

Keywords: Bimbam, killer protein, Bam,CEM-C7H2-2C8,GC

Discovery and characterization of post-translationally myristoylated proteins in cells undergoing apoptosis via chemoselective ligation: PKCε; a case study

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The 14-carbon fatty acid myristate can be covalently attached to proteins either co- or post-translationally. In those proteins, the myristoyl moiety promotes weak protein-membrane interactions that are essential to their function. In many cases, a polybasic or palmitoylated cysteine residue(s) complement myristoylation to further promote membrane anchoring. In co-translational myristoylation, the removal of the initiator methionine precedes the addition of myristate on the amino terminal glycine residue by N-myristoyltransferase (NMT). Myristoylation can also occur post-translationally upon the exposure of an internal glycine after caspase cleavage during apoptosis. Interestingly, the pro-apoptotic protein BID and the cytoskeletal proteins actin, gelsolin and p21-activated protein kinase 2 (PAK2) are all new examples of post-translational myristoylation during apoptosis. Myristoylation has, therefore, emerged as an important novel regulator of apoptosis. Until recently, myristoylation could only be detected by labelling with [3H]-myristic acid, a laborious, biohazardous and time consuming methodology. Consequently, we have replaced the need of radioactivity by using a bioorthogonal azidomyristate analogue that can be incorporated into proteins, chemoselectively ligated to tagged triarylphosphines and detected by western blotting in a matter of seconds. This has decreased detection times over 5 million fold in some cases. Using computational prediction analysis and metabolic labelling with azidomyristate analogue, we identified five potentially post-translationally myristoylated proteins following caspase cleavage during apoptosis. One of which is the constitutively active C-terminal kinase domain of Protein Kinase C epsilon (ctPKCε). Exogenously expressed ctPKCε-HA was shown to incorporate the azidomyristate label. In contrast, when the glycine residue essential for myristoylation was substituted to alanine there was no detection of incorporation of the azido-myristate analogue. Indirect immunofluorescence confocal microscopy indicates that myr-ctPKCε-HA localizes to endoplasmic reticulum and Golgi structures or to transitional regions between the two organelles. The non-myristoylatable form remained primarily cytosolic. This suggests that post-translational myristoylation of ctPKCε promotes membrane tethering of PKCε during apoptosis and that membrane localization of the constitutively active caspase cleaved product of ctPKCε is likely important for its function.

Keywords: post-translational myristoylation, chemical biology, chemoselective ligation, azidomyristate, triarylphosphine

Increasing the efficacy of ER stress-induced apoptosis for the therapy of metastatic melanoma through combined treatment with fenretinide and velcade.

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Malignant melanoma, the most aggressive form of skin cancer, is one of the most difficult cancers to treat with an increasing incidence in developed countries which has risen faster than any other malignancy in the past 40 yeras. Single agent chemotherapy is largely the treatment of choice for systemic therapy of metastatic melanoma but survival rates rarely exceed 12 months. In recent years, little progress has been made towards an effective treatment for metastatic melanoma and novel adjuvant and systemic therapies are urgently required. The phenomenon of endoplasmic reticulum (ER) stress in tumour cells has emerged as a potential therapeutic target and two relatively new drugs, fenretinide and velcade (bortezomib), each acting via different cellular mechanisms, have been shown to induce ER stress leading to apoptosis in melanoma cells. The aim of this study was to test the hypothesis that ER stress-induced apoptosis of melanoma cells may be increased by combining clinically-achievable concentrations of fenretinide and velcade. In three human melanoma cell lines (CHL1, A375 and WM2664), fenretinide synergistically increased apoptosis and decreased viability in response to velcade. This effect was reflected by increased expression of GADD153, a marker of apoptosis induction in response to ER stress. The efficacy of fenretinide in combination with velcade was also assessed in vivo using A375 cells in a xenograft model and resulted in a significant reduction in tumour volume compared with the response to fenretinide or velcade alone. Furthermore, analysis of tumour samples at 10 days resulted in both a significant decrease in proliferation (Ki67 staining) as well as increased apoptosis in tumours after treatment with fenretinide and velcade compared to control. These results suggest that fenretinide and velcade, both of which are available in clinical formulations, may be an effective combination therapy for the treatment of metastatic melanoma.

Keywords: Melanoma, ER stress, fenretinide, velcade, synergy, apoptosis

Pharmacological inhibition of caspase activity - impact on cell death in explant cultures

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Explant cultures are widely used to follow 3R principles in laboratory animal science in functional experiments and have been modified also for cell death research. Limb explant cultures are applied to study cell death in interdigital segments during digitalization, mandibular explant cultures to study cell death in developing tooth primordia. Apoptosis takes place in all stages of tooth development but the most distinguishable apoptotic populations can be found in the signalling centres of the enamel knots.
Pharmacological inhibitor of general caspases (zVAD) was delivered in the culture medium or on soaked bead to target specific structures. To trace penetration of the inhibitor within the tissue, biotinylated pan caspase inhibitor (R&D Systems) was used. Apoptosis inhibition was achieved in both experimental systems as demonstrated by histological examination of the cultures (apoptotic bodies) and TUNEL test negativity, however, the effect was limited in the time course.
As recently published, zVAD inhibition of apoptosis may divert cells into an autophagic or necrotic fate. However, zVAD seems to inhibit also lysosomal proteases and thereby autophagy. Moreover, autophagy is likely to function in cell survival promotion. To follow fate of the primary enamel knot cells in developing molar teeth after zVAD caspase inhibition, different experimental groups were designed. 1) control, with no treatment, 2) control, with 1% DMSO (corresponding to DMSO concentration for the inhibitor dilution), 3) general caspase inhibition for 96 h, 4) general caspase inhibition for 48 h followed by 48 h recovery with no treatment. Mouse mandibular cultures from E13.25 embryos were applied for this investigation.
Our pilot study is based on transmission electron microscopy to distinguish autophagic vs. apoptotic and necrotic morphological features, Beclin1 immunohistochemistry to demonstrate biochemical markers of autophagy and caspase-3 and TUNEL tests to detect apoptotic cells.

Supported by the Grant Agency of the Academy of Sciences (B500450802).

Keywords: development, apoptosis inhibition, alternative cell death pathways

Presenting Author: Paola Matarrese

Mitochondria regulate platelet metamorphosis induced by opsonized zymosan A: activation and long term commitment to cell death

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Background. Changes of mitochondrial membrane potential play a key role in determining cell fate. Mitochondria membrane hyperpolarization has in fact been found after cell activation, e.g., in lymphocytes, whereas depolarization has been associated with apoptosis execution.
Objectives. To investigate the effects of an immunologic stimulus, i.e. opsonized zymosan A, on human platelet mitochondria.
Methods. The effects of opsonized and non-opsonized zymosan on human platelet rich plasma were evaluated by means of flow and static cytometry analyses as well as by biochemical methods.
Results. We found that opsonized zymosan induced, at early time points (90 minutes), significant changes of platelet morphology. This was associated with increased reactive oxygen species production and, intriguingly, mitochondrial membrane hyperpolarization. Later (24 hours) opsonized zymosan induced: i) increased CD47 adhesion molecule expression, ii) platelet aggregation; iii) mitochondrial membrane depolarization, and iv) phosphatidylserine externalization. Although in nucleated cells these late events usually represent signs of apoptosis execution, in opsonized zymosan-treated platelets they were not associated with membrane integrity loss, changes of Bcl-2 family protein expression and caspase activation. In addition, pre-treatment with low doses of a “mitochondriotropic” protonophore counteracted mitochondrial membrane potential alterations, reactive oxygen species production and phosphatidylserine externalization induced by opsonized zymosan.
Conclusions. Mitochondrial hyperpolarization seems to represent a key event in platelet activation and remodeling under opsonized zymosan immunological stimulation. This model system may contribute to the understanding of the pathogenetic role of platelets in vascular complications associated with metabolic and autoimmune diseases.

Keywords: platelets, mitochondrial, immunological stimulus, zymosan

Presenting Author: Lidia Mazur

Cell death - inducing potential of new generation oxazaphosphorines and antimetabolites

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Cell death plays an essential role in chemotherapy. A wide range of anticancer drugs induce programmed cell death. The term 'programmed cell death' nowadays refers to any kind of cell death mediated by an intracellular death program, irrespective of the trigger. Apoptosis, necrosis, autophagy and mitotic catastrophe are accepted to be the distinct cell death pathways. A better understanding of the fundamental mechanisms and the nature of the molecular and biochemical links between the various pathways of cell death induced by the chemotherapeutic drugs, is of key importance in necrobiology of cancer hematopoietic cells. The oxazaphosphorines - mafosfamide cyclohexylamine salt (D-17272), 4-hydro-peroxy-cyclophosphamide (D-18864), glufosfamide (D-19575) and the antimetabolites - cladribine (2-CdA) and fludarabine (FA) are new generation chemotherapeutic agents. Scant information on death induction in pathological hematopoietic cells by these anticancer drugs is available. The aim of the present study was to determine and compare the effects of three oxazaphosphorines D-17272, D-18864 and D-19575 (NIOMECH) and two antimetabolites – cladribine (Biodribin, Bioton) and fludarabine (Fludara, Schering) on cell death induction in pathological hematopoietic cells. The experiments were performed in vitro on human HL-60 and U-937 leukaemic cell lines. Temporary morphological and functional changes occurring in leukemic cells after their exposure to the oxazaphosphorine and antimetabolite agents, were studied. A potential anticancer activity of these new generation drugs was analysed. The mode of cell death triggered in leukemic cells by the oxazaphosphorines and antimetabolites was dependent on the drug given and its dose, the time intervals after exposure of cells to the chemotherapeutic drug, and the type of cells being stimulated to die. The chemotherapeutic agents were able to trigger apoptotic, necrotic, autophagic, and mitotic catastrophe pathways. The different leukemic cell - killing potential of the oxazaphosphorines D-17272, D-18864, and D-19575 as well as the antimetabolites 2-CdA and FA, was shown.

Supported by DS/BiNoZ/IZ/FZ/777 and WRBW/BiNoZ/IZ

Keywords: apoptosis, necrosis, autophagy, mitotic catastrophe, HL-60 and U-937 cells, new generation anticancer drugs, oxazaphosphorines, antimetabolites

Presenting Author: Marie-Thérèse Melki

Impact of NK-DC crosstalk on the destruction of DCs - Influence on the control of viral infections

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Introduction: Dendritic cells (DCs) and natural killer (NK) cells are key innate effectors playing a critical role in early defenses against infections. Evidence of an NK-DC crosstalk has emerged recently. This crosstalk is bidirectional and may lead to both NK cell activation and differentiation into killer cells, DC maturation, or DC apoptosis, depending on the activation state of both cell types. DCs are required for the priming of helper CD4 T cells into Th1 effectors, and the chronic expression of uncontrolled viruses, such as HIV, may induce impaired maturation and destruction of DCs. In this study, we addressed the question of the impact of NK–DC interaction on the destruction of DCs, and the influence of HIV on this crosstalk.
Methods: Immature DCs (iDCs) were prepared from sorted monocytes of healthy donors, cultured for 6 days in the presence of IL-4 and GM-CSF. In some experiments, iDCs were infected with R5-HIV-1 (1 ng/ml of p24). Coculture experiments with autologous purified aNK cells (activated by PHA+IL-2) were performed at various NK: DC ratios. The influence of NK:DC interaction on DC's maturation and apoptosis was analyzed using multiparametric flow cytometry, combining 7-AAD staining with membrane and intracellular stainings with mAbs specific for HLA-DR, DC-SIGN, CD80, CD86, DR4, DR5, mTRAIL, Bcl-2 etc…Viral replication was quantified both by ELISA p24 detection into culture supernatants, and determination of the frequency of infected cells following intracellular p24 staining and FACS analysis.
Results: We report that aNK cells induce apoptosis of uninfected iDCs when cocultured for 24hrs at the 5:1 NK:DC ratio. This cell death process involves TRAIL, detected both in NK:DC coculture supernatants, and at the membrane of CD56bright NK cells. TRAIL's DR4 receptor is induced on DCs as soon as they interact with aNK cells, and NK-dependent apoptosis of DCs is completely abrogated by neutralizing anti-DR4 antibodies.
Following R5-HIV-1 infection of iDCs, aNK-dependent DC's apoptosis is dramatically decreased. Gene array analysis suggests that the survival of infected DCs may be the consequence of upregulation of anti-apoptotic proteins (Bcl-2, Bcl-xl, Bcl-xs-xl, IAPs) and downregulation of caspases (Casp-7 and -9), as a consequence of viral infection of DCs. The possible contribution of aNK cells to the survival of infected DCs needs to be determined.
Conclusion: This study shows that opposite outcomes (apoptosis vs survival) are induced by the crosstalk between aNK cells and iDCs, depending on the infectious status of DCs. Thus, NK cells may be physiologically involved in the homeostatic control of non infected iDC, while they may contribute to the survival of infected DCs, therefore constituting HIV-1 reservoirs in these target cells.

Keywords: Apoptosis, dendritic cells, NK cells, HIV, TRAIL

Presenting Author: Cristina Claudia Mihalache

GM-CSF enhances CD44-mediated neutrophil cell death

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CD44 is a transmembrane glycoprotein involved in cell-cell and cell-matrix interactions. It is expressed in multiple cell types and implicated in a wide range of physiological and pathological processes, such as morphogenesis, organogenesis, haematopoiesis, leukocyte activation, lymphocyte homing, wound healing, cell migration, tumour growth and metastasis. Ligation of CD44 with the monoclonal antibody A3D8 has been shown to induce leukeamic cell death and initial studies on neutrophils showed that GM-CSF increases the CD44 mRNA level. Thus, we hypothesized that CD44 might induce cell death in neutrophils.

In a concentration-dependent manner, ligation of CD44 resulted in a specific induction of neutrophil apoptosis, which was even enhanced upon priming of the cells with the pro-inflammatory cytokine GM-CSF. Similarly to GM-CSF, additional pro-inflammatory cytokines (e.g. IL-1 and IL-6) as well as the chemotactic peptide fMLP were able to enhance CD44-mediated cell death of neutrophils. Moreover, the pan-caspase inhibitor, z-VAD, could hardly block CD44-mediated apoptosis suggesting that mainly caspase-independent apoptosis is launched by CD44 ligation and GM-CSF priming. Morphologic characterization of CD44-mediated apoptosis resulted in an aberrant morphology, including cytoplasmic vacuolization. The chromatin of neutrophils appeared to be more disintegrated and less condensed as compared to classical apoptotic neutrophils, suggesting that autophagic-like apoptosis is occurring under these conditions. By isolating neutrophils from CGD patients, which have a genetic mutation in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase impeding the generation of reactive oxygen species (ROS), we could demonstrate that ROS is a major player in neutrophil CD44-mediated apoptosis. Finally, neutrophils derived from patients suffering from different inflammatory diseases such as sepsis, rheumatoid arthritis and cystic fibrosis, demonstrated increased CD44-mediated cell death. Additional priming with GM-CSF had no further increase of neutrophil cell death, indicating that neutrophils had already been primed in vivo.

Taken together, this data suggest that a ROS-dependent but largely caspase-independent autophagic-like cell death is initiated by CD44 ligation in neutrophils. These findings emphasize CD44 receptors as possible therapeutic targets in inflammatory diseases.

Keywords: CD44, cell death

Presenting Author: Laura Mitrofan

Targeting of apoptotic pathways in cancer cells by zoledronic acid

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Biphosphonates are potent inhibitors of osteoclast function widely used to treat excessive bone resorption associated eg. to tumour bone metastases. They have also anti-tumour activity. However, it is unclear whether this reflects an indirect effect via inhibition of bone resorption or a direct anti-tumour effect. A better understanding of diverse modes of cell death induced by this class of drugs will provide new strategies to target tumors associated with excessive bone resorption.
Nitrogen-containing biphosphonates (N-BPs) act by inhibiting farnesyl pyrophosphate synthase (FPPs), one of the key enzymes of the intracellular mevalonate pathway. The pathway is blocked and the accumulation of a pathway intermediate, isopentenyl pyrophosphate (IPP), consequently occurs. IPP is conjugated to AMP to form a novel ATP analogue (ApppI). The only characteristic of ApppI to promote apoptosis is so far linked to its potential to inhibit the mitochondrial adenine nucleotide translocase (ANT) (1) in a cell free system. However its significance in apoptosis is unclear even though the ability of zoledronic acid (ZOL) and other biphosphonates to induce apoptosis in different cell types have been widely investigated.
Objectives: The present study was undertaken to clarify whether IPP and/or ApppI has a direct involvement in cellular death caused by ZOL.
Strategy: There are marked differences in ZOL induced ApppI formation between different cancer cell lines (2). On this basis we selected four cancer cell lines that differ significantly from each other in their ZOL-induced IPP and ApppI accumulation: human estrogen-dependent (MCF7) and estrogen-independent (MDAMB436) breast cancer cell lines, human myeloma (RPMI826) and human follicular lymphoma cell line (HF28RA).
Results: The amount of IPP/ApppI is correlated with the capacity of cells to undergo apoptosis. Additionally, ZOL induces S-phase arrest. Geranylgeraniol (GGOH), an intermediate of mevalonate pathway, blocks both IPP and ApppI formation and in some degree ZOL induced apoptosis. Our conclusion is that IPP/ApppI formation induces apoptosis but also other mechanisms should be considered. Moreover, the mitochondrial potential is completely preserved in BclXL overexpressing HF28RA cells upon ZOL treatment, suggesting that ApppI is required at a pre mitochondrial or mitochondrial level. However, more experiments are needed to elucidate the exact mechanism. Furthermore, lovastatin, an inhibitor of the enzyme HMGCoA reductase (the major regulatory enzyme of the mevalonate pathway) completely blocks IPP/ApppI formation as determined by mass spectrometry analysis, but enhances apoptosis.
In conclusion, IPP/ApppI accumulation via inhibition of mevalonate pathway seems to correlate with cellular death, but is not an absolute requirement for apoptosis. It seems obvious that multiple pathways are involved in ZOL induced cancer cell death.

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Keywords: cancer, bisphosphonate-induced ATP analog, mitochondria

Pivotal role of caspases in p21 cleavage and stress MAPKs activation during 3-hydrogenkwadaphnin -induced apoptosis of U937 cells

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Despite the depth of knowledge concerning the pathogenesis of acute myeloblastic leukemia (AML), long-term survival remains unresolved. Therefore, new agents that act more selectively and more potently are required. In that line, we have recently characterized a novel diterpene ester, called 3-hydrogenkwadaphnin (3-HK), with capability to induce both differentiation and apoptosis in various leukemia cell lines. After 3-HK (15 nM) treatment, a portion of U937 cells remained in suspension and underwent apoptosis, as confirmed using sub-G1 peak and Annexin-V/PI double staining. The kinetics of caspases activation in the drug-treated U937 cells showed that an increase in activities of caspase-8 and -3 at 12 h were followed with an increase in activity of caspase-9 at 36 h. According to colorimetric assays, when U937 cells were co-treated with caspase-9 inhibitor and 3-HK, the increase in caspase-8 and -3 activities also began at 12 h, suggesting that the apoptotic mechanism was amplified via the involvement of the mitochondria. More detailed investigations revealed that both p21 Waf1 cleavage and activation of stress MAPK pathways (JNK1/2 and p38) were also occurred among apoptotic cells. Interestingly, caspase inhibitors impeded p21 cleavage and JNK1/2 and p38 activation. Therefore, caspases activities are required for 3-HK-mediated apoptosis, p21 cleavage and JNK1/2 and p38 activation. This novel signaling pathway and the efficiency of 3-HK may be useful to improve therapeutic options in AML.

