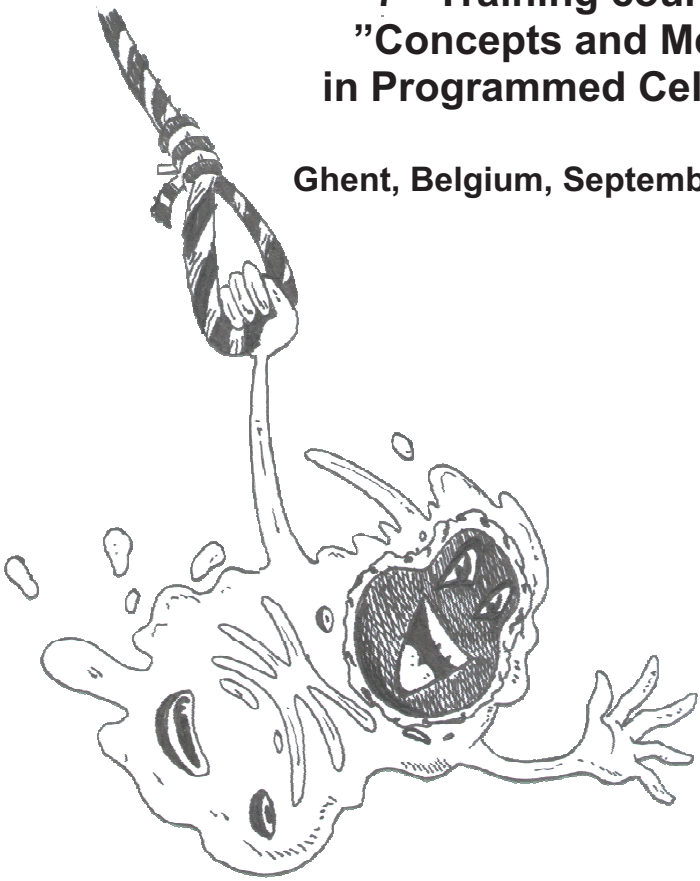
18th Euroconference on Apoptosis
&
7th Training course on
"Concepts and Methods in Programmed Cell Death"

Ghent, Belgium, September 1-4, 2010



Programme & Book of Abstracts

European Cell Death Organization
ECDO

Table of Contents

Welcome

Dear participants,

It is our extreme pleasure to welcome you to this year's edition of the cell death conference annually organized by the European Cell Death Organization (ECDO) – the 18th Euroconference on Apoptosis - in the splendid city of Ghent. The aim of the ECDO is to advance the field of fundamental, experimental and clinical cell death research in Europe. Scientific meetings like these certainly help to achieve this goal. Each ECDO meeting gives the floor to established and starting researchers in the field of cell death from all over the world, to showcase their research. Cell death research is no longer a hidden niche, as it has been for decades, but became in the mean time a major converging point of fundamental and clinical research. As cell death research nowadays has so many connections with different research domains in life sciences, ECDO invites and selects every year excellent speakers (43 this year) who report on the multifaceted aspects of cell death research such as biochemistry of cell death modalities, caspases, imaging of cell death, connection with metabolism, signal transduction, developmental biology, immunology, cancer, etc. The ECDO meeting this year (just like last 6 editions) is preceded by a Training Course on 'Concepts and Methods in Programmed Cell Death' during which experts will provide critical overviews on major areas and technologies in cell death research. This year we have paid extra attention to the poster sessions – first of all we will display continuously all posters (about 150) during the whole meeting in order to provide maximal exposure and secondly we have provided ample time for active poster presentations in two separate sessions on Thursday evening and Friday afternoon. We invite all participants and speakers to contribute towards the success of these poster sessions. Besides excellent science and discussions the ECDO meetings also stimulate socializing activities between cell death researchers, young and old, by organizing receptions, common lunches and a gala dinner all included in the participation fee. Also this year, ECDO provided 25 scholarships for young researchers and reduced registration fees for members. Interested to become an ECDO member yourself? Contact Veronique Vandevoorde at the registration desk or have a look at our website http://www.ecdo.eu.

You will undoubtedly also enjoy the historical, cultural and contemporary atmosphere of Ghent, which is present all around the city in the beautiful medieval buildings, the romantic riversides, the cosy plazas ideal for beer tasting, the 'Jazz in the Park' festival for early Indian summer feeling, the Museum for Fine Arts, SMAK Museum for Contemporary Art, Design Museum, MIAT Museum for Industrial Archaeology and Textile, the art nouveau buildings of the socialist movement in the 19th and beginning of the 20th century, the fish market on Friday morning, etc. Ghent is of course more than rich history and marvellous buildings, there is also the Ghent harbour, the steel and chemical industry, biotechnology companies, car manufacturing. Have a look at the website (http://www.visitgent.be) to plan your non-scientific activities during, before or after the meeting.