Keywords: 3-hydrogenkwadaphnin, caspase, MAPK, p21, U937

Epoxyeicosatrienoic acids induce growth inhibition and calpain/caspase-12 dependent apoptosis in PDGF cultured 3T6 fibroblast

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Arachidonic acid can be metabolised by the epoxygenase activity of cytochromes P-450-producing epoxyeicosatrienoic acids (EETs). Then, cytosolic epoxide hydrolases catalyse the hydration of the EETs to produce dihydroxyeicosatetraenoic acids (DHETEs). EET synthesis has been identified in several tissues and cells, and these arachidonic acid metabolites exert numerous physiological effects including the regulation of cell growth and differentiation.
Our results show that PDGF stimulates 3T6 fibroblast proliferation while the addition of 5,6-EET, 8,9-EET, 11,12-EET or 14,15-EET (0.1-1 µM) inhibit these process. Furthermore, 5,6-DHETE and 11,12-DHETE (0.1-1 µM) also inhibit cell growth. Interestingly, this growth inhibition was correlated with an induction of apoptosis. Thus, we observed that in presence of PDGF, EETs or DHETEs (0.1-1 µM) induce phosphatidylserine externalization and DNA fragmentation. Our results also show that calpain, as well as caspase-12 and caspase-3, are involved in these events. Considering that we previously reported that EETs were able to induce a marked calcium influx in 3T6 fibroblast, we propose that EETs/DHETEs cause apoptosis through Ca2+-dependent calpain-dependent caspase-12 activation.

Supported by BFU2004-04960 and BFU2007-61727 (MEC)

Keywords: arachidonic acid, epoxyeicosatrienoic acids, apoptosis, caspases, calpain, fibroblast

Presenting Author: Annick Mühlethaler-Mottet

Caspase-10 can not functionally substitute caspase-8 in neuroblastoma cells to activate apoptosis in response to TRAIL

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in most tumour cells. Neuroblastoma (NB), the second most common solid childhood tumour, is a particularly heterogeneous and aggressive disease. The silencing of caspase-8 expression in invasive NB cell lines was shown to be responsible for their resistance to TRAIL-induced apoptosis. We have previously demonstrated that stable restoration of caspase-8 expression fully restored TRAIL sensitivity in the caspase-8/10 silenced IGRN-91 cell line.
Caspase-10 is also frequently down-regulated in aggressive NB cell lines. As the precise role of caspase-10 and its ability to substitute for caspase-8 in TRAIL-induced apoptosis is still controversial, we analysed the particular contribution of caspase-10 in the mechanisms of TRAIL-induced cell death in NB cells.
In contrast to caspase-8, stable re-expression of caspase-10 by retroviral infection in the TRAIL-resistant IGR-N91 cells was not sufficient to restore TRAIL sensitivity. Reverse experiments were performed in TRAIL sensitive caspases-8/-10 positive NB cells using RNA interference strategy. While caspase-10 silencing failed to increase NB cells resistance to TRAIL, caspase-8 silencing resulted in complete resistance to TRAIL-induced apoptosis, indicating that caspase-10 on its own was unable to substitute caspase-8 to activate downstream caspases, Bid and ultimately cell death.
In conclusion, the down-regulation of caspase-10 in NB cells does not contribute to TRAIL resistance. Caspase-10 is not able to substitute caspase-8 in NB to initiate a full apoptotic cascade in response to TRAIL. The precise function of caspase-10 in apoptosis initiation in NB cells remains to be determined.

Keywords: neuroblastoma, caspase-8, caspase-10, TRAIL

Presenting Author: Kert Mätlik

Applying plasmid-based RNAi to the model of trophic factor deprivation-induced neuronal death

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We have developed an RNA interference technique for the in vitro model of trophic factor-deprived apoptotic neurons using microinjection of constructs expressing short hairpin RNA (shRNA). When deprived of nerve growth factor (NGF), the sympathetic neurons from superior cervical ganglion die asynchronously during several days. Thus, a relatively long period of time is required between introducing the shRNA-constructs and assaying survival. Overexpression of shRNAs targeting different sequences resulted in the death of NGF-maintained sympathetic neurons. This toxicity could not be prevented by co-expression of Exportin-5, the component of miRNA maturation pathway which is involved in the nuclear export of pre-miRNAs. By adjusting the expression levels of shRNA we have worked out conditions in which shRNA overexpression is tolerated by these cells. To show that such level of shRNA expression is sufficient for down-regulation of a specific target, we silenced the pro-apoptotic molecule Bax that is critically required for the apoptosis of NGF-deprived sympathetic neurons.

Keywords: neuronal apoptosis, RNAi

Influence of 5-lipoxygenase gene silencing on rat neonatal cardiomyocytes survival at modeling of anoxia-reoxygenation

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5-lipoxygenase (5-LO) is known to play an important role in pathogenesis of anoxia-reoxygenation injury. The aim of our study was to investigate the level of different cell death types (necrosis, apoptosis and autophagic cell death) in rat neonatal cardiomyocytes culture at modeling of anoxia-reoxygenation in conditions of 5-LO gene silencing and also to investigate the changes in 5-LO gene expression in cultured cardiomyocytes at anoxia-reoxygenation. The cardiomyocytes were obtained from ventricles of 2-3 days old rats by several cycles of collagenase-pancreatine digestion. After overnight cultivation we performed 30 minutes of anoxia (5 % CO2 and 95 % of N2) and 60 minutes of reoxygenation (5 % CO2 20 % of O2 and 75 % of N2). The level of 5-LO gene expression was estimated by RT-PCR with sybr green. The si-RNA to 5-LO gene were entered into the cells with use of electroporation procedure. The number of living, necrotic and apoptotic cells was determined by staining of the cardiomyocytes with Hoeñhst 33342 and propidium iodidum (8.75 microM), and the number of autophagic cells was determined by monodansylcadaverine staining.
Primary culture of neonatal rat cardiomyocytes in our experiments contained 90,0 ± 2,8 % living (L), 3,3 ± 1,09 % necrotic (N), and 5,5 ± 2,27 % apoptotic (Ap) cells. The number of cardiomyocytes with autophagic cell death was 4.3 % ± 0.23 After anoxia-reoxygenation we had: L -78,5 ± 5,31% , N – 7,8 ± 3,06 % , Ap – 12,8 ± 5,13 % ($P<0.05$) and 14.2 ± 0.96 % ($P1 < 0.001$,) of autophagic cells. The level of 5-LO gene expression was decreased after anoxia-reoxygenation in 2.6 times. The silencing of 5-LO gene gave the decreasing in its expression in 51.7 times. Decreasing of the living cells number by development of necrosis and apoptosis was observed in 24 hours after 5-LO gene silencing. At modeling of anoxia-reoxygenation in these conditions we had the increase of living cells population compare to anoxia-reoxygenation group. Thus, the inhibition of 5-LO transcription has a protective effects in rat neonatal cardiomyocytes culture at anoxia-reoxygenation.

Keywords: cell death, 5-lipoxygenase expression, anoxia-reoxygenation

Bcr-Abl mediated redox regulation of the PI3k/Akt pathway

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Bcr-Abl causes chronic myelogenous leukemia, a myeloproliferative disorder characterized by clonal expansion of hematopoietic progenitor cells.
In this study, inducible expression of Bcr-Abl in TonB.210 cells is associated with increased production of intracellular reactive oxygen species (ROS), which is thought to play a role in survival signalling when generated at specific levels. Elevated ROS in Bcr-Abl expressing cells were found to increase activation of PI3k/Akt pathway members such as Akt and GSK3beta as well as downstream targets such as beta-catenin and Mcl-1. The up-regulation of these proteins was inhibited by the NADPH oxidase (Nox) inhibitor DPI suggesting that increased ROS might be related to increased activity of one isoform of the Nox family. Knock down experiments using siRNA showed that Nox-4 is probably the main source of increased ROS following Bcr-Abl expression. We showed that Bcr-Abl induced ROS could also increase survival pathway signaling through oxidative inhibition of PP1α, a serine threonine phosphatase that negatively regulates the PI3k/Akt pathway.
Overall our results demonstrate that Bcr-Abl expression increases Nox-4 generated ROS which in turn increases survival signaling through PI3k/Akt pathway by inhibition of PP1α, thus contributing to the high level of resistance to apoptosis seen in these Bcr-Abl expressing cells.

Keywords: Bcr-Abl, ROS, PI3k/Akt, Phosphatases.

Cobalt chloride induced different cell death pathways in neuroblastoma cell lines according to their p53 status.

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In many solid cancer, such as neuroblastoma, during tumor growth, hypoxia is known to induce ROS production, HIF-1α stabilization and p53 upregulation, leading to cell death. Cobalt chloride (CoCl2) is commonly used as a chemical hypoxia-mimetic agent, in particular by HIF-1α stabilization. Nevertheless, divergent data (especially about its transcriptionnal activity) are described concerning p53 implication in CoCl2-induced cell death. In order to determine the precise p53 role, we investigated CoCl2 cell death pathways into two neuroblastoma cell lines, respectively SHSY5Y (wild type- and siRNA- p53) and SKNBE(2c) with a deficient p53 transcriptional activity.
As expected, CoCl2 treatment of SHSY5Y cells (p53wt), induced an apoptotic response triggered by an early Bax and p21 expression enhancement correlated to p53 up-regulation and a phospho-p53 ser 15 translocation to the mitochondria. In SHSY5Y cells down-regulated for p53 expression by RNAi then CoCl2 treated, we observed a cell death signalization. As expected, p53-induced protein expression, such as p21 and Bax, were not up-regulated. Nevertheless, a mitochondrial membrane potential collapse and a caspase-3 activation appeared later. Thus, CoCl2-induced apoptosis was initiated by tBid mitochondrial translocation, indicating a regulator recruitment of cell death receptor pathway.
On the other hand, in hypoxia-like treated SKNBE(2c) cells, cell death features such as mitochondrial transmembrane potential collapse and nuclear fragmentation were evidenced later (after 15 h). The protein p53 was detected at a high level in no-treated cells and significantly abolished in a manner time-dependent. No p53 phosphorylation at serine 15 nor mitochondrial location was observed in those cells. Data suggest an autophagic cell death pathway for SKNBE(2c) cells with a mutated p53. Results confirmed that p53 plays an essential role in the apoptotic response following a hypoxia-like stress, but that its lack or nonfunctionnal status activates alternative cell death pathways.

Keywords: Neuroblastoma, P53, siRNA-p53, SHSY5Y, SKNBE2(c), apoptosis signalization

Presenting Author: Erik Norberg

An increase in intracellular Ca2+ is required for activating mitochondrial calpain to release AIF during cell death

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Deregulation of apoptosis contributes to the development of cancer, as well as to tumor resistance to treatment with anti-cancer agents. Non-small cell lung carcinomas (NSCLCs) are characterized by the resistance to both drug- and radiation-induced apoptosis. Recently, we have shown that staurosporine might reactivate the apoptotic machinery in NSCLC cells and that malfunction of AIF release from mitochondria was essential for the chemoresistance of U1810 NSCLC cells. This suggests an important role for the AIF-mediated cell death pathway in sensitivity/resistance of NSCLCs. However, the mechanism of AIF release is not fully understood. Here we report that an increase in intracellular Ca2+ level is a prerequisite step for AIF release during cell death. Import of Ca2+ from the extracellular store activates mitochondrial calpain, located in the intermembrane space of mitochondria. The activated calpain, in turn, cleaves the membrane-bound AIF and the soluble form can be released from mitochondria upon outer membrane permeabilization through tBid-induced Bax/Bak-mediated pores or through the induction of Ca2+-dependent mitochondrial permeability transition. Inhibition of calpain, or chelation of Ca2+, but not the suppression of caspase activity, prevented processing of AIF. Combined, these results provide novel insights into the mechanism of AIF release.

Keywords: Apoptosis-inducing factor, Calcium, Calpain, Mitochondria, Apoptosis

Presenting Author: Ulla-Maija Nuutinen

Regulation of mitochondrial dependence in TRAIL-induced apoptosis

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Based on Bcl-XL overexpression studies we have identified type I and type II human follicular lymphoma cell lines in response to TRAIL. In type II cells overexpression of Bcl-XL completely blocked TRAIL-induced mitochondrial changes and apoptosis. In type I cells, overexpression of Bcl-XL prevented the loss of mitochondrial membrane potential but only decreased DNA fragmentation and release of cyt c. Interestingly, Bcl-XL did not prevent activation of caspase-8 or cleavage of Bid in either of the cell types. Furthermore, an inhibitor of NF-κB, PDTC, enhanced TRAIL-induced caspase-8 activation, disruption of mitochondrial membrane and DNA fragmentation in type II cells but had no effect on type I cells. In addition, PDTC switched TRAIL-induced apoptosis from type II pathway to type I pathway as demonstrated in Bcl-XL overexpressing type II cells. However, a potent and selective inhibitor of IKK, BMS-345541, enhanced TRAIL-induced apoptosis in both cell types and did not switch apoptosis to type I pathway in Bcl-XL overexpressing type II cells indicating that NF-κB is not the target of PDTC being responsible for the switch. In conclusion, our results show that neither amount of caspase-8 activation nor Bid cleavage, can define if a cell is mitochondria-dependent or -independent in response to TRAIL. Instead, the dependence of mitochondria in TRAIL-induced apoptosis might be regulated by still unknown target of PDTC.

Keywords: TRAIL, mitochondria, apoptosis, human, follicular lymphoma, Bcl-XL, caspase-9, NF-κB

Signaling pathways activated by mild hypoxia- and UVB in cutaneous malignant melanoma

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Cutaneous malignant melanoma are the most lethal of all dermatological cancers. Recent studies suggest that the mild hypoxia (1-5 %O2) of the human skin represents a tumour promoting environmental factor in melanomagenesis (1). In combination with other environmental stresses, such as UVB exposure, and mutations causing defects in cell death and/or survival pathways the hypoxic state of the skin could aid melanoma cell survival by increasing adaptation to cellular stress, induce a more aggressive phenotype and also affect the notorious chemoresistance of melanoma. Since the molecular elements of the cellular response to mild hypoxia and UVB are not completely understood, we set out to investigate the signalling pathways underlying the response of human melanocytes and melanoma cells to these relevant cellular stresses. Because of their crucial involvement in hypoxia and UVB signalling, and their role in melanomagenesis, we focused on the role and cross-talk between Hif-1 (hypoxia inducible factor-1) and MAPKs (mitogen activated protein kinases).
Exposure of melanocytes and melanoma cell lines to mild hypoxia or UVB results in the upregulation and nuclear translocation of Hif-1á, albeit with different intensity and kinetics. Hif-1á upregulation by both stress factors involves a redox-sensitive mechanism, but only for mild hypoxia are the mitochondria-generated ROS crucially important. The transient and biphasic Hif-1á upregulation following UVB is correlated with the rapid and sustained activation of the p38MAPK and JNK-pathways and with a transient activation of ERK- and Akt- pathways. The selective inhibition or knockdown of p38MAPKá significantly attenuates Hif-1á accumulation in normal and cancer cells, indicating that p38MAPKá is a key molecular element of the cascade linking UVB irradiation to Hif-1á stabilization. Moreover, Hif-1á modulation following UVB is associated with the induction of Noxa, concomitant downregulation of Bip/GRP78, and cell death, which occurs through both caspase-dependent and –independent pathways. Conversely, enhanced Hif-1á upregulation in melanoma cells and melanocytes following mild hypoxia does not involve p38MAPKá or JNK-signals, and correlates with Akt activation, sustained Bip/GRP78 levels and cell survival. Moreover, mild hypoxia stimulates autophagy, which is possibly coordinated through Hif-1-dependent BNIP3 upregulation. These results indicate that mild hypoxia and UVB modulate the activity of different redox pathways impinging on Hif-1 upregulation, and which can eventually lead to cell survival (e.g. mild hypoxia) or cell death (e.g. UVB). We are currently characterizing the functional role of Hif-1-dependent pathways in the response of normal melanocytes and melanoma cells to these skin microenvironmental stress factors, in order to further define their role in melanoma development.

1.Bedogni et al., 2005, Cancer Cell 8:443-54

Keywords: melanoma, hypoxia, UVB, signal transduction

Presenting Author: Tracey O'Donovan

Drug resistant oesophageal cancer cell display features of autophagy in response to 5Fluoruracil and Cisplatin.

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Cancers of the oesophagus are highly malignant tumours with five-year survival rates of less than twenty percent. Although combinations of chemotherapy and surgery can effectively remove the primary tumour, in more than 80% of cases this is not curative, due to the presence of drug resistant disseminated cells. It is therefore necessary to improve the anti-tumour efficacy of chemotherapy which ultimately relies on regimes that can induce maximum cancer cell death.

Apoptosis (Type I cell death) is often regarded as the central mechanism of programmed cell death in response to chemotherapeutic agents. Its induction is believed to confer chemo-sensitivity in many tumours, however in recent years, research has identified alternative non-apoptotic cell death programmes in eukaryotic cells (Type II-autophagic cell death). The aim of this work was therefore to understand the death mechanisms that exist or co-exist in oesophageal cancer cells, and assess how they may be more effectively activated.

We took a panel of oesophageal cancer cell lines, two adenocarcinoma (OE19/OE33) and two squamous carcinoma (OE21/KYSE450), and investigated their chemosensitivity and associated cell death pathways. Our results reveal that two of the four oesophageal cancer cell lines display increased resistance to both cisplatin and 5-fluorouracil (5-FU), an affect that is not attributed to original tumour histological subtype. The more drug sensitive cell lines (OE21/OE33) show predominantly, classical apoptotic morphology, which was confirmed by the detection of active caspase-3 and mitochondrial depolarization, in response to both drugs tested.

The more resistant cell lines (OE19 and KYSE450), failed to show any signs of apoptosis, but induced a non-apoptotic cell death mechanism, with features that include pyknosis of the nuclear material and vacuolization of the cytoplasm. Examination, by electron microscopy (EM) revealed morphological changes similar to those described for autophagic type II cell death. Therefore, all cell lines were examined for makers of increased autophagy in response to cisplatin and 5-FU. The formation of acidic vesicular organelles, associated with the induction of autophagy was observed in both resistant cell lines using an autophagy specific dye-Monodansylcadaverine (MDC). In addition, we observed increased levels of the autophagy specific protein LC3-II (Western blotting and fluorescent microscopy) in both OE19 and KYSE450 following drug treatment.

Neither OE21 or OE33 cell lines showed markers of enhanced autophagic cell death. However, inhibition of apoptosis with a caspase inhibitor, lead to a switch from apoptosis to a non-apoptotic cell death mechanism resembling autophagy. This suggests that both cell death mechanisms may co-exist, with apoptotic competent cells showing a preference for apoptotic cell death.

Modulation of the autophagic response in OE19 and KYSE450, with pharmacological inhibitors of autophagy, 3-methyladenine (3-MA) and LY294002, failed to modulate their drug sensitivity. These inhibitors inhibit class III PI3-kinase, which is involved in the formation of the autophagosome, but they can also alter class I PI3-kinase, thus inducing non-specific and possibly opposing signalling effects in cell death pathways.

This data shows that oesophageal cancer cells that fail to undergo apoptosis can under go autophagy, and this is associated with a more drug resistant phenotype. It is anticipated that more specific modulators of this process could improve chemotherapeutic regimes in the future.

Keywords: Oesophageal cancer, Drug resistance, apoptosis, autophagy

Knockdown of Histone Deacetylase 10 Induces Autophagy in Neuroblastoma Cells

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Background: The control of histone acetylation and deacetylation by histone acetyl transferases and histone deacetylases (HDACs) plays an important role in regulating transcriptional programs in normal cells. Deregulation of these epigenetic mechanisms have been linked to different human cancers We want to dissect the contribution of class I, II and IV HDAC family members to the malignant properties of neuroblastoma cells. Here, we investigated the functional role of histone deacetylase 10 for neuroblastoma tumor biology and compared it to the roles of two class I family members, HDAC2 and 8.
Methods: Expression of HDAC2, 8 and 10 in BE(2)-C neuroblastoma cells was inhibited by the use of two siRNAs targeting different regions of the respective HDAC mRNA. Upon HDAC10 knockdown, tumor cell proliferation (automated cell counting) and clonogenic growth (soft agar assays) were measured and different types of cell death were characterized by caspase-3-like activity assay, PARP cleavage and acridine orange staining. Furthermore, differentiation marker expression was investigated following HDAC8 knockdown.
Results: Silencing of the class IIb family member HDAC10 with RNA interference revealed that this enzyme plays a role in BE(2)-C neuroblastoma cell death. Knockdown of HDAC10 decreased both the population doubling time and the ability for anchorage independent growth and induced autophagic cell death (acridine orange staining).Treatment of several neuroblastoma cell lines with pan-HDAC inhibitors also induced autophagic cell death (acridine orange staining). In contrast, knockdown of class I family member HDAC2 induced predominantly apoptosis via caspase-3 activation and knockdown of HDAC8 induced both cell cycle arrest and neuronal differentiation.
Conclusions: Our data show that HDAC10 participates in the regulation of autophagic processes in neuroblastoma cells. HDAC10 is neither involved in the regulation of caspase-dependent apoptosis, nor in the regulation of differentiation of neuroblastoma cells, which distinguishes it from the class I family members HDAC2 and HDAC8.

Keywords: HDAC, neuroblastoma

Velcade/Bortezomib- and Ná-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-induced apoptosis in the T-cell leukemic Jurkat cell line is apoptotic protease-activating factor-1 (Apaf-1)-dependent

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Velcade/Bortezomib is a highly selective inhibitor of the 26S proteasome and has been shown to induce apoptosis by inhibiting the transcription factor NF-êB with down-regulation of the expression of several endogenous apoptosis inhibitors. Velcade/Bortezomib was recently approved for clinical use in the treatment of relapsing and refractory multiple myeloma. Clinical trials are also underway to assess the role of Velcade/Bortezomib in other human malignancies including leukemia. However, the molecular mechanisms responsible for the cell death-inducing ability of Velcade/Bortezomib have not fully been elucidated. Ná-tosyl-L-phenylalanine chloromethyl ketone (TPCK) is a serine protease inhibitor that has been shown to possess both pro- and anti-apoptotic properties in different cell types. We have previously shown that certain B lymphoma cell lines fail to undergo 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). We also observed that the proteasome inhibitor lactacystin induces apoptosis in B cell lines in an apoptosome-dependent manner (Sun et al., Leukemia, 2007). Here, we used human T-cell leukemic Jurkat cells stably transfected with (pSUPER-Apaf-1) or without (pSUPER) siRNA against Apaf-1 (Franklin and Robertson, Biochem J, 2007) to assess the role of Apaf-1. We provide evidence that Apaf-1-deficient Jurkat T cells are resistant to Velcade/Bortezomib-induced apoptosis, as assessed by caspase-3-like enzyme activation, phosphatidylserine (PS) externalization, and percentage of cells with hypodiploid DNA content, but not a loss of mitochondrial transmembrane potential. Furthermore, Apaf-1-deficient Jurkat T cells are resistant to TPCK-induced apoptosis. These studies show that apoptosome activation is required for the pro-apoptotic effects of Velcade/Bortezomib and TPCK, and suggest that apoptosome activation may be a relevant parameter to assess in studies focusing on the effects of these drugs in human leukemia.

Keywords: Velcade/Bortezomib; Apaf-1; Apoptosis; Leukemia

Inhibition of Notch in T cell leukemia and hepatoma cell lines results in cell cycle arrest, but not apoptosis

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Notch signaling is a well conserved pathway playing crucial roles in regulating cell fate decision, proliferation and apoptosis during development of multiple cell lineages. Aberration in Notch signaling is associated with tumorigenesis of tissues from various origins. To investigate the role of Notch signaling in proliferation of cancer cell lines, the expression profiles of Notch1 in six human cancer cell lines (Jurkat, HepG2, SW620, KATOIII, A375, BT474) were examined. All cell lines differentially expressed Notch1, and only Jurkat and SW620 expressed cleaved Notch1 (Val1744). Among six cell lines tested only Jurkat and HepG2 showed decreased in cell proliferation during 4 days of treatment with gamma-secretase inhibitor (GSI). These two cell lines expressed Notch1-3, Jagged1, Jagged2, Dlk1 and Hes1. GSI treatment led to decrease in Hes1 expression in both cell lines. Surprisingly, GSI treatment resulted in accumulation of Notch1 protein from 24 hr upon treatment. During this period, GSI treatment did not induce apoptosis but caused cell cycle arrest in both cell lines, in correlation with decreased c-myc expression. Forced expression of activated intracellular Notch1 completely abrogated GSI sensitivity in both cell lines. These results clearly demonstrate that Notch signaling positively regulates cell proliferation in Jurkat and HepG2 cell lines and GSI treatment inhibit tumor cell proliferation through suppression of Notch signaling.

Keywords: Notch, gamma secretase inhibitor, cell cycle arrest, c-myc

Clearance of RBAc-PDT derived apoptotic and autophagic cells

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In vivo the meaning of apoptosis is the fast removal of dead cells by neighbours and macrophages to prevent inflammatory response. Indeed, phagocytosis of apoptotic cells is a complex and very important process to which a number of genes greater than those involved in the induction of the apoptosis itself concur. Impaired phagocytosis of apoptotic cells is the cause of disease as Lupus erythematous, cystic fibrosis, chronic obstructive pulmonary disease (Munoz et al. 2008; Sabroe et al. 2007; Hodge et al. 2003). Any clinical approach based on the induction of apoptosis for the cancer therapy must face with the problem of the removal of the apoptotic cells. A new and promising therapeutic approach for the treatment of cancer is the photodynamic therapy (PDT). PDT is based on the action of photosensitizing drugs (PS) and light irradiation. PS are able to accumulate preferentially in tumour cells, where they induce cytotoxic reactions when they are irradiated by light of the appropriate wavelength. Photochemical generation of ROS can directly kill tumour cells or destroy endothelium vessels, promoting ischemia of tumour and consequently its death. We have recently reported that Rose Bengale Acetate (RBAc), iodinate xanthenic derivative modified chemically by addition of acetate groups, is very efficient in the production of O2 singlet that leads to the apoptotic and autophagic cell death of HeLa cells (Panzarini et al. 2006; 2008). Our in vitro experimental conditions, RBAc 10-5M and irradiation with a green LED DPL 305, emitting at 530¡Ó15 nm to obtain 1.6 J/cm2 as total light, allowed to obtain 30% of apoptotic and 15% autophagic HeLa cells at 72 h post irradiation. Since cell surface modifications (i.e. presence of blebs, roundness etc), on which is based the specific recognition between phagocytes and dead cells, are dependent on the apoptotic inducer (Wiegand et al. 2001), we studied the surface modifications induced on the apoptotic and autophagic HeLa cells after RBAc-PDT and the efficiency of their phagocytosis. Data were compared with those achieved with puromycin, a well studied inducer of apoptosis. Expression of sugar residues, ICAM-3, HCAM and CD47 have been visualized with a panel of FITC-conjugated lectins and specific antibodies respectively. Data have shown that when HeLa cells underwent PDT, the modifications detected on the cell surface of apoptotic and autophagic cells were more marked when compared to puromycin-induced apoptotic HeLa cells. An extensive exposure of normally hidden sugar residues and CD47 was found. Moreover, RBAc-PDT apoptotic HeLa cells translocated soluble Hsp70 to plasma membrane as alternative signalling. RBAc-PDT apoptotic and autophagic HeLa cells were phagocytized within 4 h by macrophages with a 150% efficiency than puromycin treated HeLa cells. The index and the rate of phagocytosis were 45% and 2,3 respectively for RBAc-PDT treated HeLa cells versus 30% and 1,7 for puromycin treated HeLa cells. No inflammatory cytokine were released. Indeed, TNF-α, a potent inflammatory mediator, was not found in the culture medium of Raw 264.7 macrophages challenged with RBAc-PDT as well as puromycin treated HeLa cells. Contrary, phagocytosis of necrotic HeLa cells (45¢XC for 1 h) induced a strong release of TNF-α soon after 1 h. All these data further encourage the use PDT for the cure of tumours, also because PDT-derived apoptotic and autophagic cells can be engulfed by macrophages at a very high rate without release of TNF-α. Thus, the in vivo therapy of tumours with PDT prevents the onset of not wanted unhealthy side effects.

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Keywords: phagocytosis, photodynamic therapy, authophagy, apoptosis

Resveratrol Regulates the Expression of NHE-1 by Repressing its Promoter Activity: Critical Involvement of Intracellular H2O2 and Caspases 3 and 6 in the Absence of Cell Death

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Overexpression of the Na+/H+ exchanger-1 (NHE-1) is associated with carcinogenesis and hence an attractive target for intervention. Here we report that the cancer chemopreventive agent resveratrol (RSV) downregulates the expression of NHE-1 in a caspase dependent manner, but without overt signs of cell death. RSV treatment resulted in early activation of caspase 3 and late activation of caspase 6, which were independent of each other and did not involve upstream initiator caspases. Whereas capase 3 activation appeared to be a direct effect of RSV, caspase 6 activation was mediated via intracellular hydrogen peroxide (H2O2) production and iron. In addition, downregulation of NHE-1 expression was a function of RSV-induced repression of NHE-1 gene promoter activity. RNAi-mediated gene silencing of caspase 3 or 6 blocked the effect of RSV on NHE-1 expression, however the effect on NHE-1 promoter activity was observed at distinctly different phases of promoter repression with caspase 3 controlling the early phase (4-12 hours) and caspase 6 regulating the late phase (12-24 hours). Scavenging H2O2 or iron only reversed the late phase of RSV-induced NHE-1 promoter repression. We also identified an AP-2 binding region within the promoter as the target of RSV-induced inhibition of NHE-1 promoter activity. These data demonstrate NHE-1 as a target of RSV, and could explain the cancer chemopreventive and chemotherapeutic activity of RSV in the light of the association of increased NHE-1 expression with carcinogenesis.

Keywords: Resveratrol, Caspases, NHE-1, Hydrogen peroxide, AP-2

Presenting Author: Karine Pilon-Larose

AIF interacts with Opa1 to maintain survival and mitochondrial function

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AIF is a mitochondrial oxido-reductase protein responsible for caspase-independent cell death. We have recently shown that AIF also plays a pro-survival role by controlling mitochondrial structure. Here, we are proposing that AIF interact with Opa1, a protein implicated in mitochondrial fusion and cristae structure, to control mitochondrial structure. This novel functional and physical interaction between the two proteins is indispensable for proper mitochondrial structure, ATP production and cell survival. AIF co-localize with Opa1 oligomers and is required for their assembly. During cell death, AIF release from the mitochondria and coincide with Opa1 oligomers breakdown, which can be rescued by the expression of an anchored form of AIF. Overexpressing Opa1 can also rescue cell death. Furthermore, Opa1 isoforms expression is modified during cell death, which could explain for the oligomers breakdown. By using AIF-/- MEFs, we determined that AIF deficient cells undergo cell death by inducing autophagy. To conclude, we propose a novel function of AIF as a signaling molecule to induce cristae remodeling via Opa1 during cell death.

Keywords: AIF, Opa1, neuronal survival, mitochondrial structure and function

Effect of the antidiabetic drug ciglitazone on bladder cancer cell growth arrest and death through PPAR gamma independent mechanisms

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Ciglitazone is a synthetic PPARgamma (peroxisome proliferator-activated receptor gamma) ligand belonging to the thiazolidinedione (TZD) class of antidiabetic drugs. Recent evidences indicate that TZD exhibit antiproliferative, differentiation and apoptotic activities in various cancer cells. As known, defects in cell death pathways confer a survival benefit to tumor cells and a resistance to anticancer treatment. Several studies demonstrated that TZD sensitize cancer cells to TRAIL-induced apoptosis. The purpose of this study was to evaluate the cytostatic / cytotoxic effects of the PPARgamma agonist ciglitazone on two bladder cancer cell lines, RT4 (derived from a grade I papillary tumor) and T24 (derived from a grade III undifferentiated carcinoma) and its influence on TRAIL-induced apoptosis. Incubation of RT4 cells for 24 hours with ciglitazone resulted in a G2/M cell cycle arrest and in increased levels of p27 and p53 and decreased levels of cyclin B1. In T24 cells, ciglitazone induced a dose-dependent cell death characterized by an increase in Sub-G1 fraction, the proteolytic cleavage of procaspase-8, procaspase-3 and PARP. These results suggest that ciglitazone-induced cell death is due to apoptosis. In regard to caspase 8 activation, ciglitazone affected components of the TRAIL (TNF-related apoptosis-inducing ligand) signaling pathway. Interestingly, ciglitazone up-regulated DR4 at the protein level only in RT4 cells and TRAIL only in T24 cells. RT4 cells were sensitive to TRAIL-induced apoptosis whereas T24 cells were resistant. We next performed experiments with the combined treatment, ciglitazone plus TRAIL. Ciglitazone restored the sensitivity of TRAIL-resistant T24 cells to TRAIL-induced apoptosis. We also examined the antiapoptotic protein survivin expression level upon treatment with ciglitazone and/or TRAIL. A marked decrease of survivin was observed in the presence of either the antidiabetic drug or the combination of both molecules at concentrations for which we observed apoptosis. In addition, ciglitazone effects in RT4 and T24 cells were not abolished by a potent PPARgamma inhibitor, GW9662, suggesting that growth arrest and apoptosis induced by this antidiabetic drug involved PPARgamma independent mechanisms. Collectively, these data suggest that combining PPARgamma agonist with TRAIL can produce significant antitumor effect by inducing apoptosis through a down-regulation of survivin and may provide an exciting novel therapeutic approach for the treatment of bladder cancer.

Keywords: bladder cancer, PPAR gamma, apoptosis, TRAIL

Mitochondrial regulation of myofibroblast differentiation

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There is no doubt that reactive oxygen species (ROS) produced by mitochondria and other cellular sources are not only injurious by-products of metabolism but also essential mediators of cell signaling during proliferation, differentiation and apoptosis. We have investigated the role of mitochondrial ROS in intracellular signaling using synthetic mitochondria-targeted antioxidants, 10-(6`-ubiquinolyl) decyltriphenilphosphonium (MitoQ) and its more effective structure analog SkQ1. It was found that mitochondria-targeted antioxidants as well as classical antioxidants (trolox, or N-acetyl-cystein) induced myofibroblast differentiation of humane skin fibroblasts (HSF). We have investigated earlier step of myofibroblast differentiation - stabilization of actin stress-fibers. It was found that cofilin/phospho-cofilin system regulate this process and it depends on Rho- and Rac1- signaling and presence of Transforming Growth Factor b1 (TGF-b1) in cultural medium. We have detected no change of TGF-b1expression or secretion, but we made visible short-time increasing of activation of this cytokine in 1 hour after SkQ1 addition. We also observe gate triggering of TGF-b1-dependent signaling in 2 hours after SkQ1 addition. It was suggested that scavenging of mitochondrial ROS by SkQ1 initiated activation of TGF-b1 which is a well-known inducer of myofbrablast differentiation. We suppose that it may be the main mechanism of SkQ1 induced stress-fibers stabilization and myofibroblast differentiation.

Keywords: mitochondria, ROS, myofibroblast, cytoscelet

Presenting Author: Michele Purrello

The apoptotic machinery as a biological complex system: Analysis of its *Omics*, identification of candidate genes for fourteen major types of cancer and experimental validation in CML and neuroblastoma

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Apoptosis is a critical biological phenomenon that allows the physiologic elimination of differentiated, senescent or diseased cells. Based on this, we have performed the characterization of the Omics of the Apoptotic Machinery (AM) in Homo sapiens: Genomics, Transcriptomics including microRNAs (MIRNs), Proteomics including post-translational modifications and Natively Unfolded Proteins (NUPs), Interactomics, Oncogenomics. The evolution of the system was analyzed by comparing its Omics in 90 different Model Organisms (from Homo sapiens to Bacteria and Archaea). Homo sapiens'AM comprises 342 protein-encoding genes (that possess either anti- or pro-apoptotic activity, or a regulatory function) and 110 MIRN-encoding genes: some have a critical role within the system (core AM nodes), others perform pathway-, tissue-, or disease-specific functions (peripheral AM nodes). By overlapping the cancer type-specific mutation maps of AM genes in the fourteen most frequent cancers in western societies (Breast, Colon, Kidney, Leukaemia, Liver, Lung, Neuroblastoma, Ovary, Pancreas, Prostate, Skin, Stomach, Thyroid, Uterus) to their transcriptome, proteome and interactome in the same tumour type, we identified the most prominent molecular alterations within each specific neoplasia. By overlapping the fourteen mutated AM networks (both protein- as MIRN-based), we pinpointed those hubs with a critical role in tumour development and conversely on cell physiology. Some of these had already been used as targets for pharmacological anticancer therapy, whereas others are new targets to be tested. The expression time course of 84 core AM genes in two model cell lines (the CML line K562 and the Neuroblastoma line SH-SY5Y) was determined through HT Real Time PCR after treatment with two drugs commonly used for anticancer therapy (Imatinib and Fenretinide, respectively). Through our studies we have characterized a critically important biological machinery and identified a series of new candidate genes, that could represent specific cancer biomarkers upon which innovative anticancer therapies could be focused.

Keywords: Apoptosis, Apoptotic Machinery, Biological Complex Systems, Cancer, Cellular Networks, High Throughput Analysis, MicroRNAs, Natively Unfolded Proteins, Omic Molecular BioMedicine, Pharmacological Anticancer Therapy, Signal Biology

RNAi downregulation of uPAR and MMP-9 induces apoptosis via the caspase 9 apoptosome cascade and cause the activation of the JUN pathway in human glioma xenograft cells

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Glioblastomas are resistant to treatment due to the tendency of the tumor cells to invade normal brain tissue. We have previously demonstrated that the simultaneous downregulation of uPAR and MMP-9 resulted in the regression of pre-established intracranial tumors in nude mice. In this study, we have attempted to further evaluate the molecular mechanisms involved in glioma tumor regression via the simultaneous downregulation of uPAR and MMP-9. Human glioma xenograft cells simultaneously downregulated for uPAR and MMP-9 caused the activation of caspase 9 and a collapse in the mitochondrial delta chi. We also observed release of cytochrome c and the activation of the apoptosome complex. The dephosphorylation of ERK1/2 and a decrease in the nuclear and cytoplasmic levels of NFkB p65 and 50 were also observed. Mobility shift assay with oligos specific for NFkB further confirmed NFkB downregulation. The real time RT-PCR array results showed that MEK levels did not change, whereas the levels of MAP4K1 levels increased, thereby indicating the activation of the JUN pathway. Previous studies have speculated that MAP4K1 may play a role in response to environmental stress. It is also speculated that the decrease in the kinase activity of these molecules may be related to cell surface components associated with the target molecules uPAR and MMP-9. Taken together, it is evident that glioma cells, which over express uPAR and MMP-9, have the potential to undergo apoptosis upon the downregulation of uPAR and MMP-9. Hence, the simultaneous targeting of uPAR and MMP-9 holds promise for glioma therapy.