We wish to gratefully acknowledge the hard work of Veronique Vandevoorde (ECDO secretariat, Ghent) to organize this meeting, Jelle Verspurten for IT help and Tim Deceuninck for art work, as well as the generous financial support by the Faculty of Sciences and the accommodation support by the Ghent University. We are also grateful to the other sponsors and exhibitors (see next pages), and to the people of our research units.

On behalf of the organizers and ECDO,
Welcome to the 18th Euroconference on Apoptosis in Ghent!

Peter Vandenabeele, Wim Declercq and Patrizia Agostinis

Chairman Collective of the Conference

LOCAL ORGANISATION (CHAIRS)

Peter Vandenabeele
Department for Molecular Biomedical Research
Ghent University
Technologiepark 927
9052 Ghent
Belgium
Phone: +32-9-3313682
E-mail: Peter.Vandenabeele@dmbr.UGent.be

Wim Declercq
Department for Molecular Biomedical Research
Ghent University
Technologiepark 927
9052 Ghent
Belgium
Phone: +32-9-3313660
E-mail: Wim.Declercq@dmbr.UGent.be

Patrizia Agostinis
Department of Molecular Cell Biology
Catholic University of Leuven
Herestraat 49
3000 Leuven
Belgium
Phone: +32-16-345715
Email: patrizia.agostinis@med.kuleuven.be

ECDO AND CONFERENCE SECRETARIAT

Veronique Vandevoorde
ECDO secretariat
Department for Molecular Biomedical Research
VIB-University of Ghent,
Technologiepark 927
9052 Ghent (Zwijnaarde), Belgium
Phone. +32-(0)9-3313682
Fax. +32-(0)9-3313511
e-mail: Veronique.Vandevoorde@dmbr.ugent.be
http://www.ecdo.eu

ECDO SCIENTIFIC COMMITTEE

Patrizia Agostinis
Catholic University of Leuven, Belgium
Klaus-Michael Debatin
University Children's Hospital, Ulm, Germany
Wim Declercq
VIB/Ghent University, Ghent, Belgium
Marie-Lise Gougeon
Institut Pasteur, Paris, France
Marja Jäättelä
Institute for Cancer Biology, Danish Cancer Society, Copenhagen, Denmark
Peter Krammer
German Cancer Research Centre, Heidelberg, Germany
Guido Kroemer
Institut Gustave Roussy, Villejuif, France
Seamus Martin
Trinity College, Dublin, Ireland
Gerry Melino
MRC Toxicology Unit, Leicester, United Kingdom
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University of Rome "Tor Vergata", Rome, Italy
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Jozef Stefan Institute, Ljubljana, Slovenia
Peter Vandenabeele
VIB/Ghent University,Ghent, Belgium
Boris Zhivotovsky
Karolinska Institutet, Stockholm, Sweden

CONFERENCE LOCATIONS *(see map in conference bag)*

Conference venue *(all events except lunches and gala dinner)*:

Aula Academica
Voldersstraat 9
9000 Gent

Lunches *(02/09/2010 and 03/09/2010):*

Het Pand
Onderbergen 1
9000 Gent

Gala dinner *(03/09/2010):*

Monasterium PoortAckere
Oude Houtlei 56
9000 Gent

REGISTRATION

Please register at the Conference Secretariat in the Foyer of the Aula complex to get your conference material and gala voucher.

Opening hours of the registration desk:

September 1 (Wednesday)	13:00 – 18.00
September 2 (Thursday)	08.00 – 18.00

If you need any help during the conference, you can find the local organisers or the ECDO secretary at the registration or information desk. They will try to be of help in all practical aspects of conference participation.

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

INTERNET CONNECTION

PCs with internet access will be available to the conference delegates in the conference venue. Also Wireless Internet access will be available in the Foyer, Peristilium and the parallel projection room (Academieraadzaal) of the Aula complex. Please contact the registration desk for more information.

MEALS AND REFRESHMENTS

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

After the last lecture of the Training Course, **a sandwich snack** will be provided to the conference delegates who attended the Training Course, since the time slot between the end of the training course and the official opening of the meeting is rather tight to go out for dinner.

The Welcome reception on Wednesday evening **September 1** will take place in the Peristilium of the Aula, Volderstraat 9, Ghent.

Lunches on Thursday and Friday will be served in the Pand, Onderbergen 1, Ghent. Lunch tickets for accompanying persons can be bought at the registration desk (50 euro for both lunches).

The **gala dinner** on Friday evening **September 3** will take place at the Monasterium PoortAckere, Oude Houtlei 56, 9000 Gent. **Please pick up your voucher at the registration desk by Thursday morning at the latest** (in case you checked the gala dinner option in your online registration form). Gala dinner tickets for accompanying persons can be bought at the registration desk (60 euro).