Keywords: caspase 9, apoptosome, uPAR, MMP-9, apoptosis

Proteomic approaches to identify novel binding partners of the survival factor Bcl-xL

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Overexpression of the Bcl-xL protein protects a variety of cell types from apoptosis by sequestering initiators (BH3-only proteins) as well as effectors (Bax, Bak) for outer mitochondrial membrane perforation and subsequent cytochrome c release and caspase activation. Endogenous Bcl-xL is crucial for the homeostasis of the immune system as well as for embryonic development and the formation and maintenance of the neuronal network. However, despite intense research, it has remained elusive which proteins/factors regulate endogenous Bcl-xL under surviving and apoptotic conditions. We therefore established proteomic techniques to identify novel binding partners of Bcl-xL in the cytosol and on mitochondria of healthy and apoptotic monocytes and fibroblasts. On the one hand, Bcl-xL protein complexes were purified by gel filtration analysis followed by a combination of 2-dimensional blue native and SDS-polyacrylamide gel electrophoresis, on the other hand by Bcl-xL immunoprecipitation or tag affinity purification after expressing FLAG- or Strep-tagged Bcl-xL on a Bcl-x-/- background. Preliminary data indicate that Bcl-xL is present in protein complexes in the range between 40 – 120 kD, both in the cytosol as well as on mitochondria. Since known Bcl-xL binding partners such as Bax, Bak or BH3-only proteins exhibit molecular masses between 20 – 30 kD, these data indicate that ca. 28 kD Bcl-xL is either present as a multimer or bound to other, yet unidentified proteins.

Keywords: Bcl-xL, mitochondria, binding partners, proteomics

Presenting Author: Danielle Read

Identification of a caspase-12 splice variant in rat PC12 cells and its upregulation during endoplasmic reticulum stress induced-apoptosis

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Stress in the endoplasmic reticulum (ER) can induce apoptosis, which is associated with many neurodegenerative disorders. ER stress involves processing of the ER-localised caspase-12, which contains a caspase-recruitment domain (CARD) and p20/p10 domains. However, the role of caspase-12 is yet to be fully characterised during ER stress-induced apoptosis. This study investigates the mRNA expression of rodent caspase-12 and a novel splice variant during ER stress-induced apoptosis in rat PC12 cells. Interestingly, expression of the variant was much greater than normal caspase-12, and the mRNA levels of both were significantly increased following treatment of PC12 cells with thapsigargin (1.5 microM), tunicamycin (2 microg/mL) and 6-hydroxydopamine (200 microM) over a 24 h time-course. Furthermore, we have shown an absence of caspase-12 induction following the initiation of non-ER stress-induced apoptosis with etoposide (50 microg/mL) and staurosporine (1 microM). The variant has been fully cloned and sequenced to reveal a 26 amino acid insert situated after the CARD domain. This does not interrupt the reading frame nor introduce a premature stop codon. We have investigated the mRNA levels of the variant in a wide range of adult rat tissues, revealing that it is widely expressed and always at a higher level than normal caspase-12. Tissues which demonstrated particularly high levels of normal and variant caspase-12 included the adrenal gland, stomach, rectum and liver, while the spleen, small intestine and kidney had low expression. Functional analyses are currently underway to determine whether the variant can be cleaved in vitro by a recombinant active caspase-12 (deltaCARD mutant) that we have generated that is capable of cleaving pro-caspase-12.

This work was financially supported by Enterprise Ireland, Science Foundation Ireland and the Irish Research Council for Science, Engineering and Technology.

Keywords: Endoplasmic reticulum (ER) stress, caspase-12

Presenting Author: Chris Redfern

Increasing Melanoma Cell Death Using Inhibitors of Protein Disulphide Isomerases to Abrogate Survival Responses to Endoplasmic Reticulum Stress

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Introduction
Exploiting vulnerabilities in intracellular signalling pathways of tumor cells is a key strategy for drug development. The activation of cellular stress responses mediated by the endoplasmic reticulum (ER) allows cancer cells to survive outside their normal environment. Many proteins that protect cells against ER stress are active as protein disulfide isomerases (PDI) and the aim of this study was to test the hypothesis that apoptosis in response to ER stress can be increased by inhibiting PDI activity.
Methods
The response of melanoma cell lines to fenretinide, velcade or PDI inhibitor was evaluated using biochemical markers of ER stress and assays for viability and apoptosis. The role of PDI inhibition was assessed by transfection with both wild-type and mutant PDI.
Results
The novel chemotherapeutic drugs fenretinide and velcade induced ER stress-mediated apoptosis in melanoma cells. Both the stress response and apoptosis were enhanced by bacitracin, a macrocyclic dodecapeptide antibiotic which inhibits PDI activity. Over-expression of the main cellular PDI, procollagen-proline, 2-oxoglutarate-4-dioxygenase-beta subunit (P4HB), resulted in increased PDI activity and abrogated the apoptosis-enhancing effect of bacitracin. In contrast, over-expression of a mutant P4HB lacking PDI activity did not increase cellular PDI activity or block the effects of bacitracin.
Conclusion
These results provide proof-of-principle for the concept that ER-stress-induced apoptosis can be enhanced using PDI inhibitors. Furthermore, the data imply that small-molecule PDI inhibitors designed to bind to the CXXC motif of the PDI active site may have significant potential for enhancing the efficacy of chemotherapy in a wide range of cancers.

Keywords: Melanoma, ER stress, protein disulphide isomerase inhibitors, apoptosis

Presenting Author: Matthias Regner

Granzymes A and B are dispensable for in vivo killing by natural killer and cytotoxic T cells

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Natural killer (NK) and cytotoxic T (Tc) cells can destroy virus-infected and transformed target cells via exocytosis of granules containing perforin (perf) and granzymes (gzm). In vitro, gzm delivery into the target cell cytosol results in apoptosis, and in the absence of the most abundantly expressed gzm, A and B, NK and Tc cell-mediated induction of apoptosis is severely impaired. However, using in vivo cytotoxicity assays, we find that virus-infected gzmAxB double deficient mice are fully competent to rapidly eliminate adoptively transferred NK and Tc cell target cells in several organs. Gene expression analysis of less frequently expressed orphan gzm did not reveal compensatory expression of any of these. Thus, neither gzmA nor gzmB are required for rapid and efficient in vivo cytotoxicity by NK and Tc cells, indicating that their biological function is not cell death induction per se, but need yet to be identified.

Keywords: granzymes, natural killer cells, cytotoxic T cells, cytotoxicity

Real-time kinetics of pore formation in the outer mitochondrial membrane and spatial waves of cytochrome-c release during apoptosis

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In response to pro-apoptotic stimuli, cells within a population individually undergo apoptosis at distinct times. We established a novel imaging technique that allows prediction of the onset of mitochondrial outer membrane permeabilisation (MOMP) in individual HeLa cells. Previous MOMP-imaging approaches achieved a resolution of minutes and suggested invariable kinetics of this rapid process. Our technique enables fast sampling of MOMP at seconds resolution while avoiding phototoxic damage, and provides novel spatiotemporal information.
Depending on the type of apoptotic stimulus and the size of the cell, fast sampling of cytochrome-c release using cyt-c-GFP imaging detected spatial release waves in up to 63 % of cells. Release waves propagated at velocities of 0.58 µm/s in response to TRAIL and 0.17 µm/s in response to staurosporine, and mathematically could be re-modeled to be a consequence of cytosolic diffusion of MOMP-inducers. High-speed sampling also allowed quantification of the kinetics of the pore generation process for the first time in living cells. Pore formation associated with activator BH3-only proteins proceeded approximately three times faster than pore formation predominantly induced by enabler BH3-only proteins.

Keywords: Systems Biology, Mitochondria, Cytochrome-c, Live cell imaging

Molecular cloning and characterisation of sea bass (Dicentrarchus labrax) caspase-8.

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In fish, information on the apoptotic process is scarce and apoptotic related genes only recently started to be sequenced. Sea bass (Dicentrarchus labrax) is one of the most important species for aquaculture in Southern Europe, and here it is described the sequence and characterization of its caspase-8 gene. A 1924 bp cDNA of sea bass caspase-8 was obtained, consisting of 1455 bp open reading frame coding for 484 amino acids and several distinctive features of caspase-8 were identified in the generated sequence. The sequence exhibits a very close homology to the sequences of caspase-8 from other vertebrates. The sea bass caspase-8 gene exists as a single copy gene and is organized in 11 introns and 12 exons. A low expression of caspase-8 was detected by RT-PCR in various tissues of non-stimulated sea bass.

Keywords: sea bass; caspase-8

Presenting Author: Thibaud Renault

Regulation of Bax pro-apoptotic function on mitochondria through phosphorylation.

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The pro-apoptotic protein Bax is known to play a major role in the release of mitochondrial apoptotic effectors like cytochrome c or Smac/Diablo during mitochondrial apoptosis.
It has been demonstrated that Bax is phosphorylatable on S184 [1,2] and S163 [3] residues.
The heterologous expression of human Bax in the yeast S. cerevisiae was used to investigate the molecular mechanisms of its effects on mitochondria. Indeed, wild type human Bax is not addressed to mitochondria in yeast, whereas activated mutants are addressed, inserted into mitochondrial outer membrane and promote cytochrome c release. Thus yeast is a good reporting system to assess Bax mutants activity in absence of any other Bcl-2 or apoptosis-related proteins.
Substitution of alanine/aspartate residues for Bax potentially phosphorylatable serine residues respectively simulates the lack/presence of a phosphorylation [4]. We provide arguments for repression of Bax addressing to mitochondria when serine 184 is phosphorylated.
We set out to characterize the regulation of Bax at the level of serines 184, 163 and 60 by kinases AKT/PKB, GSK-3-beta and PKA, by analyzing the mitochondrial addressing and membrane insertion of Bax mutants. Such a regulation could explain transition from cytosolic inactive to mitochondrial active conformations of the protein.
Furthermore we have gathered evidence showing the impact of Bax phosphorylation on its interaction with the anti-apoptotic protein Bcl-xL, leading to Bax inhibition. Recent models [5] based on in vitro experiments suggest that Bcl-xL may inhibit Bax by other means than retaining it in the cytosol. Our preliminary data, obtained in the yeast paradigm are in accordance with this proposition.

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Keywords: Apoptosis, Mitochondria, Bax, Bcl-xL, phosphorylation, S. cerevisiae.

Presenting Author: Katharina Ross

TOM-independent complex formation of Bax and Bak in mammalian mitochondria during TNFα;-induced apoptosis

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The Bcl-2 family proteins Bax and Bak are activated in response to many apoptotic stimuli. As a consequence of activation, Bax and Bak oligomerize and permeabilize the outer mitochondrial membrane to permit the release of apoptosis inducing factors. It still remains unclear whether these proteins require components of the mitochondrial protein import machinery, namely the TOM and the SAM complex, for their function at the mitochondria. We addressed this question by using inducible RNA interference for the study of Bax and Bak protein import in mammalian mitochondria.
From our study we conclude that, in contrast to other reports, Bax and Bak translocation, oligomerization and complex assembly during TNFα;-mediated apoptosis is not dependent on the components of the mitochondrial import and assembly machineries in mammalian cells, the TOM and the SAM complex. Tom22, Tom40 and Sam50 are not components of Bax and Bak complexes in the outer mitochondrial membrane, since their absence does not influence the size or the amount of formed Bax and Bak complexes.

Keywords: Bax, Bak, mitochondria, TOM, apoptosis.

Presenting Author: Ausra Sasnauskiene

Programmed cell death induced by mitochondria-targeted photodynamic treatment

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Cell death triggered by oxidative damage has been studied in human epidermoid carcinoma cell line A431. Oxidative damage was induced by photodynamic treatment targeted to mitochondria. Two photosensitisers have been used: meso-tetrahydroxyphenyl chlorine (mTHPC) localized to cell membranes, including those of mitochondria, and Safranine O (Safr) accumulated to mitochondrial matrix. Photodynamic treatment with membranously localized mTHPC induced the apoptotic cell death. Safr-mediated photodynamic treatment at low cytotoxic dose induced the cell growth arrest, but at higher light doses apoptosis was initiated. Autophagy (determined by LC3-I conversion to LC3-II) was detected following any of the treatment modalities. Especially high level of autophagy was detected at high PDT doses, when apoptosis was observed.

Keywords: oxidative damage, mitochondria, apoptosis, autophagy

Presenting Author: Karen Schrader

Investigating new Strategies for Identifying Serine Proteases Implicated in ER stress induced Apoptosis

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We have previously shown that apoptosis induced by ER stress (brefeldin A, tunicamycin and thapsigargin) proceeds via caspase-dependent and -independent processes in rat embryo fibroblasts (R6), 3T9- and SV40-immortalized mouse embryo fibroblasts (MEFs) and factor (IL-3) dependent monocytes (FDMs). The caspase-independent step involves (a) serine protease(s) upstream of mitochondrial membrane permeabilization. To identify the still elusive serine protease we have taken several approaches with one limiting factor always being the broad specificity of the available inhibitors, such as TPCK, TLCK and AEBSF. We have addressed this question by screening a small peptide library, predicted to target serine proteases. We are currently testing several promising candidates, which show higher specificity than for example AEBSF. Furthermore we have synthesised a “click probe” of the most promising inhibitor. Click chemistry is a technique, which allows us to “click” two compounds irreversible together in a copper-catalysed azide-alkyne reaction in vitro. Here, we use click chemistry to click on a biotin tag to the modified inhibitor to perform a pull down and subsequent mass-spectrometry analysis.
In parallel we are trying to identify serine proteases implicated in ER stress induced apoptosis using an siRNA library approach. We have screened an siRNA library targeting 123 serine proteases, using reduced DEVDase activity as a read out. We are now in the process of further characterising their role and involvement in ER stress induced apoptosis.

Keywords: ER stress induced apotosis, serine proteases, serine protease inhibitors, click chemistry

Hif1-Alpha is required for Apoptosis inhibition by Chlamydia trachomatis infected cells

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Chlamydia are obligate intracellular bacteria that cause 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 have earlier shown
that apoptosis resistance in C. trachomatis infected cells requires the up-regulation of anti- apoptotic proteins Mcl-1 and cIAP2.
Hypoxia inducible factor-1 (HIF-1) regulates crucial genes involved in the adaptation to low oxygen concentrations, cell metabolism and the innate immune response. HIF-1 is a heterodimeric complex constituting of a hypoxically inducible subunit HIF1-α; and a constitutively expressed HIF1-β. In this study we show that Chlamydia trachomatis infection leads to the stabilization and activation of Hypoxia Inducible Factor 1 α, which is required for the downstream up-regulation of Mcl-1 and cIAP2. Our data suggest that there is an infection dependent increase in the levels of HIF-1α; in the cells. We also observe that during infection, HIF1-α; translocates from the cytoplasm to the nucleus where it can transcriptionally activate its target genes.
We propose a model whereby Chlamydia activate the MAPK pathways to stabilize and activate HIF1-α; which in turn leads to the up regulation of prominent anti apoptotic proteins – Mcl-1 and cIAP2, thereby conferring apoptosis resistance to the infected host cell.

Keywords: Apoptosis inhibition, Chlamydia trachomatis, Hif 1a, Mcl-1

Presenting Author: Varda Shoshan-Barmatz

The N-terminal domain of VDAC1 is essential for mitochondria- mediated apoptosis

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Apoptotic signaling to mitochondria causes an efflux of apoptotic regulators from the intermembranal space to the cytosol, triggering caspases activation and apoptosis. Accumulating evidence indicates that the voltage-dependent anion channel (VDAC) plays a central role in mitochondria-mediated apoptosis. Here, we demonstrate that the N-terminal domain of VDAC1 controls release of cytochrome c, apoptotic cell death, and regulation of apoptosis by anti-apoptotic proteins, such as hexokinase (HK) and Bcl2. Expression of murine N-terminal truncated VDAC1 in cells either expressing endogenous human VDAC1 or silenced for its expression revealed the N-terminal region as required for cytochrome c release and apoptosis induction. Furthermore, a synthetic peptide corresponding to the VDAC1 N-terminal region bound to HK-I and Bcl2, and when expressed in cells over-expressing HK-I, HK-II or Bcl2, prevented their anti-apoptotic effects. These results demonstrate that the VDAC1 N-terminal region is required for VDAC1-mediated apoptosis. We also demonstrated that apoptosis induction is associated with VDAC oligomerization and propose that cytochrome c release is mediated by a protein-conducting channel formed within a VDAC1 homo-oligomer or hetero-oligomer containing VDAC1 and pro-apoptotic protein. These results show VDAC1 to be a critical component of the apoptosis machinery and that its N-terminal region controls cytochrome c release and offer new insight into the mechanism of d how anti-apoptotic proteins regulate apoptosis and promote tumor cell survival.

Keywords: Apoptosis, Mitochondria, VDAC, Porin, hexokinase, Bcl2

Thiazolides inhibit growth and induce Glutathione-S-tranferase pi (GSTP1)-dependent cell death in human colon cancer cells

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Thiazolides are a novel class of broad-spectrum anti-infective drugs with promising in vitro and in vivo activities against intracellular and extracellular protozoan parasites. The nitrothiazole-analogue nitazoxanide (NTZ; 2-acetolyloxy-N-(5-nitro 2-thiazolyl) benzamide) represents the thiazolide parent compound, and a number of bromo- and carboxy-derivatives with differing activities have been synthesized. Here we report that NTZ and the bromo-thiazolide RM4819, but not the carboxy-thiazolide RM4825, inhibited proliferation of the colon cancer cell line Caco2 and non-transformed human foreskin fibroblasts (HFF) at or below concentrations the compounds normally exhibit anti-parasitic activity. Thiazolides induced typical signs of apoptosis, such as nuclear condensation, DNA fragmentation and phosphatidylserine exposure. Interestingly, the apoptosis-inducing effect of thiazolides appeared to be cell cycle-dependent and induction of cell cycle arrest substantially inhibited the cell death-inducing activity of these compounds. Using affinity chromatography and mass spectrometry glutathione-s-transferase P1 (GSTP1) from the GST class Pi was identified as a major thiazolide-binding protein. GSTP1 expression was more than 10 times higher in the thiazolide-sensitive Caco2 cells than in the less sensitive HFF cells. The enzymatic activity of recombinant GSTP1 was strongly inhibited by thiazolides. Silencing of GSTP1 using siRNA rendered cells insensitive to RM4819, while overexpression of GSTP1 increased sensitivity to RM4819-induced cell death. Thiazolides may thus represent an interesting novel class of future cancer therapeutics.