During coffee breaks and **poster sessions** the exhibition stands from the sponsoring companies can be visited.

INSTRUCTIONS FOR SPEAKERS

We support OFFICE 2003-2007. 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. Your Powerpoint presentation should be on a USB stick or a CD. Slide presentations are not supported.

Because we have a tight schedule, all speakers will have to stick strictly to the time allocated for their talk.

Short orals take 15 minutes (discussion included). Hence, you should try to prepare a talk of 10-12 min.
Invited speakers should prepare a talk of 25 minutes + 5 minutes discussion.

You will be able to upload your presentation before the start of each session in the lecture room. A technician will be available to assist you.

INSTRUCTIONS FOR POSTER PRESENTERS

Posters can be mounted from the beginning of the meeting and will be displayed throughout the meeting.

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:
Thursday, September 2: 17:30-19:00: Uneven poster numbers (P-1,-3,…)

Poster session 2:
Friday, September 3: 14:30-16:00: Even poster numbers (P-2, -4,…)

Posters should be made in portrait, posterboard measurements are **200 cm (height) x 100 cm (width)**. Please prepare your poster accordingly.

Posters should be readable by viewers one meter away. The poster should contain the title of the submitted abstract, the author(s) name(s) and affiliation at the top. The organisers will provide mounting materials to fix posters.

This year's Poster Prize is sponsored by the **Biochemical Journal**, and consists of a monetory amount of 250 GBP.

BJ BIOCHEMICAL JOURNAL

Delegates are not allowed to take pictures from slides during the presentations, nor from the posters without the consent of the authors.

Posters should be removed by Saturday September 4 at noon (end of meeting). We cannot take responsibility for the posters not removed.

SMOKING

Smoking is NOT allowed in the Aula complex.

TRANSPORT TO/FROM THE AIRPORT

Please consult the conference website (Venue>How to get there) for practical information. Train schedules can be checked at http://www.b-rail.be

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 Belgian currency is the **Euro** (EUR). Cash retrievers are available throughout the city and near the conference venue. Usual opening hours of Belgian banks are: Monday-Friday: 9:00-12:00 and 14:00-17:00.

SPONSORS

The local organisers and ECDO wish to thank the **SPONSORS** listed below for their financial support and/or active contribution.

Faculty of science and University of ghent

VIB

Het Fonds Wetenschappelijk Onderzoek

Poster price: Biochemical Journal

The local organisers and ECDO wish to thank the **EHIBITORS** listed below for their financial support and active contribution.

BD

A leading global medical technology company that manufactures and sells medical devices, instrument systems and reagents, is dedicated to improving people's health throughout the world. BD is focused on improving drug therapy, enhancing the quality and speed of diagnosing infectious diseases, and advancing research and discovery of new drugs and vaccines. The Company's capabilities are instrumental in combating many of the world's most pressing diseases. Founded in 1897 and headquartered in Franklin Lakes, New Jersey, BD employs approximately 28,000 people in approximately 50 countries throughout the world. The Company serves healthcare institutions, life science researchers, clinical laboratories, industry and the general public. For more information, please visit www.bd.com.

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At PerkinElmer we're taking action to create a better tomorrow. We share our customers' commitment to finding answers to the mysteries of human and environmental health. PerkinElmer is a leading provider of cellular imaging and analysis solutions. From in-depth to high throughput biology and from assay development to high content screening, we let biology drive our solutions. Based on the Company's core expertise and knowledge in cellular sciences, PerkinElmer is helping pharmaceutical, biotechnology and academic research organizations around the world to gain the highest quality image data to turn into quantifiable results that advance research. Comprising hardware and software, our cellular imaging and analysis solutions are used in key applications such as cancer, stem cells, developmental biology, neuroscience and infectious diseases.

7th Training Course on Concepts and Methods in Programmed Cell Death

September 1, 2010, Ghent, Belgium

Training Course Programme

Welcome word (local chairs)

Chair: Boris Turk (Ljubljana, Slovenia)

14:00-14:45	**Markus Rehm** (Dublin, Ireland) *Imaging subcellular events during cell death*
14:45-15:30	**Francis Blankenberg** (Stanford, USA) *In vivo imaging of cell death*
15:30-16:00	Coffee
16:00-16:45	**Laurence Calzone** (Paris, France) *Systems biology of cell death*
16:45-17:30	**Lorenzo Galluzzi** (Villejuif, France) *Methods for the detection of cell death*
17:30-18:15	**Klaus-Michael Debatin** (Ulm, Germany) *Therapeutic targeting of apoptosis, an overview of different strategies and clinical trials*

18th Euroconference on Apoptosis

September 1-4, 2010, Ghent, Belgium

Conference Programme

Wednesday evening September 1
Welcome and ECDO Keynote Lecture

Chair: **Peter Vandenabeele** (Ghent, Belgium)

19:30-19:55	**Official Opening** 19.30-19.35 **Peter Vandenabeele**, chairman of the conference 19.35-19.45 **Paul Van Cauwenberghe**, Rector UGent 19.45-19.55 **Jo Bury**, Managing Director VIB, Ghent
19:55-20:40	**ECDO keynote lecture: Guy Salvesen** (La Jolla, USA) *Caspase activation, specificity and substrates*
20:40-21:00	**Tom Balthazar**, Deputy Mayor for environmental and social affairs *Ghent, then and now*
21:00-22u30	**Welcome reception** (Aula)

September 2: Thursday morning

Chair: Patrizia Agostinis (Leuven, Belgium)

09:00-09:30 **Andreas Villunger** (Innsbruck, Austria)
A role of the PIDDosome in tumor suppression?

09:30-09:45 **Delphine Merino** (Melbourne, Australia)
Bim indirectly activates Bax/Bak in ABT-737 induced cell death

09:45-10:00 **Donat Kögel** (Frankfurt, Germany)
(-)-gossypol targets Mcl-1 for degradation and induces autophagic cell death in glioma cells

10:00-10:30 **Rosario Rizzuto** (Ferrara, Italy)
Calcium, mitochondria and cell death

10:30-11:00 Coffee break

Chair: Wim Declercq (Ghent, Belgium)

11:00-11:30 **David Wallach** (Rehovot, Israel)
The 'Apoptotic' Caspases as Regulators of Inflammation: New Lessons from the Study of Caspase-8 Function

11:30-12:00 **Manolis Pasparakis** (Cologne, Germany)
Inflammatory and apoptotic signalling pathways in the regulation of epithelial homeostasis

12:00-12:15 **Geert van Loo (**Ghent, Belgium)
A20/tnfaip3 : a master brake on apoptosis

12:15-12:30 **Sandrine Jouan-Lanhouet** (Rennes, France)
An acidic extracellular pH switches TRAIL-induced apoptosis to a RIPK1/RIPK3/PARP-1 dependent programmed necrosis in human colon cancer cells

12:30-13:00 **Thirumala Kanneganti** (Memphis, TN, USA)
Caspase-1 inflammasomes in innate immunity, cell death and inflammation

13:00-15:00 **Lunch** (The Pand)

September 2: Thursday afternoon

Chair: Simone Fulda (Frankfurt, Germany)

15:00-15:30 **Pascal Meier** (London, UK)
Ubiquitin-mediated regulation of apoptosis

15:30-16:00 **John Silke** (Victoria, Australia)
Appointment with death - and other cell death mysteries

16:00-16:20 **Emmanuel Dejardin** (Liège, Belgium)
TNFR-induced activation of MAP3K14/NIK enhances TNFR-1-induced cell death

16:20-16:45 **Henning Walczak** (London, UK)
A physiological role for linear ubiquitination in innate and adaptive immune signalling

16:45-17:00 **Nele Vanlangenakker** (Ghent, Belgium)
c-*IAP1 and TAK1, modulators of TNF-induced necroptosis*

17:00-17:30 **Phil Barker** (Montreal, Quebec, Canada)
SMAC'd by the IAPs - Molecular Interplay in Cell Death Signaling

17:30 **Poster viewing session #1: Uneven poster numbers**

18:30-19:30 ECDO board meeting

20:30 Dinner for invited speakers

September 3: Friday morning

Chair: Mauro Piacentini (Rome, Italy)

09:00-09:30 **Frank Madeo** (Graz, Austria)
Spermidine for a long life

09:30-09:45 **Joel Beaudouin** (Heidelberg, Germany)
Spatiotemporal characterization of caspase activity in single cells

09:45-10:00 **Nieves Peltzer** (Lausanne, Switzerland)
A caspase- and RasGAP fragment N-activated survivin-dependent protective response in the skin

10:00-10:30 **Ana Maria Cuervo** (New York, USA)
Selective autophagy: fueling and cleaning in the same comparment

10:30-11:00 Coffee break

Chair: Peter Krammer (Heidelberg, Germany)

11:00-11:30 **Cosima Baldari** (Siena, Italy)
p66Shc: a novel player in lymphocyte apoptosis

11:30-12:00 **Judy Liebermann** (Boston, USA)
Granzyme A-mediated cell death: using proteomics to dissect the anatomy of a murder

12:00-12:25 **Kris Gevaert** (Ghent, Belgium)
Positional proteomics in cell death research

12:25-12:55 **Marion MacFarlane** (Leicester, UK)
Selectively Targeting Death Receptor Signalling in Cancer

12.55-13.10 **Alexander Pintzas** (Athens, Greece)
Oncogenes can sensitise tumour cells to TRAIL induced apoptosis: The paradigm of RAS and BRAF oncogenes in colorectal tumours, from models to the clinic