Keywords: apoptosis; colon cancer, cell cycle, oncogene

Presenting Author: Micol Silic-Benussi

The p13 protein of HTLV-1 controls mitochondrial function, reactive oxygen species levels and cell death

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In previous studies we showed that the p13 protein coded by Human T-cell Leukemia/Lymphoma virus type-1 (HTLV-1) is targeted to mitochondria, affects cell turnover in vitro and exerts antitumor effects in experimental animal models.
The present study was aimed at understanding the mechanism of p13 function. Assays employing full-length synthetic p13 and isolated mitochondria showed that p13 triggers an inward K+ current which results in inner membrane depolarization, increased respiratory chain activity and accumulation of reactive oxygen species (ROS). Similar effects were induced by the K+ ionophore valinomycin, while the protomophore FCCP reduced ROS production, suggesting that depolarization induced by K+ vs. H+ currents has opposite effects on ROS. To pursue these findings, we next studied the effects of p13 expression on mitochondrial ROS production in HeLa cells and T-cells (the latter being the main target of HTLV-1 infection in vivo). Results demonstrated that p13-expressing cells exhibit increased ROS levels. Interestingly, these effects ensued when cells were subjected to glucose deprivation, and were abrogated by treatment with ROS scavengers. Having found interesting differences in ROS levels in p13-expressing vs. control cells, we next compared the viability of these cells after short-term (24 hours) culture in standard conditions and under glucose starvation. Results showed that p13-expression induced a 5.5-fold (HeLa) or 14 -fold (Jurkat) increase in cell death specifically triggered by glucose deprivation (specific cell death) compared to control cells. In addition, treatment with ROS scavengers completely abrogated the effects of p13 expression on glucose-deprivation-induced specific cell death. Taken together, these findings indicate that by remodelling mitochondrial function, p13 may control mitochondrial ROS production and cell turnover under specific metabolic conditions.

Keywords: HTLV-i, mitochondria,ros, cell death,

Presenting Author: João Pedro Silva

Nitrogen compounds as protectors of oxidative damage induced in H9c2 myoblasts

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We have been investigating the potential of new synthetic compounds in different conditions where oxidative stress is involved, as the first step in the development process of drugs that can improve human health. Reactive oxygen species (ROS) are produced during normal cell metabolism and in response to various stimuli, and also frequently occur during cardiac ischemia/reperfusion situations. In this study we evaluated the protective role of four of these new synthetic compounds (FMA4, FMA7, FMA762 and FMA796), against oxidative damage induced to H9c2 myoblasts, by the pro-oxidant tert-butylhydroperoxide (t-BHP). These compounds, namely FMA762 and FMA796, were able to decrease t-BHP-induced cell death, as evaluated both by sulforhodamine B assay and by counting the number of apoptotic nuclei stained with the DNA dye Hoechst 33342. Triple labelling of H9c2 cells with TMRM, calcein-AM and Hoechst probes showed that two of these compounds (FMA762 and FMA796) prevent the alterations in cellular and mitochondrial morphology induced by t-BHP. Moreover, they reduced the t-BHP-induced increase in the activities of caspases 3 and 9, suggesting their involvement in the regulation of apoptotic mechanisms, probably mediated by an action at the mitochondria level. Although the more precise mechanisms underlying the action of these compounds in the apoptotic pathways are currently under investigation, it can be stated that the compounds' ROS scavenging ability seems to be involved in their protective effects, as a significant decrease in t-BHP-induced intracellular ROS formation was observed in their presence, namely for FMA762 and FMA796. The role of these compounds against oxidative damage induced in cardiac cells encourages their further development for clinical practice, namely as protectors of ischemia/reperfusion conditions.

JPS is supported by the FCT grant SFRH/BD/17174/2004.

Keywords: nitrogen compounds, myoblasts, oxidative damage, ischemia/reperfusion, apoptosis

Rab32 Regulates Mitochondria-Associated Membrane Targeting and Apoptosis Onset

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The mitochondria-associated membrane (MAM) orchestrates calcium signaling during the onset of apoptosis, but also is a site of lipid synthesis and even influences oxidative protein folding within the ER. Consistent with these multiple functions, proteins of the MAM include lipid transfer proteins, calcium channels and ER chaperones. Our laboratory aims to identify proteins targeting to the MAM and sorting proteins mediating this targeting. Currently, two cytosolic proteins are known that mediate the formation of the MAM: PACS-2 and GRP75 that bridges IP3R and VDAC. We have recently shown that the ER chaperone calnexin targets to the MAM depending on the cytosolic sorting protein PACS-2. PACS-2 also targets the Bcl2 family member Bid to mitochondria.
Our recent, unpublished results have now identified a novel cytosolic protein that mediates MAM targeting. Rab32, a cytosolic Ras-like GTPase that is found on mitochondria, regulates the distribution of calnexin along the secretory pathway and in particular the MAM. However, Rab32 does not influence targeting of mitochondrial proteins such as Bim or the mitofusins. Instead, the activity of Rab32 impacts on mitochondrial structure and the mitochondrial membrane potential. Through these diverse functions, Rab32 overexpression delays the onset of apoptosis, triggered by ER stress.

Keywords: endoplasmic reticulum, mitochondria-associated membrane, apoptosis, calnexin, breast cancer

MCL-1 is an important determinant of the apoptotic response to the BH3-mimetic molecule HA14-1 in cisplatin resistant ovarian carcinoma cells

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Chemoresistance and in vitro recurrence of ovarian carcinoma have been previously associated to the absence of down-regulation of Bcl-xL expression in response to cisplatin. Our team is therefore developing various strategies to impede Bcl-xL activity and/or expression, among which the use of BH3-mimetic molecules. These compounds reproduce the structure of the BH3 domain of the Bcl-2 family members; they are able to induce apoptosis by dissociation of bax-like pro-apoptotic multidomain proteins from their anti-apoptotic partners.
We evaluated the interest of one of them, HA14-1, on various ovarian carcinoma cells lines resistant or sensitive to cisplatin. Differences were observed in response to HA14-1 in these cell lines, one of them undergoing strong cell death (apoptotic or/and autophagic) (IGROV1-R10) whereas the others presented only a partial response (IGROV1, SKOV3, A2780) or an absence of response (OAW42). However, the sensitivity to HA14-1 was unrelated to the level of sensitivity to cisplatin, and the expression of HA14-1 targets (Bcl-2 and Bcl-xL) was not correlated to these different responses. In contrast, the lost of MCL-1 seemed associated with cell death in response to HA14-1, whereas maintenance or increase of MCL-1 expression led to resistance to this agent.
We therefore attempted about the importance of MCL-1 in the response to HA14-1 in SKOV3 resistant cells, and decided to inhibit its expression using a siRNA targeting MCL-1. Our results showed that siMCL-1 did not induced apoptosis on its own in these cells, whereas its association with HA14-1 induced a massive cell death. These results suggest that MCL-1 could cooperate with others Bcl-2 family members (e.g. Bcl-xL) to protect ovarian carcinoma cells against oncogenic stress-induced apoptosis.
We also demonstrated that in SKOV3 cells (both resistant to cisplatin and to HA14-1), cisplatin was able to decrease MCL-1 expression, and that the association of HA14-1 with cisplatin induced a massive cell death and delayed the recurrence, whereas cisplatin or HA14-1 alone was only transiently cytostatic.
These results suggest that MCL-1 is essential for the response to various apoptotic stimuli (oncogenic stress or conventional chemotherapy), and that resistant ovarian carcinomas constitute pertinent targets for the use of BH3-mimetics. Our work also showed that a siRNA directed against MCL-1 could interestingly reinforce the action of such molecules for the treatment of ovarian cancers refractory to conventional chemotherapy.

Keywords: chemotherapy, Bcl-2 family, autophagy, mitochondrial pathway, sensitization

Skeletal Muscle Differentiation Evokes Endogenous XIAP to Restrict the Apoptotic Pathway

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Myotube apoptosis occurs normally during muscle development and aging but it can lead to destruction of skeletal muscle in neuromuscular diseases. We have investigated skeletal muscle and report a striking increase in resistance to apoptosis following differentiation. We find that mitotic C2C12 cells (myoblast-like cells) are sensitive to cytosolic cytochrome c microinjection. However, differentiated C2C12 cells (myotube-like cells) and primary myotubes are markedly resistant. This resistance is due to endogenous X-linked inhibitor of apoptotic proteins (XIAP). Importantly, the selective difference in the ability of XIAP to block myotube but not myoblast apoptosis is not due to a change in XIAP levels but rather a decrease in Apaf-1 expression. This change in Apaf-1 levels tightly links XIAP to caspase activation and death. By requiring not only cytochrome c release but also the inhibition of XIAP to activate caspases, endogenous XIAP serves as a safety brake that could prevent cells from unwanted apoptosis in the case of accidental cytochrome c release. Our findings show that in order for myotubes to Importantly, we identify a role for endogenous Smac in overcoming XIAP to allow myotube death.

This work was supported by National Institutes of Health grants NS42197 (to M.D.).

Keywords: Apaf-1, Apoptosis, Cytochrome c, Myotubes, Smac, XIAP

Presenting Author: Cyril Sobolewski

Effects of COX-2 inhibition on cell proliferation and survival to etopside treatment on human hematopoietic cancer cell lines

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The cyclooxygenases (COX) are a family of enzymes which catalyse the rate limiting step in prostaglandin biosynthesis. COX-2, the inducible isoform of COX, is typically upregulated during inflammation. In addition, this enzyme was described being constitutively expressed in different adherent tumors, where a role for COX-2 in inhibiting cell proliferation and chemosensitizing cancer cells to apoptogenic treatments has been suggested (1), thus implying possible therapeutic applications.
In this study, we have analyzed a possible role for COX-2 in cell proliferation and survival on a panel of leukemic and lymphoblastic cell lines, including cells expressing COX-2 at high (U937, Jurkat, Hel and Raji) and low (K562) levels, focusing our investigations mainly on U937 and K562 cells. To approach the question, we used two specific COX-2 inhibitors : nimesulide and Ns-398.
Here, we show that both COX-2 inhibitors strongly decrease the rate of cell proliferation of COX-2 positive cell lines. Results correlate with an accumulation of the cells in the G1 phase of cell cycle and the up-regulation of p27, a cyclin-dependent kinase inhibitor, involved in the block of G1/S transition. No cytostatic effect is observed on K562, which practically does not express COX-2, thus suggesting that this phenomenon is due to the specific COX-2 inhibition.
In the second part of our study, we show that COX-2 inhibition, that per se does not affect viability, leads to a strong protection against etoposde-induced apoptosis in U937 but not in K562 cells. This effect is associated with the prevention of caspase-3, caspase-8 and caspase-9 cleavage and by the prevention of Bcl-2 degradation, occurring during apoptosis (2).
Our findings show a novel anti-apoptotic effect of COX-2 inhibitors on hematopoietic cancer cells, which recommends cautions in the use of anti-inflammatory agents as chemoadjuvant in the treatment of such tumors.

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Keywords: cyclooxygenases, hematopietic cancer cells, cell proliferation, etopside

Presenting Author: Gabriel Sollberger

New insights into caspase functions in UV-irradiated keratinocytes

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The epidermis as the outermost layer of the skin is continuously exposed to pathogens, toxins or other danger signals such as UV radiation. Therefore, the epidermis is an important component of innate immunity, mediating immune responses not only by Langerhans cells/ dendritic cells but also by keratinocytes, the most abundant cell type in the epidermis. Our laboratory recently demonstrated the presence of an active inflammasome in keratinocytes. The inflammasome is a macromolecular complex that assembles upon various danger signals, resulting in the activation of caspase-1 and the processing and/ or secretion of cytokines such as IL-1beta, IL-18, IL-33 and pro-IL-1α. Keratinocytes respond to UVB irradiation by secreting high amounts of IL-1beta, pro-IL-1α and a variety of other proteins by a Golgi-independent pathway, depending on the activity of caspase-1. Caspase-1 forms, together with caspases -4, -5, -11, -12 and -13, the group of inflammatory caspases. In contrast, there is a second group of caspases, the apoptotic caspases, that themselves are divided into initiators (caspase-2, -8, -9, -10) and executioners (caspase-3, -6, -7) of apoptosis. We analyzed the roles of various caspases in the response of human primary keratinocytes to UVB irradiation by siRNA-mediated knock-down or specific inhibition of the proteases. Our results demonstrate an essential role of caspase-4 in inflammasome assembly and IL-1beta processing/ secretion as either knock-down or inhibition of caspase-4 resulted in markedly decreased IL-1beta secretion of UVB-irradiated keratinocytes. Furthermore, knock-down but not inhibition of caspase-4 and caspase-1 strongly affects migration of keratinocytes, suggesting a function of these inflammatory caspases in this process independently of their enzymatic activity. In addition, we were able to demonstrate a role for caspase-1 in UVB-mediated keratinocyte apoptosis in vitro. Upon knock-down or inhibition of caspase-1, apoptosis of UVB-irradiated keratinocytes was markedly decreased.
Taken together, our experiments revealed novel caspase-dependent processes in UV-irradiated keratinocytes and we demonstrate new functions of these proteases in inflammation, cell migration and apoptosis.

Keywords: UV irradiation, inflammasome, apoptosis, migration

Inhibition of apoptosis by MAD1 is mediated by repression of the PTEN tumor suppressor gene

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The MYC/MAX/MAD network of transcriptional regulators controls distinct aspects of cell physiology, including cell proliferation and apoptosis. Within the network MAD proteins antagonize the functions of MYC oncoproteins, and the latter are deregulated in the majority of human cancers. While MYC sensitizes cells to proapoptotic signals, the transcriptional repressor MAD1 inhibits apoptosis in response to a broad range of stimuli, including oncoproteins. The molecular targets of MAD1 that mediate inhibition of apoptosis were not known. We describe the phosphatase and tensin homologue deleted on chromosome ten (PTEN) tumor suppressor gene as a target of MAD1. By binding to the proximal promoter region, MAD1 downregulates PTEN expression. PTEN functions as a lipid phosphatase that regulates the phosphatidylinositol 3-kinase/AKT pathway. Indeed MAD1-dependent repression of PTEN led to activation of AKT and subsequent stimulation of the antiapoptotic NF-κB pathway. Interfering with AKT function affected the control of Fas-induced apoptosis by MAD1. Furthermore knockdown of PTEN using small interfering RNA (siRNA) or the lack of PTEN rendered cells insensitive to inhibition of apoptosis by MAD1. These findings identify the PTEN gene as a target of the MYC-antagonist MAD1 and provide a molecular framework critical for the ability of MAD1 to inhibit apoptosis.
In addition to the findings that different members of the MYC/MAX/MAD network regulate apoptosis, we have shown previously that the network is also targeted by apoptotic pathways. In particular MAX, the common heterodimerization partner within the network, is hydrolyzed by caspases. We have now observed that MAD1 is degraded in response to different apoptotic stimuli. Our present work aims at understanding the signal transduction pathway(s) and the protease(s) involved in MAD1 degradation. Together our findings suggests extensive feedback regulation between apoptosis and the MYC/MAX/MAD network, supporting the notion that the latter is closely associated with controlling distinct aspects of apoptotic cell death.

Keywords: AKT, MYC, PI3K, transcription, Fas

Selective induction of apoptosis via the TNF system in hepatocellular carcinoma

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Hepatocellular carcinoma represents a prime cancer worldwide with an overall poor prognosis. The isolated hepatic perfusion (IHP) has been developed for exposing the liver to high doses of cytostatic drugs either alone or in combination with tumor necrosis factor α (TNF). However, the initiation of cell death in normal hepatocytes is a major limitation of this approach. In this project, we introduce selectivity into liver tumor therapy by exploiting the ATP dependence of hepatocyte apoptosis. Previous own work demonstrated that a high fructose load depletes metabolically healthy hepatocytes of ATP with the consequence that apoptosis induced by TNF is blocked. The ATP-depletion is due to the high activity of fructokinase that leads to an accumulation of fructose-1-phosphate. In contrast, transformed hepatic cancer cells are lacking this fructose metabolising capability, i.e. their intracellular ATP is not depleted. In pre-clinical in-vivo experiments we demonstrated that under controlled ATP-depleting conditions mouse livers but not hepatic tumor cell lines are fully protected against liver failure induced by TNF. To clarify the lack of ATP-depletion in malignant cell lines we studied differences within the fructose metabolism of primary murine and human hepatocytes and hepatoma cell lines. The specific activity of fructokinase was similar in HepG2, Huh7 and primary human hepatocytes. We detected a lower activity of aldolase b in the cell lines. Additionally, the activity of hexokinases, which catalyze the first step of fructose metabolism in muscle and other tissue, was determined. Compared to the primary cells, the specific activity of hexokinases was increased in HepG2 and Huh7 and also in the murine cell lines Hepa 1-6 and AML-12. Real-time PCR and western blot analysis revealed a stronger expression of hexokinaseII in all cell lines. The elevated expression of hexokinaseII is described as a typical feature of rapidly-growing malignant tumor cells.
Our results indicate that the high activity of hexokinaseII leads to a bypass of the liverspecific enzymes fructokinase and aldolase b and thereby prevents ATP-depletion by fructose. This metabolic difference opens up the possibility to prevent TNF-mediated cell death in healthy but not in transformed hepatocytes.

Keywords: HexokinaseII, Hepatocellular carcinoma, TNF

How does Puma regulate IL-3 deprivation-induced apoptosis?

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Apoptosis of growth factor/cytokine-dependent cells is regulated by Bcl-2 family proteins. Previously, by generating factor (IL-3)-dependent monocyte (FDM) cells from the fetal liver of mice lacking various Bcl-2 family members, we have shown that both Bax and Bak are crucial for cytochrome c release, caspase activation and apoptosis induced by IL-3 deprivation. IL-3 deprived cells lacking Bax and Bak even clonogenically survived after IL-3 re-addition. Similarly, FDMs lacking the BH3-only protein Puma showed strong apoptosis resistance, while other BH3-only knockouts such as Bim-/- and Bad-/- did not. The exact mechanism by which Puma regulates apoptosis is still unclear. To elucidate the role of Puma and its interaction with anti- (Bcl-2, Bcl-xL, Mcl-1) and pro-apoptotic (Bax, Bak) family members under endogenous conditions, we have performed co-immunoprecipitation and kinetic apoptosis analysis on three different IL-3 dependent cell lines (FDM, FL5.12 and FDC-P1). In healthy cells Bcl-2, Bcl-xL and Mcl-1 are bound to Bak. In response to IL-3 deprivation Puma and Bim are upregulated and interact with Bcl-2 survival factor in a time-dependent manner, thereby displacing Bak. Subsequently, we observe Bak and Bax oligomerization. Although these data support the model that apoptosis is triggered by the release of Bak (and Bax) from Bcl-2 survival factors, we cannot exclude the possibility that Puma and Bim can also activate Bax and Bak directly.

Keywords: apoptosis, Bcl-2 family members

Exogenous thymosin beta-4 protects human cornea epithelial cells from menadione-induced apoptosis by upregulating Cu/ZnSOD expression

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In our previous work, exogenous thymosin beta-4 (Tbeta4) has been shown to inhibit the apoptosis of SV40-immortalized human cornea epithelial (HCE-T) cells induced by H2O2 and FasL. The purpose of this study is to examine whether exogenous Tbeta4 protects similar cells against the toxic effects of a superoxide anion producing agent, menadione, and elucidate the underlying mechanism if Tbeta4 was effective. Indeed, menadione increased superoxide anion production in HCE-T cells, triggered their death dose-dependently and upregulated caspase-9 activity, and the former two effects were diminished by an antioxidant, N-acetylcysteine (NAC). In the meantime, pretreating these cells with the recombinant histidine-tagged Tbeta4 (His-Tbeta4) not only decreased the death but also inhibited the elevation of superoxide anion as well as the activation of the caspase-9 triggered by menadione. Interestingly, the aforementioned cytotoxicity of menadione could be suppressed by an oxidized Tbeta4 whose effect also depended on its internalization. Finally, we found that menadione-induced upregulation of Cu/ZnSOD mRNA, protein and activity in HCE-T cells was markedly enhanced by exogenous Tbeta4. Accordingly, Cu/ZnSOD promoter activity stimulated by menadione was also significantly enhanced by Tbeta4 pretreatment. Intriguingly, only a partial diminution in the cytoprotection of Tbeta4 was detected when Cu/ZnSOD or MnSOD was knocked down by their respective shRNAs in HCE-T cells. On the other hand, a simultaneous knockdown of both SODs in these cells completely abolished the protective effects of this peptide against menadione-induced apoptosis. Taken together, our data suggest that upregulating Cu/ZnSOD gene expression may be one of the major mechanisms for exogenous Tbeta4 (probably their oxidized adducts as well) to protect HCE-T cells from menadione-induced apoptosis.