13:10-14:30 **Lunch** (The Pand)

14.30-16.00 **Poster viewing session #2: Even poster numbers**

September 3: Friday afternoon

Chair: Klaus-Michael Debatin (Ulm, Germany)

16:00-16:30 **Peter Krammer** (Heidelberg, Germany)
Cancer, death and the immune system

16:30-16:45 **Tom Cotter** (Cork, Ireland)
Looking Cell Death in the Eye

16:45-17:00 **Sven Horke** (Mainz, Germany)
Pursuing Redox-balance modification: PON2 as a new putative anti-tumor target

17:00-17:15 **Catharina Svanborg** (Lund, Sweden)
HAMLET kills carcinoma cells but spares healthy, differentiated cells. Molecular basis of the tumoricidal effect

17.15-17.25 **Poster Awards**

ECDO Honorary Lecture

Chair: Boris Zhivotovsky, president of ECDO (Stockholm, Sweden)

17:25-18:25 **Guido Kroemer** (Villejuif, France)
The desirable death .... of the cancer cell

18:30-19:00 **ECDO General Assembly** (Aula)

20:00 **Gala Dinner** (Monasterium PoortAckere)

September 4: Saturday morning

Chair: Hans-Uwe Simon (Bern, Switzerland)

09:00-09:30 **Martin Krönke** (Cologne, Germany)
NADPH oxidase-derived ROS in cell death and autophagy

09:30-09:45 **Cristina Claudia Mihalache** (Bern, Switzerland)
Inflammation-associated autophagy-related programmed necrotic neutrophil death characterized by organelle fusion events

09:45-10:15 **Francis Chan** (Worcester, USA)
Molecular Regulation of Programmed Necrosis: From RIP Kinases and Beyond

10:15-10:30 **Kelly Jean Thomas** (Grand Junction, USA)
A lung cancer model linking apoptotic resistance and tumorigenesis via defects in mitochondrial dynamics

10:30-11:00 Coffee break

Chair: Marie-Lise Gougeon (Paris, France)

11:00-11:30 **Edward Mocarski** (Atlanta, GA, USA)
RIP3-dependent necrosis in viral infection and mammalian development

11:30-12:00 **Shigekazu Nagata** (Kyoto, Japan)
Clearance of Dead Cells

12:00-12:15 **Abhishek Garg** (Leuven, Belgium)
Cancer cells undergoing immunogenic death under photo-oxidative ER-stress, surface expose calreticulin via a "non-canonical" pathway

12:15-12:45 **Caetano Reis e Sousa** (London, UK)
Coupling of dead cell recognition to adaptive immunity by dendritic cells

Concluding remarks (local chairs)

Lecture Abstracts

Invited speakers

Abstracts are in **alphabetical** order by the name of the invited speaker.

List of Invited Speakers

Baldari, Cosima T L-1
p66Shc: a novel player in lymphocyte apoptosis
Barker, Phil L-2
SMAC'd by the IAPs – Molecular Interplay in Cell Death Signaling
Chan, Francis L-3
Molecular Regulation of Programmed Necrosis: From RIP Kinases and Beyond.
Cuervo, Ana Maria L-4
Selective Autophagy: fueling and cleaning in the same compartment
Kanneganti, Thirumala-Devi L-5
Caspase-1 inflammasomes in innate immunity, cell death and inflammation
Krammer, Peter L-6
Cancer, Death and the Immune System
Kroemer, Guido L-7 – ECDO Honorary Lecture
The desirable death .... of the cancer cell
Krönke, Martin L-8
NADPH oxidase-derived ROS in cell death and autophagy
Lieberman, Judy L-9
Granzyme A-mediated cell death: using proteomics to dissect the anatomy of a murder
MacFarlane, Marion L-10
Selectively Targeting Death Receptor Signalling in Cancer
Madeo, Frank L-11
Spermidine for a long life
Meier, Pascal L-12
Ubiquitin-mediated regulation of apoptosis
Mocarski, Edward L-13
RIP3-dependent necrosis in viral infection and mammalian development
Nagata, Shigekazu L-14
Clearance of Dead Cells
Pasparakis, Manolis L-15
Inflammatory and apoptotic signalling pathways in the regulation of epithelial homeostasis
Reis e Sousa, Caetano L-16
Coupling of dead cell recognition to adaptive immunity by dendritic cells
Rizzuto, Rosario L-17
Mitochondria, calcium and cell death
Salvesen, Guy L-18 – ECDO Keynote Lecture
Caspase activation, specificity and substrates
Silke, John L-19
Appointment with death - and other cell death mysteries
Villunger, Andreas L-20
A role of the PIDDosome in tumor suppression?
Wallach, David L-21
The 'Apoptotic' Caspases as Regulators of Inflammation: New Lessons from the Study of Caspase-8 Function

L-1 p66Shc: a novel player in lymphocyte apoptosis

Cosima T. Baldari

Department of Evolutionary Biology, University of Siena, Italy

The Shc adaptor family includes four members, known as ShcA, ShcB/Sli, ShcC/Rai and ShcD/RaLP, which share a common domain organization but differ in tissue specificity, expression and function. These proteins have emerged as stategic players in the coordinate control of cell proliferation, differentiation, apoptosis and migration. I shall summarize our findings on the role of p66Shc, the longest of the three isoforms of ShcA, as a negative regulator of survival signaling by the T-cell and B-cell antigen receptors and a promoter of apoptosis in lymphocytes. I shall discuss the relevance of the propoptotic activity of p66Shc to the control of lymphocyte activation and survival in the in vivo context of p66Shc-/- mice, which develop lupus-like autoimmunity. Finally, I shall present our recent evidence that p66Shc expression is impaired in leukemic cells from CLL patients and show that this defect underlies to a significant extent the imbalance in the Bcl-2 family which is a hallmark of this disease.

L-2 SMAC'd by the IAPs – Molecular Interplay in Cell Death Signaling

Phil Barker

Montreal Neurological Institute, McGill University, Montreal, Canada

Inhibitor of apoptosis proteins are phylogenetically conserved proteins that promote cell survival, in part by blocking caspase activation. Overexpression of IAPs occurs in many human cancers and may facilitate cancer cell survival in humans. The IAPs have therefore emerged as interesting targets for cancer development. SMAC/DIABLO, an IAP binding protein that is from mitochondria during apoptosis, disrupts IAP-caspase interactions to activate caspases and peptide mimetic compounds that target the IAP-caspase interaction reached the clinic. All IAPs possess 1-3 baculovirus IAP repeat (BIR) domains and some have a carboxy terminal RING domain. We have shown that the cIAP1 and cIAP2 function as E3 ligases that extend lysine 63 poly-ubiquination chains on RIP1 and RIP2 and established roles for cIAP1 and cIAP2 E3 ligase activity in TNF signaling and in NOD signaling. I will discuss our recent efforts to decipher regulatory mechanisms that impinge on IAP signaling activities.

L-3 Molecular Regulation of Programmed Necrosis: From RIP Kinases and Beyond.

Francis Ka-Ming Chan

Department of Pathology, Immunology and Virology Program, University of Massachusetts Medical School, S2-125, 55 Lake Avenue North, Worcester, MA 01655, USA.

Necrosis induced by death cytokines in the TNF family (also known as programmed necrosis or necroptosis) is regulated by kinases in the Receptor Interacting Protein (RIP) family. The release of endogenous “danger signals” from necrotic cells triggers inflammation and facilitates the inflammatory reaction during pathogen-induced and sterile inflammation. We previously showed that the assembly of a complex between the kinases RIP1 and RIP3 is critical for the induction of programmed necrosis. The assembly of this RIP1-RIP3 complex is tightly regulated by protein phosphorylation, ubiquitination and caspases. Emerging evidence also indicates that RIP1 and RIP3 control downstream events such as reactive oxygen species (ROS) production and energy metabolism. We will provide an update on the molecular regulation of these events as well as how certain viral inhibitors target this cell death pathway to achieve immune evasion.

L-4 Selective Autophagy: fueling and cleaning in the same compartment

Ana Maria Cuervo

Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, USA

Autophagy is the intracellular process that mediates the digestion of cellular components in lysosomes. The autophagic system fulfills two major functions in mammalian cells, serving both as an alternative source of energy, when nutrients are scarce, and as an efficient mechanism for the removal of any intracellular damage structure. Autophagic activity has been described to decline with age in almost all organisms and tissues, as wells as in several age-related disorders. On light of the prevalent functions of this catabolic process, cells with impaired autophagy are often energetically compromised and present severe problems in maintenance of cellular homeostasis and in the response to stress. Our group is interested in the study of the changes with age in different autophagic pathways and on the consequences of those changes in the aging organism. In addition, using both genetic manipulations and regulated diets, we have been able to prevent the decline in autophagic activity in old rodents and analyze the beneficial effect of this intervention both in cellular homeostasis and in their energetic balance. These models would help us evaluate the importance of maintaining proper autophagic function until advanced ages, and, on light of the observed cross-talk among autophagic pathways, the effect that repairing one autophagic pathway may have on the functioning of the others.