Keywords: Thymosin beta-4, cornea epithelial cells, oxidative stress, apoptosis, Cu/ZnSOD

Presenting Author: Manikandan Subramanian

Estrogen modulation of human macrophage apoptosis is mediated by Bax and Bcl-2

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Macrophages, the cells of the mononuclear phagocyte system play a central role in innate and adaptive immune responses. These cells express functional estrogen receptors and hence their survival and function is modulated by estrogen which has been linked to the pleiotropic effect of this hormone on the immune system and partly accounts for the gender bias observed in numerous autoimmune, cardiovascular and neurodegenerative disorders. The effect of estrogen on human macrophage survival and the mechanism of modulation of pro- and anti-apoptotic proteins by estrogen is largely unknown.

Our study reveals the dichotomous nature of estrogen signaling in human THP-1 macrophages where it provokes a death response by initiating the translocation of Bax from the cytosol to the mitochondria while a concurrent up-regulation of Bcl-2 results in cell survival. The mechanistic basis of estrogen signaling to modulate these two members of the Bcl-2 family is distinctly different. The estrogen-induced Bcl-2 up-regulation is mediated via signaling through membranous estrogen receptor α as established by the use of membrane impermeable form of estrogen (estrogen conjugated to BSA), which results in a rapid influx of Ca2+ through the L-type Ca2+ channel leading to activation of PKC/ERK pathway to initiate the transcription of Bcl-2. On the other hand, Bax translocation is independent of Ca2+/PKC/ERK pathway, but requires estrogen signaling via the intracellular pool of cytoplasmic estrogen receptor beta which results in the activation of the Na+-H+ (Sodium-hydrogen) exchanger leading to intracellular alkalinization which acts as a signal for Bax migration from the cytosol to mitochondria. The estrogen-induced Bcl-2 upregulation is crucial for the survival of human macrophages, probably by preventing Bax oligomerization in the mitochondria, as revealed by estrogen-induced cell death under siRNA mediated Bcl-2 knockdown conditions.

This study reveals the mechanistic basis of estrogen-induced Bcl-2 upregulation and Bax translocation and provides multiple targets for modulating the death pathway in human macrophages populating an estrogen-rich microenvironment. This is especially important in tumors of estrogen target tissues, as tumor associated macrophages (TAM) are known to play a pro-tumorogenic role and current anti-tumor strategy includes the targeted destruction of these cells to enhance the chemotherapeautic efficiency.

Keywords: Bax, Bcl-2, estrogen, apoptosis, macrophage

Presenting Author: Vadim Sumbayev

Cross-talk of HIF-1α protein and apoptosis signal-regulating kinase 1 (ASK1) downstream pathway is essential for protection of myeloid cells against LPS-induced toll-like receptor 4-mediated apoptosis and production of IL-6

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Sepsis is the leading cause of death in intensive care units, which reflects detrimental host response to infection where lipopolysaccharide (LPS) shared by Gram-negative bacteria acts as a potent activator of immune cells via Toll-like receptor 4 (TLR4). TLR4 is expressed in different cell types including myeloid cells, the key effectors of innate immune reactions. By now many aspects of TLR4 downstream signalling remain unclear.

In this study we have found that LPS-induced TLR4 signalling triggers cross-talk of hypoxia inducible factor 1 α (HIF-1α)ƒnand ASK1 downstream pathway in THP-1 human myeloid leukaemia cells. Both pathways are activated via redox-dependent mechanism associated with tyrosine kinase/phospholipase C-1gamma-mediated activation of protein kinase C α/beta ƒnthat activates NADPH oxidase and therefore production of reactive oxygen species that activate both HIF-1αƒnand ASK1 [1]. TLR4-dependent activation of PI3-kinase and direct S-nitrosation were found to downregulate ASK1 during TLR4 downstream signalling protecting the cells against LPS-induced, ASK1-mediated apoptosis [2]. ASK1 contributes to stabilisation of HIF-1αƒnprotein via activation of p38 MAP kinase that directly phosphorylates HIF-1α [1]. Knockdown of HIF-1α in THP-1 cells with siRNA suggested that this protein is critical for regulation of energy metabolism and protection of LPS-stimulated cells against ASK1-induced programmed death. Furthermore, HIF-1α protein supports TLR4-dependent IL-6 production by protecting the cells against depletion of ATP [3].

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Keywords: Inflammation, apoptosis, hypoxia, toll-like receptors

Effect of proteasome inhibitor low dose on cell death and genes expression in isolated neonatal cardiomyocytes culture

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The ischemia-reperfusion leads to the decrease in proteasome activity in ischemic zone. On the other hand, proteasome inhibitors prevent ischemic injury and this phenomenon seems to be of practical importance. At present, proteasome inhibitors are regarded as potential drugs that prevent negative consequences of heart and brain ischemia. The aim of this work was to investigate the cell death and genes expression in neonatal cardiomyocytes culture at proteasomal inhibitor low dose application and ischemia-reperfusion modeling. We evaluated the expression of following genes: genes of stress-proteins HSP70 and HSP90, genes of proteins taking part in realization of different types of cellular death - key autophagy regulator mTOR, antiapoptotic protein Bcl2, and gene of hypoxia-inducible factor HIF-3α. Cardiomyocytes were isolated from the ventricles of 2-3-day-old Wistar rats by serial collagenase/pancreatin digestion. After overnight plating, cells were treated with 100 nM of proteasomal inhibitor (PI) clasto-lactacystin β-lactone for 24 h. After that the cells were subjected to global anoxia for 30 minutes followed by 1 hour of reoxygenation (A/R). The number of living, necrotic and apoptotic cells was determined by staining with 8.75 μM bisbenzimide (Hoechst 33342), propidium iodide and examined by fluorescent microscopy. Total mRNA extracted from neonatal rat cardiomyocytes and subjected to reverse transcription was analyzed with the use of Fast real-time-PCR system. The A/R significantly influenced on ratio of living, necrotic and apoptotic cells. The PI in low dose protected the cells culture from the anoxia-reoxygenation injury after 24 hours. Anoxia-reoxygenation injury led to 2-fold and 3-fold increase ($P < 0.05$) of necrotic and apoptotic cells number respectively. The PI almost attenuated the effect of A/R on culture and decreased A\R-induced apoptotic and necrotic cells death. The treatment with PI in low dose for 24 h lead to 2-fold and 1,5-fold increase of HSP70 and HIF-3α, FRAP genes expression respectively. The levels of HSP90 and Bcl2 mRNA were slightly affected with insignificant tendency to reduction at PI application

Keywords: proteasome inhibitor, cell death, genes expression

Restoration of Akt activity prevents hippocampal apoptosis in experimental pneumococcal meningitis

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Apoptosis of recently post-mitotic immature neurons in the hippocampus represents an important form of brain damage in meningitis caused by the gram-positive bacterium Streptococcus pneumoniae. The death of these neurons is thought to be responsible for the learning and memory deficits presented by patients that have survived this infection. The pro-survival kinase Akt is an important determinant of neuronal cell fate in other forms of acute brain injury. Akt is activated via PI3-kinase (PI3K)-dependent formation of phosphatidylinositol 3,4,5-triphosphate (PIP3) and inactivated by de-phosphorylation of PIP3 by the lipid phosphatase PTEN. Here we show in a well-characterized infant rat model of pneumococcal meningitis that infection-induced hippocampal apoptosis coincides with a significant decrease in hippocampal PIP3 levels, phosphorylation of Akt and its downstream target Bad. At the cellular level, decreased Akt phosphorylation and nuclear translocation of the Akt downstream target FOXO1 was found in the hippocampal dentate gyrus, the region where apoptosis in pneumococcal meningitis occurs, and not in other regions of the hippocampus. Systemic treatment with the PTEN inhibitor bpV(pic) prevented the loss of Akt phosphorylation and significantly attenuated hippocampal apoptosis. Stereotaxic treatment with the PI3K inhibitor LY294002 inhibited Akt re-phosphorylation by bpV(pic) and abrogated inhibition of hippocampal apoptosis. Thus, reduced activity of the Akt pro-survival pathway contributes to hippocampal apoptosis in pneumococcal meningitis.

Keywords: Akt, hippocampus, inflammation, pneumococcal meningitis, development

Presenting Author: Karolina Swiatek-Machado

Evaluation of Anti-tumor Activity of Novel Inhibitors of JAK/STAT3 Pathway in Glioma Cells

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STAT (Signal Transducers and Activators of Transcription) signaling is crucial for regulation of cell differentiation, growth and apoptosis. Recently, constitutive activation of STATs, in particular STAT3, have been found in a large number of human tumors. Persistent signaling of STAT3 may contribute to oncogenesis by stimulating cell proliferation and preventing apoptosis, thus it emerges as promising molecular target for cancer therapy.
We developed a cell-based system for screening the small molecules inhibitors targeting STAT3 signaling and screened 18 compounds synthesized by modifying the structure of WP1066. In search for inhibitors, we evaluated drug effects on STAT-driven transcription, cell viability and STAT3 phosphorylation (determined by Western immunoblotting). Compound 2 [(E)-2-cyano-N-((S)-1-phenylethyl)-3-(pyridin-2-yl)acrylamide] was found to be the most promising STAT3 inhibitor. Comp2 at doses 25 uM significantly reduced the level of phosphorylated STAT3 (Tyr705) as well as phosphorylated upstream regulators JAK1 and JAK2. Treatment with Comp2 at doses 25 uM significantly inhibited a viability of rat C6 glioma cells. Appearance of cleaved caspase 3 and PARP, hallmarks of apoptosis, were detected 24 h after treatment with 25 uM Comp2. Noticeably, we observed a rapid (in 30 min) cell detachment from a substratum. To investigate the molecular changes induced by Comp2 downstream of STAT3, the expression of cell cycle and apoptosis regulators was evaluated by real-time PCR. The results show that the expression of genes coding for cyclin D1 and antiapoptotic protein Bcl-XL was significantly diminished in C6 glioma cells treated with 25 uM Comp2. The effect of Comp2 on MAPK and PI3K/Akt signaling pathway were determined. While Comp2 strongly elevated the level of phosphorylated p38, JNK and ERK1/2, phosphorylation of Akt remained unchanged and phosphorylation of focal adhesion kinase (FAK) decreased. The present study demonstrates that selected, newly synthesized compounds inhibit STAT3 phosphorylation and STAT-dependent transcription, affect cell attachment and viability of glioma cells.

The study is supported by Ministry of Science and Higher Education (PBZ-MIN 014/P05/2004).

Keywords: STAT3 signaling, JAK, apoptosis, glioma, Akt signaling, MAPK

Presenting Author: Zsuzsa Szondy

Transglutaminase 2 contributes to the formation of an efficient engulfing centre in macrophages taking up apoptotic cells via promoting recognition of apoptotic cells by integrin ß3 receptors

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Transglutaminase 2 (TG2) is a protein crosslinking enzyme with many additional biological functions, such as being an integrin beta3 coreceptor by facilitating its fibronecting binding and signalling during cell adhesion. We have previously shown that in TG2-/- mice the in vivo clearance of apoptotic cells is defective. Here we report that TG2 on the macrophage cell surface, in guanine nucleotide bound form and concentrated in the phagocytic cup promotes phagocytosis. We show that TG2, besides binding to integrin beta3, is also a binding partner for milk fat globulin-8, a bridging molecule between integrin beta3, a receptor known to mediate the uptake of apoptotic cells via RhoG-GTP induced Rac activation, and phosphatidylserine on the apoptotic cells. We show that in wild-type macrophages integrin beta3 is accumulated around the first attaching apoptotic cell, and induces the formation of an engulfing gate through which the additional apoptotic cells will be efficiently taken up. In the absence of TG2, integrin beta3 and consequently Rac cannot be accumulated and activated at one pole of the macrophage and the engulfing gate is not formed, though the levels of integrin beta3 are enhanced. Together, our data indicate that TG2 is a new protein member of the phagocytic cup, which together with MFG-8 is required for proper apoptotic cell recognition and integrin beta3 signalling. In vivo loss of these proteins leads to the development of autoimmunity.

Supported by OTKA T049445 and ETT 115/2006 grants.

Keywords: autoimmunity, phagocytosis of apoptotic cells, inflammation

Presenting Author: Sei-ichi Tanuma

Creation of Apoptosis Regulating Small Molecules targeting Protein Interactions by in silico Method

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Protein interactions have key roles in most biological cellular processes from cell survival to death. The ability to modulate specifically (suppression as well as activation and stabilization) with protein interactions by small molecules provides a promising and powerful means of regulating the cellular functions. Furthermore, it offers attractive opportunities for therapeutic approaches to apoptosis diseases such as cancer. However, direct screening and design of such small molecules are difficult, because of the lack of well-defined binding pockets in the target regions of protein-protein interactions. So, we developed a new in silico methodology towards drug creation targeting of protein interactions. Our concept for the creation of small molecules is the conversion of the designed optimized binding-peptide to non-peptidic small molecules by energy distribution mimetics rate study, and then auto-optimization of the selected small molecules. Here, we describe the usefulness of this strategy, named COSMOS (conversion to small molecules thorough optimized-peptides strategy), for designing of effective lead compounds for the creation of new pharmaceuticals against apoptosis diseases.

Keywords: protein interaction, in silico methodology, drug design

Presenting Author: Andrey Taranukhin

Taurine does not protect Purkinje cells against caspase-3 activation induced by ethanol in the cerebellum of 4-day-old mice

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Taurine is a sulfur-containing amino acid abundant in the nervous system. It is involved in a wide range of physiological processes such as osmoregulation, lipid metabolism, intracellular calcium regulation, neuronal development, neuromodulation and cell protection. Recently findings indicate that taurine can suppress caspase-9 activation and in this way rescue cells from ischemia-induced apoptosis. Our previous results obtained from 7-day-old male mice showed that taurine reduced ethanol-induced caspase-3 activation in cerebella granular neurons. In the present work we focused on the possible neuroprotective effect of taurine in ethanol-induced apoptosis of the Purkinje cells. The central nervous system is extremely sensitive to ethanol during its development and the periods of vulnerability are temporarily well defined. The experiments were performed on 4-day-old male mice. This age was chosen as the most sensitive to ethanol-induced apoptotic neurodegeneration for the Purkinje cells.
The animals were divided into four groups: ethanol-treated, ethanol + taurine-treated, taurine-treated and controls. Ethanol was mixed in sterile saline to 20% solution and administered subcutaneously at a total dose of 5 g/kg (2.5 g/kg at time zero and 2.5 g/kg at 2 h) to the ethanol and ethanol + taurine groups. The ethanol + taurine group also received two injections of taurine (1 g/kg each diluted with saline). The first injection was given one hour before the first ethanol injection and the second injection one hour after the second ethanol injection. The taurine-treated group received the same two taurine injections as the ethanol + taurine group. The control animals were given subcutaneously saline. Twelve hours after the first ethanol injection all animals were killed by decapitation. Their cerebella were rapidly excised, fixed in 4 % paraformaldehyde and embedded in paraffin.
To estimate apoptosis, immunostaining for activated caspase-3 were done in the mid-sagittal sections containing lobules I-X of the cerebellum. There were randomly activated caspase-3-immunoreactive cells visible in the control group, reflecting the rate at which spontaneous (physiological) cell death occurs at this age. Following ethanol administration, there was a marked increase in activated caspase-3-immunoreactive Purkinje cells in all cerebellar lobules, the increase being greatest in lobules IX and X, indicating ethanol-triggered apoptotic neurodegeneration. Contrary to our previous results on cerebellar granular neurons of 7-day-old mice, in the ethanol + taurine group taurine treatment did not affect the number of activated caspase-3-immunoreactive Purkinje cells in 4-day-old mice when compared to the ethanol group. Taurine did not either alter the number of activated caspase-3-immunoreactive Purkinje cells in the taurine-treated group when compared to the control group.
We conclude that taurine in the dose used does not protect Purkinje cells in 4-day-old mice against ethanol-induced caspase-3 activation.

This study was supported by the competitive research funding of the Pirkanmaa Hospital District and the Finnish Cultural Foundation.

Keywords: taurine, ethanol-induced apoptosis, caspase-3, Purkinje cells

Presenting Author: Svenja Tobaben

12/15-Lipoxygenases mediate Bid-dependent cell death after glutathione depletion in HT-22 neurons

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Oxidative stress is a key feature involved in neuronal degeneration and death after acute brain damage and in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. Generation of highly detrimental reactive oxygen species (ROS) in these neuropathological processes may result from glutathione depletion, membrane lipid peroxidation, and uncoupling of the mitochondrial respiratory chain, among others. In the present study we investigated the particular role of lipoxygenases and according sequence of (lipid) ROS formation, mitochondrial damage and cell death signaling in immortalized mouse hippocampal neurons (HT-22) exposed to glutamate (2-5 mM). In HT-22 cells, glutamate blocks the cysteine import thereby inducing intracellular glutathione depletion, oxidative stress and cell death.
In HT-22 cells, formation of ROS increased approximately two-fold within 8 h after glutamate exposure followed by a boost of free radical formation as measured by the fluorescent ROS-detecting dyes DCF (Dichlorodihydrofluoresceine diacetate) and Bodipy (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) at 18 h. Inhibition of 12/15-LOX by PD146176 or AA816 prevented ROS formation and significantly reduced glutamate-induced HT-22 cell death. In contrast, inhibition of cyclooxygenases by indometacin, failed to protect HT-22 cells against glutamate-induced cell death. It is interesting to note, that post-treatment with the 12/15-LOX-inhibitor PD 146176 protected HT-22-cells when applied up to 8 hours after onset of the glutamate-treatment. Cell death mechanisms downstream of glutamate-induced ROS formation involved Bid translocation to mitochondria, loss of mitochondrial membrane potential, and subsequent apoptosis inducing factor (AIF) translocation from mitochondria to the nucleus. Bid siRNA and the Bid-inhibitor Bi-6C9 rescued mitochondrial membrane integrity and prevented the secondary boost of ROS formation, but did not affect the primary lipid peroxidation within 8 h after glutamate treatment. Bid inhibition and AIF-siRNA prevented glutamate-induced cell death, whereas caspase activation was only detected at very late stages of cell death and caspase inhibitors failed to protect the cells from glutamate toxicity. It is important to note that neither the LOX inhibitors nor Trolox, a vitamin E analogon, attenuated the cytotoxic effects of tBid, suggesting that lipid peroxidation occurred upstream of Bid activation and the related caspase-independent mitochondrial mechanisms of AIF-dependent apoptosis.
In conclusion, our data suggest that glutathione depletion leads to enhanced formation of lipid peroxidation by 12/15-LOX, followed by the activation of Bid and subsequent AIF release and execution of caspase-independent cell death. Therefore, inhibition of 12/15-LOX may be a promising strategy to prevent neuronal cell death associated with glutathione depletion that has been proposed in the neuropathology of ischemic brain damage, Alzheimer's disease or Parkinson's disease.