L-5 Caspase-1 inflammasomes in innate immunity, cell death and inflammation

Thirumala-Devi Kanneganti

St. Jude Children's Research Hospital, Memphis, TN

The innate immune system comprises several classes of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), Nucleotide binding and oligomerization domain-like receptors (NLRs), and RIG-1-like receptors (RLRs). TLRs recognize microbes on the cell surface and in endosomes, whereas NLRs and RLRs detect microbial components in the cytosol. Genetic variations in several NLR members are associated with the development of a number of autoinflammatory and autoimmune disorders including Crohn's disease, vitiligo, Blau syndrome, cryopyrinopathies, FMF, etc. NLRs Cryopyrin/Nlrp3, Nlrp1 and Ipaf are critical for the activation of inflammasomes, molecular platforms that mediate the activation of caspase-1 and processing of pro-IL-1beta/IL-18 into mature IL-1beta and IL-18. The results available so far suggest that inflammasome components are critical mediators of innate immune responses, cell death and inflammation by linking intracellular recognition of microbial components and danger signals to signaling pathways and their deregulation play an important role in inflammatory and infectious diseases.

L-6 Cancer, Death and the Immune System

Peter H. Krammer

German Cancer Research Center, Heidelberg,Germany

Removal of excess cells by apoptosis is essential to ensure tissue homeostasis. Recognition of apoptotic cells is achieved via specific signals that mediate their engulfment as well as suppression of an immune response against apoptotic cell-derived self antigens. Although a number of engulfment signals have been described, immunosuppressive proteins of apoptotic cells remain largely elusive. Here, we identify annexin 1 as an endogenous, anti-inflammatory signal on the surface of early apoptotic cells. Annexin 1 did not only suppress the secretion of inflammatory cytokines but also the expression of costimulatory surface molecules of dendritic cells (DC) by antagonizing Toll-like receptor (TLR) signaling pathways and inhibiting the activation of nuclear factor κB (NF-κB) as well as phosphorylation of p38. In contrast to control T cells stimulated by mature DC, T cells stimulated by annexin 1-suppressed DC lacked secretion of Th1 cytokines like interferon-gamma(IFN-gamma) and tumor necrosis factor (TNF) and developed an anergic phenotype. These results can also be seen with Ovalbumin specific T cells in vivo. Thus, our results provide a molecular mechanism for the suppressive effect of apoptotic cells on the immune response and for tolerance to apoptotic self-cells. These data also impact on immune regulation, autoimmunity, and apoptosis in tumor therapy.

L-7 The desirable death .... of the cancer cell

Guido Kroemer (1) Laurence Zitvogel (2)

(1) Inserm U848, F-94805 Villejuif, France
(2) Institut Gustave Roussy, F-94805 Villejuif, France

The supreme goal of anticancer therapy is the induction of tumor cell death. Physiological cell death, which occurs as a continuous byproduct of cellular turnover, is non-immunogenic or even tolerogenic, thereby avoiding autoimmunity. However, cancer cell death elicited by radiotherapy and some chemotherapeutic agents such as anthracyclines and oxaliplatin can be immunogenic. Immunogenic death involves changes in the composition of the cell surface, as well as the release of soluble immunogenic signals that occur in a defined temporal sequence. This 'key' then operates on a series of receptors expressed by dendritic cells (DC, the 'lock') to allow for the presentation of tumor antigens to T cells and for the initiation of a productive immune response. Immunogenic cell death is characterized by the early cell surface exposure of calreticulin, which determines the uptake of tumor antigens by DC. The late release of the protein high mobility group box 1 (HMGB1), which acts on toll-like receptor 4 (TLR4), is required for the presentation of antigens from dying tumor cells. In addition, the release of ATP from dying cells causes the P2RX7 purinergic receptor-dependent activation of the NLRP3 inflammasome in dendritic cells, thereby allowing them to release interleukin-1ß and to polarize tumor antigen-specific CD8 T cells towards a Tc1 cytokine pattern. We postulate that the immune system determines the long-term success of anti-cancer therapies, and that this immune response is dictated by immunogenic tumor cell death. Hence we formulate two predictions: First, therapeutic failure can result from failure to undergo immunogenic cell death (rather than cell death as such). Thus, agents that fail to induce immunogenic cell death cannot yield a long-term success in cancer therapy. Moreover, tumors that are intrinsically unable to undergo immunogenic cell death are incurable. Importantly, it appears that mitochondrial events determine whether cancer cells die or not in response to chemotherapy, while an endoplasmic reticulum stress response determines whether this cell death is perceived as immunogenic. Second, therapeutic failure may result from subtle immune defects that compromise the immune system's capacity to perceive immunogenic cell death signals and/or to generate anti-cancer immune effectors. Indeed, we have found that loss-of-function alleles (that affect TLR4 or P2X7 receptors in the Caucasian population) can reduce the efficacy of conventional anti-cancer therapies, in anthracylin-treated breast carcinoma and oxaliplatin-treated colon cancer. In light of these postulates, the desirable death of a tumor cell is an immunogenic one.