Keywords: oxidative stress, mitochondria, LOX, Bid, glutamate, neuronal death

The interaction of HAMLET with α-actinin-4 triggers shedding of carcinoma cells due to inhibition of beta-1 integrin binding

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The tumoricidal HAMLET (human α-lactalbumin made lethal to tumor cells) complex consists of partially unfolded α-lactalbumin and oleic acid (Hakansson et al., Proc Natl Acad Sci U S A, 1995; Svensson et al., Proc Natl Acad Sci U S A, 2000). HAMLET kills a wide range of tumor cells in vitro and has proven clinical effects on papillomas and bladder cancers. Healthy differentiated cells have so far been resistant, suggesting that HAMLET offers a new approach to exploring mechanisms of selective tumor cell death. HAMLET rapidly enters tumor cells but the mechanism is still not fully understood. Once inside, HAMLET accumulates in the nucleus and induces rapid cell death. HAMLET-treated cells show signs of apoptosis, e.g. DNA fragmentation and cell shrinkage, but earlier studies have shown that HAMLET does not induce cell death via the classical apoptotic pathways (Svanborg et al., Adv Cancer Res, 2003). Massive shedding of tumor cells in vivo has been observed in an earlier study on patients with bladder cancer by Mossberg et al. (Mossberg et al., Int J Cancer, 2007). Tumor cell detachment is also observed in vitro while healthy cells remains adherent. The aim of this study was to identify new interaction partners for HAMLET that might explain the mechanism of tumor cell detachment.
The mechanism of tumor cell detachment in response to HAMLET was investigated, using a proteomics approach. Total cell extracts were prepared from A549 human lung carcinoma cells and membrane-associated proteins were isolated. The fractions were separated by SDS-PAGE, blotted onto a PVDF membrane and overlaid with HAMLET. Bound HAMLET was visualized using polyclonal antibodies recognizing α-lactalbumin. We observed that HAMLET bound to a 96 kDa protein, which was identified as human α-actinin-4 by Mass spectrometry. The interaction was confirmed by co-immunoprecipitation. Synthetic peptides were used to further characterize the interaction. We saw that HAMLET bound specifically to the actin- and beta-1 integrin-binding sites on α-actinin-4 thus suggesting that HAMLET may disrupt focal adhesions by competing for these binding sites on α-actinin-4. Confocal microscopy also showed a re-distribution of α-actinin-4 in tumor cells in response to HAMLET. Untreated cells show granular staining at the cell periphery and weak staining in the cytoplasm. After HAMLET, the staining in tumor cells was stronger at the cell periphery and more uniform. Healthy cells, in contrast, maintained their granular staining pattern. A western blot showed reduced FAK and ERK1/2 phosphorylation, thus further suggesting that the interaction between HAMLET and α-actinin-4 disrupts focal adhesions.
HAMLET was shown to influence α-actinin-4 staining in vivo as well. Tumor sections from HAMLET treated mouse bladders were subjected to immunohistochemistry, using α-actinin-4 specific antibodies. An increase in α-actinin-4 staining was detected in tumor tissue adjacent to the urinary tract lumen. Alpha-actinin-4 staining was localized to the periphery of the tumor cells, with a pattern similar to that observed in HAMLET-treated carcinoma cells in vitro. This intense, membrane α-actinin-4 staining was not detected in healthy bladder tissue from the same mouse.
We conclude that HAMLET interacts with α-actinin-4 and triggers cell detachment.

Keywords: cancer, HAMLET, α-actinin-4, detachment

Presenting Author: Thomas Unterkircher

Velcade mediated sensitization to TRAIL induced apoptosis in glioblastoma

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Glioblastoma multiforme (GBM), the most common primary brain tumor, is highly refractory to current treatment approaches. As even highly aggressive chemotherapy is not sufficient to raise median survival of GBM patients beyond one year, new targets for anticancer therapy need to be established. Such resistance to chemotherapy is often associated with defects in the apoptotic signaling networks, rendering cells unable to undergo programmed cell death. One novel target for cancer therapy is the ubiquitin-proteasome system. Velcade, also known as Bortezomib, was the first agent targeting the proteasome used in clinical trials and is approved for treating relapsed multiple myeloma, in this tumour complete clinical responses have been obtained in patients with otherwise refractory or rapidly advancing disease. Interestingly, it was recently observed that proteasome inhibition is able to sensitize TRAIL-resistant glioblastoma cells to TRAIL-triggered apoptosis independently of NF-κ-B. However, it is not well understood in which way proteasome inhibition leads to sensitization to this death inducing ligand. We found that combinatory treatment of glioblastoma cells with Velcade and TRAIL markedly reduces viability and enhances apoptosis of glioblastoma cell lines, whereas treatment with either agent alone does not. Cells treated with Velcade and TRAIL showed rapid activation of the extrinsic apoptotic pathway and cleavage of Caspase-8 and Caspase-3. Both TRAIL-treated and combinatory treated cells formed a Death Inducing Signaling Complex with recruitment of FADD and Caspase-8. Further studies indicated that the mitochondrial apoptotic pathway might be important for the sensitization process. When treating glioblastoma cells with both Velcade and TRAIL we observed reduction of full-length Bid, activation of Bax, loss of the mitochondrial membrane potential and activation of Caspase-9. Also the BH-3-only protein Noxa was strongly induced in Velcade treated cells. Knockdown of Noxa by shRNA however was not sufficient to rescue cells from Velcade and TRAIL triggered apoptosis. Taken together our results suggest that cotreatment of glioblastoma cells with Velcade and TRAIL might be a feasible strategy to break apoptosis resistance in this highly malignant tumor.

Keywords: Velcade, Proteasome Inhibition, Glioblastoma, TRAIL

Death with Semliki Forest (SFV), a positive strand RNA virus that requires RNA replication and mitochondrial Bak for apoptosis induction

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The RNA αvirus Semliki Forest (SFV) triggers apoptosis in various mammalian cells but it has remained controversial at what infection stage and by which signalling pathways host cells are killed. Both RNA synthesis dependent and –independent initiation processes and mitochondrial as well as death receptor signalling pathways were implicated. Here we show that SFV-induced apoptosis is initiated at the level of RNA replication or thereafter. Moreover, by expressing anti-apoptotic genes from recombinant SFV (replicons) and using neutralizing reagents and gene-knock-out cells, we provide clear evidence that SFV does not require CD95L, TRAIL or TNF-mediated signalling but mitochondrial Bak to trigger cytochrome c release, Apaf-1/caspase-9 apoptosome formation and caspase-3/-7 activation. Of seven BH3-only proteins tested, only Bid was partially important for effective SFV-induced apoptosis. However, caspase-8 activation and Bid cleavage occurred downstream of Bax/Bak indicating that tBid formation serves to amplify rather than to trigger SFV-induced apoptosis. In an attempt to identify the molecular link between virus replication and mitochondrial membrane perforation, we focussed on the intracellular signalling pathways induced by dsRNA, an intermediate in the replication cycle of SFV. Out of the three intracellular dsRNA sensing systems, which are also used to regulate innate immunity, dsRNA-dependent protein kinase (PKR) signalling is not required for SFV-induced apoptosis. By using various knock-out cells lines and siRNA techniques, we are now looking at the implication of the two other signalling systems (RIG-I and MDA-5) in apoptosis induction by SFV.

Keywords: αvirus, mitochondria, Bak

Presenting Author: Alena Vaculova

TRAIL and small cell lung carcinoma: Is there any rationale for therapeutic use?

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TRAIL (TNF-related apoptosis-inducing ligand), a member of tumour necrosis factor (TNF) family, is known to induce apoptosis in many cancer cells but not in most normal cells and thus represents a promising anticancer agent. However, some cancer cells including small cell lung carcinoma (SCLC) have been shown to be non-responsive to TRAIL. Understanding molecular mechanisms of SCLC resistance, along with finding agents that are capable of sensitizing the cells to TRAIL effects are important prerequisites for proper therapeutic strategies.

Majority of human SCLC cells are resistant to death receptor (DR)-mediated apoptosis because of defects in key signalling molecules such as DRs and/or initiator caspases. Caspase-8 is frequently inactivated by epigenetic silencing (hypermethylation of gene regulatory region) in this type of cancer. Comparison of six SCLC cell lines with different caspase-8 status (3 of them containing and 3 lacking caspase-8 protein) revealed that both cell groups are equally resistant to TRAIL-induced apoptosis. We investigated the expression of other components of TRAIL apoptotic signalling pathway - TRAIL receptors (TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1, TRAIL-R4/DcR2), FADD, caspase-10, and FLIP in these cells. Although 3 of cell lines expressed DR4 mRNA and intracellular protein, only in 1 out of the 6 cell lines DR4 was presented at the cell surface. On the other hand, 5 out of 6 cell lines were characterized by surface and intracellular DR5 expression. While no surface DcR1 was present in any of the cell lines, DcR2 was always detectable. All cell lines also expressed a relatively high level of FADD protein, and lacked caspase-10 expression. Finally, we observed significant differences in FLIPL/S level, which were not related to the presence/absence of caspase-8.

In order to sensitize the caspase-8 containing SCLC cells to TRAIL-induced apoptosis, selected chemotherapeutic agents we used in combination with TRAIL. We found that TRAIL and doxorubicin or TRAIL and etoposide acted cooperatively to kill these cells and that the cooperation involved increase of caspase-8 processing, followed by effector caspase activation and enhanced apoptosis execution. Restoration of caspase-8 expression by gene transfer or demethylation agents in caspase-8-lacking cells was not sufficient for their sensitization to TRAIL-induced apoptosis. Moreover, combined treatment with TRAIL and chemotherapy (doxorubicin) did not seem to be effective in overcoming the resistance to TRAIL in cells with restored caspase-8 expression. As no caspase-8 processing was apparent in these cells, we suggest additional block(s) at and/or above the caspase-8 level. Thus, obtained data have important therapeutic implications, suggesting that (a) TRAIL monotherapy is generally inefficient in treatment of SCLCs, and (b) combination of TRAIL and anticancer agents might be beneficial for elimination of caspase-8-containing SCLC cells.

Keywords: TRAIL, apoptosis, lung cancer

Presenting Author: Helin Vakifahmetoglu

Mitotic catastrophe: A phenomenon that precedes necrosis or apoptosis

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The term “mitotic catastrophe” (MC) is used to explain a mechanism of a delayed mitotic-linked cell death, a sequence of events that results from premature or inappropriate entry of cells into mitosis caused by chemical or physical stresses. Initially MC was depicted as the main form of cell death induced by ionizing radiation, but today it is known to be triggered also by treatment with agents influencing the stability of microtubule, various anticancer drugs and mitotic failure caused by defective cell cycle checkpoints. MC has been considered as a mode of cell death and various descriptions explaining mitotic catastrophe exist. However, there is still no general accepted definition of this phenomenon. Some reports suggest that MC shares several biochemical hallmarks of apoptosis, in particular, mitochondrial membrane permeabilization and caspase activation. In spite of these observations, important differences between the two cell death modalities have emerged.

Investigation of the contribution of p53 and caspase-2 to apoptotic cell death and MC in cisplatin-treated ovarian carcinoma cell lines revealed that both functional p53 and caspase-2 were required for the apoptotic response, which was preceded by translocation of nuclear caspase-2 to the cytoplasm. In the absence of functional p53, cisplatin treatment resulted in caspase-2-independent MC followed by necrosis. In these cells, apoptotic functions could be restored by transient expression of wt p53. Hence, in this experimental model p53 appeared to act as a switch between apoptosis and MC followed by necrosis-like lysis. Further, we show that upon treatment with DNA-damaging agents, cells that are sensitized to undergo MC by checkpoint inhibition or deficiency still die through apoptosis.

Thus, we demonstrate that the final mode of cell death triggered by DNA damage in ovarian carcinoma cells is determined by the profile of proteins involved in the regulation of the cell cycles. Accumulated data indicate that death-associated MC is not a separate mode of cell death, rather a process (“pre-stage”) preceding cell death, which can occur through necrosis or apoptosis.

Keywords: Apoptosis, Cell cycle, Necrosis, mitotic catastrophe

Presenting Author: Marianne van Dijk

Study of the mechanism of TRAIL resistance in non-transformed cells.

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Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumour necrosis factor family of death ligands. TRAIL is able to bind to two death inducing receptors, DR4 and DR5, and to two decoy receptors, DcR1 and DcR2. Through binding to DR4 and DR5, TRAIL can induce apoptosis in tumour cells. Normal, untransformed cells are resistant to TRAIL, however, very little is known about the mechanism of their resistance. Approximately 50-60% of tumour cells are resistant to TRAIL. To sensitise these cells to TRAIL without causing toxicity to normal cells, the mechanism that protects non-transformed cells from TRAIL should be identified.

As a typical non-transformed cell type, primary human fibroblasts were used. Fibroblasts mainly express DR5 on the cell surface and small amounts of DR4, DcR1 and DcR2. Ligation of the DR5 receptor with an agonistic antibody failed to induce apoptosis, indicating that the decoy receptors are not the sole reason for resistance, instead, the intracellular part of the signalling pathway is not functional. TRAIL treatment also failed to induce pro-caspase-8 cleavage, suggesting that the pathway is inhibited upstream of pro-caspase-8 processing. To investigate whether an anti-apoptotic protein is expressed that maintains the resistance towards TRAIL, cells were treated with the protein synthesis inhibitor cycloheximide. Cycloheximide could not sensitise the cells to TRAIL-induced apoptosis, however, it could sensitise the fibroblasts to Fas-induced apoptosis. This indicates that blocking the synthesis of an anti-apoptotic protein is sufficient to re-open the Fas-, but not the TRAIL-induced apoptotic pathway. Our current studies examine what is the additional factor required for TRAIL-mediated apoptosis, focusing on the mechanism of receptor activation.

Keywords: TRAIL, normal cells

Presenting Author: Miroslav Varecha

Interactions of apoptotic proteins AIF and endonuclease G analyzed by bioinformatic predictions, molecular docking, and fluorescent microscopy

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During apoptosis several mitochondrial proteins are released. Some of them participate in caspase-independent nuclear DNA degradation, especially apoptosis-inducing factor (AIF) and endonuclease G (endoG). We studied the structure, cellular localization, and interactions of several proteins in silico and in vitro using fluorescent microscopy. Bioinformatic predictions were conducted to analyze the presence of interaction sites in the studied proteins. We conducted molecular modeling of proteins with unknown 3D structure: endonuclease G, CPS-6, WAH-1, and heat shock protein 70-1. These models were then refined by MolProbity server and employed together with experimentally known 3D structures of other proteins like AIF and cyclophilin A in molecular docking simulations of interactions. Fluorescence resonance energy transfer (FRET) technique and consequent image analysis was used to evaluate the interactions of fluorescently labeled proteins in cells. Our results represent new information about the structure, cellular localization, and interactions of several proteins involved in caspase-independent apoptotic death.

This work was supported by The Ministry of Education, Youth and Sports of the Czech Republic (projects number MSM0021622419, 2B06052, and LC535).

Keywords: AIF, endonuclease G, heat shock protein, cyclophilin A, CPS-6, WAH-1, colocalization, image analysis, FRET, interaction prediction, protein structure modeling, molecular docking

Presenting Author: Tom Verfaillie

The unfolded protein response as a modulator of cancer cell death pathways after ROS-mediated ER damage

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One of the major obstacles for the effective treatment of human cancers is the activation of adaptive or protective responses in cancer cells after therapy. An emerging stress-mediated response with a putative role in carcinogenesis and in therapy resistance is the Unfolded Protein Response (UPR), which is initiated following perturbations of the endoplasmic reticulum (ER) functions. In its initial phase the UPR is a pro-survival response activated in order to restore the folding capacity of the ER. If ER stress persists, the UPR is able to promote cell death which occurs mostly in the form of apoptosis. However, a clear molecular understanding of how the UPR sets apoptosis in motion after ER stress is missing. Recently, we reported that hypericin-mediated photodynamic therapy (PDT), a promising anticancer modality for the treatment of superficial bladder cancer, promotes ER stress in cancer cells. The generation of highly cytotoxic reactive oxygen species (ROS) caused by light activation of the ER-associated hypericin, leads to immediate injury to the sarco(endo)plasmic Ca2+-ATPase (SERCA) pump and subsequent ER-Ca2+ depletion, which is followed by induction of (macro)autophagy and Bax/Bak-dependent mitochondrial apoptosis [1].
Here we report that ROS-mediated ER injury in murine embryonic fibroblasts (MEF) and in cancer cells results in the activation of the UPR, which is accompanied by an extensive ER dilatation in vitro as well as in vivo. A dominant activation of the PERK-eIF2alfa-ATF4 branch of the UPR required for CHOP expression in our study is indicated at the transcript- and protein levels. The PERK-eIF2α-ATF4 pathway appears to be central for the induction of the BH3-only protein Bim, which occurs with kinetics matching caspase-3 activation both in WT MEF cells and T24 bladder cancer cells in response to ROS-induced ER stress. Moreover, the upregulation of Bim is prevented in CHOP- and, albeit to a lesser extent, in PERK-deficient cells in which CHOP upregulation is ablated. chop-deficient MEFs demonstrate increased survival after treatment, suggesting a possible role for Bim in this paradigm of ER stress. We are currently exploring the functional role of Bim, and other BH3-only proteins, in conveying apoptosis following ROS-mediated ER stress and the mechanisms underlying their regulation at the transcriptional and posttranslational levels by members of the MAPK family.

(1)Buytaert et al, (2006) FASEB J. 20:756-8

Keywords: cancer, hypericin PDT, ROS, ER stress, UPR

Parenteral administrations of ascorbate achieve blood pharmacological concentrations that exhibit both in vitro and in vivo antitumoral effects.

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The biological effects of ascorbic acid (vitamin C) are still subject of controversy. Recent human pharmacokinetic data suggest that pharmacological concentrations (1-20 mM) of vitamin C can be reached by intravenous injections (1, 2). Intrigued by these results we decide to study the pharmacokinetic of ascorbate in mice, as well as its putative anticancer activity. Our results show that pharmacological doses of ascorbate achieved by either intravenous or intraperitoneal administrations (1g/kg) lead to blood ascorbate concentrations in the mM range as compared to the 10 µM observed in control mice. On the opposite, the oral supplementation of the same dosage in drinking water induced only a three-fold increase of basal concentrations (36 µM). The cytotoxicity of ascorbate was then assessed in vitro by using two-hours exposure, a protocol which mimics a parenteral use. We observed that pharmacological concentrations of ascorbate killed very efficiently various cancer cell lines, with EC50 ranging from 3 to 7 mM. The mechanism of cytotoxicity is based on the production of reactive oxygen species, leading to necrotic cell death. According to our pharmacokinetic data, parenteral administrations of ascorbate were able to slow down (but did not inhibit) tumor growth in a tumor-bearing mice model, whereas oral administration of the same dosage had no effect. Importantly, ascorbate did not interfere with the activity of five chemotherapeutic drugs, but rather reinforced their cytotoxic effects. Taken together, these results suggest that high concentrations of ascorbate exhibit interesting anticancer properties and highlight the putative interest of redox-based strategies in cancer therapy.

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Keywords: cancer, oxidative stress, ascorbate

Presenting Author: Valerie Voss

The PINK1 mutant G309D specifically enhances sensitivity of PARK6 fibroblasts to apoptosis induced by proteasomal stress

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Proteasomal dysfunction and apoptosis are major hallmarks in the pathophysiology of Parkinson's disease (PD). PARK6 which is caused by mutations in the mitochondrial protein kinase PINK1 is a rare autosomal-recessively inherited disorder mimicking the clinical picture of PD. To investigate the cytoprotective physiological function of PINK1 in PARK6, we used primary fibroblasts from three patients homozygous for G309D-PINK1 as well as SHEP neuroblastoma cells stably overexpressing GFP-tagged wild type PINK1. Here we demonstrate that overexpression of GFP-PINK1 in SHEP cells inhibits release of cytochrome C, significantly diminishes proteolytic activation of caspase 9 and downmodulates effector caspase activity after induction of proteasomal stress induced by the two proteasome inhibitors (PIs) MG132 and Epoxomicin. Similiarly, effector caspase activation induced by proteasomal stress was potently increased in primary fibroblasts from homozygous PARK6 patients in comparison to those of heterozygous carriers or unaffected siblings. In contrast to their response to PIs, fibroblasts from homozygous patients and SHEP GFP-PINK1 cells did not exhibit major differences in their sensitivity to the two unrelated apoptotic stimuli staurosporine and etoposide in comparison to their respective controls. Interestingly, overexpressed GFP-tagged PINK1 localized to the mitochondria of SHEP cells, but was redistributed to the cytoplasm after treatment with proteasome inhibitors. Our data indicate that PINK1 plays an important and specific physiological role in protecting cells from proteasomal stress and suggest that PINK1 might exert its protective effects upstream of the mitochondria. They also underscore the applicability of peripheral cells for future studies on the molecular mechanisms underlying PD and the development of novel Parkinson therapies.

Keywords: Parkinson, proteasome, mitochondria, apoptosis, fibroblasts

Presenting Author: Lasse Neumann Waehrens

Imaging of phoshatidylserine on apoptotic plasma membranes utilizing bovine lactadherin

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Scrambling of the plasma membrane phospholipid asymmetry is an early feature of apoptosis. As a result, the phospholipid phosphatidylserine is exposed on the outer surface of the cell membrane, providing the recognition signal for the engulfment by phagocytes. We have found that lactadherin, a widely distributed glycoprotein isolated from bovine milk, is a useful probe to monitor early phosphatidylserine exposure on apoptotic cells. This is ascribed to its bifunctional binding properties, which includes tandem domains with homology to the phosphatidylserine binding domains of blood coagulation factor V and factor VIII. Like factor V and factor VIII, lactadherin specific phosphatidylserine binding is Ca2+-independent, has a stereoselective preference for phosphatidyl-L-serine, and prefers membranes of high curvature. In contrast to the two blood coagulation factors, lactadherin binds to membranes with low phosphatidylserine content (2%), regardless of the overall phospholipid composition in the membrane. By comparison, the membrane-binding motifs of annexin V, another phosphatidylserine binding protein, is not homologous with the C domains of lactadherin, factor V and factor VIII. Accordingly, annexin V's membrane binding specificity markedly differs form that of lactadherin. I.e. annexin V requires Ca2+, does not have the capacity to fully compete for phospholipid binding sites utilized by lactadherin, has a preference for membranes when the phosphatidylserine content exceeds 8% and holds a limited affinity for curved membranes. The presence of excess phospatidylethanolamine reduces the detection threshold to 2,5%.
Microscopy of costained apoptotic K562 cells with annexin V and lactadherin reveals a contrasting pattern. Lactadherin diffusely stains the plasma membrane of cells progressing through the cell death program and the intensity increases with progressive apoptosis. Annexin V stains internal bodies of the cells with intact nuclei and stains discrete membrane patches on these cells.
Taken together, lactadherin opens new avenues for complimentary detection of phosphatidylserine exposure at earlier stages of apoptosis than established methodology.

Keywords: Lactadherin, phosphatidylserine, imaging

Presenting Author: Dorothée Walter

Switch from type II to I Fas/CD95 death signaling upon in vitro culturing of primary mouse hepatocytes

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Apoptosis displays a crucial event for the maintenance of liver health, allowing hepatocytes to die through a highly controlled and regulated process. Previously it was shown that the injection of the agonistic anti-Fas antibody Jo-2 into wild type mice causes fatal hepatitis in vivo. However, mice lacking the BH3-only protein Bid were completely protected from liver failure indicating an absolute requirement for the type II apoptosis signaling pathway after Jo-2 challenge. In this work we present the remarkable finding that freshly isolated primary mouse hepatocytes, either cultured on collagen type I or BD MatrigelTM, switch CD95/Fas- induced apoptosis from type II to type I signaling. These cells undergo apoptosis in response to a physiological type of FasL, irrespective of the presence or absence of Bid. Crucial events of the type II pathway as Bid cleavage, Bax translocation, Bak activation and cytochrome c release do not occur in primary mouse hepatocytes in culture. Moreover, XIAP levels remain constant, limiting full caspase-3 activation. Indeed, hepatocytes isolated from XIAP-/- mice reveal a more rapid and extensive caspase-3 activation. Compared to hepatocytes plated on extracellular matrices, hepatocytes in suspension exhibit a dependency on Bid. The switch is specific for the CD95/Fas system as Bid-/- hepatocytes on collagen are protected from TNFa/ActD-induced cell death. Our results indicate that extracellular matrix components such as collagens or laminins trigger signaling cascades that specifically crosstalk with FasL signaling to abrogate its dependence on mitochondria for apoptosis induction.

Keywords: Apoptosis, hepatocytes

Presenting Author: Jana Wendt

Resistance of Bax deficient tumours to TRAIL can be overcome by Mcl-1 down-regulation

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TRAIL (Tumour necrosis factor related apoptosis-inducing ligand) is a promising anticancer agent in view of its preferential killing of tumour cells with limited cytotoxicity against non-malignant cells. The vast majority of tumours are of “type II” where death receptor signals require amplification via the mitochondrial apoptosis pathway. We previously established that TRAIL-induced cell death entirely depends on the proapoptotic Bcl-2 family member Bax but not the homologous Bak. Bax is often lost due to epigenetic inactivation or, in mismatch repair deficient cancers, due to frameshift mutations. Consequently, Bax deficiency confers resistance against TRAIL induced cell death. Here we provide evidence that this Bax dependency of TRAIL-induced apoptosis is determined by Mcl-1, an antiapoptotic Bcl-2 family protein, that keeps the Bak in check. Despite expression of Bak, Bax deficient DU145 prostate and HCT116 colon cancer cells were fully resistant to TRAIL induced apoptosis. Targeted knock-down of Mcl-1 overcomes inhibition of Bak, enables TRAIL to activate Bak and renders Bax deficient DU145 and HCT116 cancer cells susceptible to TRAIL-induced apoptosis. Downregulation or inhibition of Mcl-1 therefore provides an opportunity to sensitize Bax-deficient tumours for TRAIL-induced apoptosis via a Bak dependent pathway.

Keywords: TRAIL, Mcl-1, Bak, apoptosis

Presenting Author: Gwyn Williams

GAS5 encodes non-coding RNA which controls apoptosis and growth arrest in human cells

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Our laboratory strategy is to identify novel molecular controls on apoptosis directly by using the effects of such unidentified apoptosis-controlling genes on cell survival (1-5). This forward genetics strategy of functional expression cloning in mammalian cells has identified several genes playing important regulatory roles in apoptosis which had not been detected by other methods (1-5). The strategy does not depend on interactions with previously identified apoptosis control molecules and, since this approach does not even require that the sequence identified produces a protein product, can identify non-coding RNA transcripts which control apoptosis.
A partial GAS5 transcript was isolated through its suppression of apoptosis induced by gamma radiation (6). GAS5 transcripts show complex splicing patterns but do not encode a significant polypeptide; instead snoRNAs (small nucleolar RNAs) encoded in the introns of GAS5 transcripts have been conserved during evolution and appear to carry out the functions of this unusual gene (7). Our experiments show that over-expression of several GAS5 transcripts induces apoptosis and inhibition of the cell cycle in both T-leukaemic cell lines and primary human peripheral blood T-cells. Down-regulation of GAS5 using siRNAs inhibits the apoptosis and cell cycle extension which normally accompanies the transition from log phase to stationary phase of T-cell cultures, demonstrating that GAS5 is both necessary and sufficient for normal growth arrest in these cells (8).
Several GAS5 transcripts also induce apoptosis in breast cancer cell lines, and the levels of expression of GAS5 detected by real-time RT-PCR are highly variable between different breast cancer cell lines. GAS5 transcript levels are significantly reduced in clinical breast cancer tissue relative to matched unaffected breast epithelial tissue from the same patient. These observations suggest that abnormalities in GAS5 expression may play an active role in the development of breast cancer. The potential importance of snoRNAs like GAS5 in cancer has been further highlighted recently by the independent observation that mutations within a related snoRNA, U50, are significantly associated with progression in prostate cancer (9).

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Keywords: snoRNA, non-coding RNA, T-cells, breast cancer, growth arrest

Presenting Author: Jen-Leih Wu

Transcription of Regulation of Apoptotic Genes in the Pathogenesis of Infectious Pancreatic Necrosis Virus

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Infectious pancreatic necrosis virus (IPNV) is a fish-specific pathogen and possess a bi-segmented, double-stranded RNA genome. Apoptosis precedes necrosis of fish cell line with IPNV infection. Viral proteins of IPNV promote the down-regulation of the survival factor Mcl-1 during viral-induced apoptotic cell death. Expression of bad gene was induced by IPNV infection through a tyrosine kinase signaling pathway, and promoting host apoptotic death. By using zebrafish 14K oligo-microarray and quantitative RT-PCR, we identified the differential expression of a defined subset of genes involved in apoptosis and immune response at 6-, 12- and 24 h.p.i.. Most of pro-apoptotic Bcl-2 family members were up-regulated after IPNV infection. FasL and TRADD involved in death-receptor mediated apoptosis were also up-regulated. We found that IPNV induced apoptosis through activated caspase 3, 8 and 9 in ZF4 cells. Activation of pro-apoptotic members can disrupt the membrane potential of mitochondria and leads to the mitochondria and death-receptor mediated apoptosis in the late stage of IPNV infection. Anti-apoptotic genes BIRC2, BIRC4, annexin I and Bcl-xL were up-regulated, and counteracted to apoptosis during IPNV infection. IPNV VP5 is an anti-apoptotic gene of the Bcl-2 family, regulates Mcl-1 and viral protein expression. IPNV can utilize VP5 and some anti-apoptotic genes of host to perform the apoptosis-off system during IPNV replication to fulfill replication cycle.

Keywords: Infectious pancreatic necrosis virus, Oligo-microarray, Bcl-xL, anti-apoptotic gene VP5, BIRC2, Fish cell

Sustain ERK/MAPK activity is required for the expression of cyclin D1 in megakaryocytic differentiation induced by 3-hydrogenkowadaphnine

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3-Hydrogenkowadaphnine (3-HK), a novel daphnane-type diterpene ester from Dendrostellera lessertii (Thymelaeaceae), is an effective inducer of megakaryocytic differentiation in chronic myelogenous leukemia (CML) K562 cells without any adverse effect on normal cells. Megakaryocytic differentiation is accompanied by nuclear polyploidization as a result of DNA replication in the absence of mitosis which is prevented in the presence of MEK inhibitor. Ploidy analyses revealed more than 40% of cells reached to the state of 4N DNA content and some of the cells even reached to higher ploidities In contrast, when K562 cells were treated with 3-HK in the presence of PD98059, the cell fraction with 4N or higher polyploidization decreased by almost 20%. Immunoblot analyses also showed that 3-HK induced sustained activation of ERK1/2 from early exposure times, before the onset of differentiation, up to 72 h. Moreover, our results revealed that suppression of ERK activation by PD98059 decreased the expression of cyclin D1 among the 3-HK-treated cells. Consequently, it is concluded that up-regulation of cyclin D1, accompanied by the persisted activation of ERK/MAPK activity, is involved in the megakaryocytic differentiation of K562 cells under the influence of 3-HK.

Keywords: 3-Hydrogenkowadaphnine, megakaryocytic differentiation, ERK/MAPK

Presenting Author: Ji Hye Yim

GLTSCR2 induces cell death and suppress of cell growth by divergent pathway from PTEN.

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The tumor suppressor phosphatase and tensin homologue deleted from chromosome 10 (PTEN) gene is a negative regulator of the phosphatidylinositol-3-kinase (PI3K) protein kinase B (Akt/PKB) signaling pathway. PTEN functions as both a protein and lipid phosphatase in cells that it can inhibit cell proliferation and tumorigenicity via induction of cell cycle arrest at G1 phase and apoptosis. Recently, GLTSCR2 has been reported that it (PICT-1) phosphorylates and stabilizes PTEN.

The purpose of this study was to investigate the molecular mechanisms of GLTSCR2 in cell death pathways in association with its binding partner PTEN. In this work, we show that GLTSCR2 is a nucleus localized protein with a discrete globular expression pattern. In add to it induces caspase-independent apoptotic cell death and its death pathway are divergent from PTEN. GLTSCR2 inhibits growth of cancer cells via diverse range of pathway such as cell cycle regulation or induction of apoptosis.

So, we suggest that GLTSCR2 induced cell death through cell cycle G2/M phase of one of the putative tumor suppressor mechanism activity.

Keywords: GLTSCR2 , PTEN, cell death, apoptosis

TRAIL-induced lysosomal pathway of apoptosis in oral cancer

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TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is a potent apoptosis inducer that limits tumor growth without damaging normal cells and tissues in vivo. We present evidence for a central role of lysosomal cathepsin proteases in TRAIL-induced cell death in oral squamous cell carcinoma (OSCC) cells. TRAIL treatment induced activation of cathepsins (B, L), caspases (-3 and -9), Bid cleavage, release of Bax and cytochrome c, and DNA fragmentation. These events were blocked by cell-permeable cathepsin inhibitors, consistent with the involvement of lysosomal cathepsins in cell death. Also, lysosome stability and mitochondrial membrane potential were reduced during TRAIL-induced apoptosis. However, TRAIL treatment under hypoxic condition resulted in diminished apoptosis rates compared to normoxic condition, showing that hypoxia had an inhibitory effect on TRAIL-induced apoptosis in tumor cells. Our data show that TRAIL triggered lysosome destabilization and cathepsin activation leading to apoptosis. Our work suggests a novel tumor-preventing function for cathepsins in oral cancer, which opposes their established tumor-promoting role as pro-invasive proteases. Thus, drug-mediated lysosome destabilization and induction of cathepsin-mediated cell death may be a promising novel cancer therapy strategy, especially for highly apoptosis-resistant tumors.

Keywords: oral cancer, cathepsins, TRAIL, lysosomes, apoptosis

Structural and biochemical changes during cell death modes determined by IR vibrational spectroscopy.

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Background:
Apoptosis and necrosis are two distinctly different death modes. Both play a major role in various physiological and pathological conditions. In the last decade, both cell death types were found to be intertwined causing the determination of cell death type to become a complicated issue. To deal with this problem, Fourier transform infrared (FTIR) spectroscopy is suggested as it is a rapid, reagent free and simple optical method for simultaneously monitoring multiple biochemical changes.
Working hypothesis and aims:
The proposed research aims to investigate FTIR utilities for characterizing apoptotic and necrotic death using leukemic cell lines as a model system.
Methods:
CCRF-CEM and U937 cells were treated with Ara-C and doxorubicin apoptosis inducers in different concentrations as well as by KCN, saponin, freezing-thawing and H2O2 necrosis inducers. Apoptotic and necrotic death were evaluated by Annexin V, acridine orange and ethidiume bromide staining, Trypan blue staining, LDN release and HMGB-1 release. In parallel, the treated cells were spotted on ZnSe IR transparent substrates and measured by an FTIR microscope.
Results:
FTIR spectral analysis revealed several markers for apoptosis and necrosis. Apoptosis at early and late stages was characterized by an increase in overall lipid absorption and a decrease in infrared detectable DNA, while necrosis was characterized by an increase in both lipid absorption and detectable DNA. In addition, FTIR was able to identify an unclassified mode of cell death induced under several conditions.
Conclusions:
The results indicate the possibility of determining the mode of cell death by analyzing FTIR spectra of cellular components. Our study may provide a simple solution for a complicated problem in medicine and biochemistry in identifying and characterizing cell death in different complex systems.

Keywords: Apoptosis, Necrosis, FTIR-microscopy, Leukemia.

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Multiple Apoptotic Pathways Are Inhibited in Human Melanoma Cells upon ER Stress

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Many mechanisms have been proposed to lead to apoptotic cell death of cells under intense or prolonged endoplasmic reticulum (ER) stress. However, most human melanoma cell lines are not sensitive to apoptosis induced by the classic ER stress inducers tunicamycin or thapsigargin. We show here that although ER stress can potentially initiate multiple apoptosis signaling pathways, they are inhibited by various survival mechanisms, either constitutively activated, or induced by the ER stress response, otherwise referred to as the unfolded protein response (UPR), in melanoma cells. Perhaps the most prominent of these is up-regulation of the anti-apoptotic Bcl-2 family member Mcl-1, which plays a critical role in neutralizing the BH3-only proteins PUMA and Noxa that are also up-regulated by ER stress. Inhibition of Mcl-1 by siRNA rendered melanoma cells sensitive to ER stress-induced apoptosis, but the sensitization was partially reversed by siRNA knockdown of PUMA or Noxa as demonstrated in Mcl-1 deficient melanoma cell lines. Up-regulation of PUMA and Noxa was mediated by both p53-dependent and -independent transcriptional mechanisms. Similarly, up-regulation of Mcl-1 was also due to increased transcription that involved the IRE1α and ATF6 signaling pathways of the UPR. Another important anti-apoptotic factor involved in protection of melanoma cells against ER stress-induced apoptosis is the “master UPR regulator” glucose-regulated protein 78 (GRP78). When GRP78 was inhibited by siRNA, ER stress induced caspase-dependent apoptosis that involved caspase-4, -9 and -3. Among them, caspase-4 appears to be the apical caspase in that caspase-4 activation occurred prior to activation of caspase-9 and -3, and that inhibition of caspase-4 blocked casapse-9 and -3 activation, but inhibition of the latter had only minimal effects on activation of caspase-4. An additional mechanism that protects against ER stress-induced apoptosis in melanoma cells is the apoptosis repressor with caspase recruitment domain (ARC) protein. ER stress induced apoptosis in melanoma cell lines deficient in ARC expression by activating caspase-8, which was otherwise bound to and inhibited by ARC. Significantly, activation of the MEK/ERK pathway appears to be not only required for maximal induction of GRP78, but also necessary for optimal up-regulation of Mcl-1, suggesting an essential role of this pathway in survival of melanoma cells upon induction of ER stress.

Keywords: endoplasmic reticulum, endoplasmic reticulum stress, Mcl-1, caspase-4, GRP78

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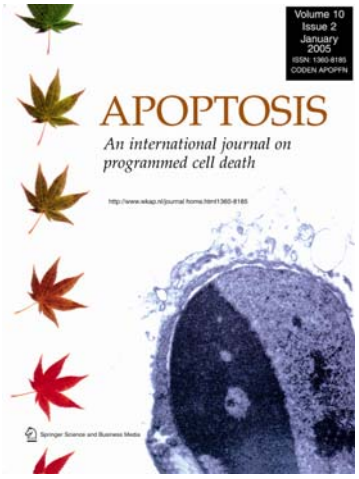

IMPACT FACTOR (2006) 3.421

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2008. Volume(s) 13. 12 Issues.
ISSN: 1360-8185 (print version)
ISSN: 1573-675X (electronic version)
Title No. 10495