L-8 NADPH oxidase-derived ROS in cell death and autophagy

Martin Krönke

Institute for Medical Microbiology, Immunology and Hygiene
University of Cologne, Goldenfelsstr. 19-21, 50935 Köln, Germany

The family of NADPH oxidases (Nox) is a major source of reactive oxygen species (ROS). Besides their physiological functions in host defense, Nox enzymes are involved in many cellular responses including cell death and autophagy. Seven human homologs of the NADHP oxidase have been described, Nox1-5, and Duox1, and -2, which exhibit similar structural and functional features. Nox/Duox isoenzymes exert both overlapping and specific functions by temporally and spatially controlling ROS production in select tissues. A further layer of control of ROS production by Nox oxidases is brought about by the complex activation requirements of Nox/Duox isoenzymes. A role of ROS in cell death and autophagy seems well established yet the involvement of specific Nox isoenzymes remain to be dissected. As to TNF-induced cell death, transfection of HeLa cells with either siNox1 or siNox2 confers partial resistance and the combination of siNox1 and siNox2 almost completely blocks TNF-induced cell death, indicating functional redundancy of Nox1 and -2. TNF-induced cytochrome c release and PARP cleavage are markedly reduced by the ROS scavenger NAC as well as in Nox-deficient HeLa cells. These data suggest that Nox1 and Nox2 but not other Nox/Duox enzymes enhance caspase-mediated apoptosis in TNF treated HeLa cells.

Autophagy represents a primordial defense mechanism against intracellular bacteria and viruses. However, some pathogens like *Staphylococcus (S.) aureus* exploit autophagy to remodel phagosomes into a replication-supporting environment. Virulent strains of *S. aureus* escape from autophagosomes into the cytoplasm, which results in caspase-independent host cell death. Inhibition of ROS production in both professional (RAW267.4) and non-professional (HeLa) phagocytes leads to inhibition of *S. aureus*-induced autophagy. Inhibition of ROS production in *S. aureus* infected cells was also associated with improved host cell survival indicating a contribution of Nox isoenzymes to *S. aureus*-induced cell death. The contribution of a given Nox isoenzyme to *S. aureus*-induced autophagy and cell death is currently assessed by various genetic mouse models for defined Nox deficiencies.

L-9 Granzyme A-mediated cell death: using proteomics to dissect the anatomy of a murder

Danielle Jensen (1), Denis Martinvalet (2), Michael Walch (1) and Judy Lieberman (1)

(1) Immune Disease Institute, Harvard Medical School, 200 Longwood Avenue, Boston, 02115, USA
(2) University of Geneva, Geneva, Switzerland

Granzyme A, an abundant death-inducing protease in immune killer cells, activates a novel caspase-independent programmed cell death pathway that has all the morphological features of apoptosis. Granzyme A induces reactive oxygen species and dissipates the mitochondrial transmembrane potential without permeabilizing the mitochondrial outer membrane. We previously identified NDUFS3, a component of electron transport complex I, as a granzyme A mitochondrial substrate by comparing the proteome of isolated intact mitochondria briefly treated or not with granzyme A. Granzyme A concentrates in the nucleus of target cells, and most of the known granzyme A target proteins reside at some point in the nucleus, including SET, HMGB2, APE1, PARP1, Ku70, Histone H1 and core histones, and the lamins. To identify new granzyme A nuclear substrates, we analyzed the nuclear proteome of intact nuclei treated or not with granzyme A. We resolved 1554 spots, of which only 92 were reduced in intensity by exposure to granzyme A. Amongst these were known granzyme A targets (lamins A, B and C, histone H2A). We now present the results of this analysis, which has helped us to uncover a new pathway that is inactivated by granzyme A and the caspases.

L-10 Selectively Targeting Death Receptor Signalling in Cancer

Davina Twiddy(1), Shambhavi Naik(1), Michelle Hughes(1), Nick Harper(1), Kelvin Cain(1), Rosemary Walker(2), Gerald Cohen(1), Marion MacFarlane(1)

(1) MRC Toxicology Unit, Hodgkin Building, Lancaster Road, Leicester, LE1 9HN, UK
(2) Cancer Studies & Molecular Medicine, University of Leicester, Leicester, UK

Acquisition of resistance to apoptosis is a defining feature of tumour cells, thus strategies that bypass nodes of apoptotic resistance may be used to trigger cell death selectively in tumour cells. One strategy to selectively induce apoptosis is to activate 'death receptors'. Dissecting the molecular mechanisms that determine death or survival of tumour cells following exposure to members of the TNF family of 'death receptor' ligands, and in particular regulation of the signalling pathways activated by the selectively toxic and potential cancer therapeutic, TRAIL/Apo2L is therefore of key importance. To address this, we have employed both mechanistic and translational-based approaches.

Firstly, we have reconstituted in vitro the prototype CD95 Death-Inducing Signalling Complex (DISC), using only three core components, namely CD95, FADD and procaspase-8. Using this model, we have then further delineated a critical switch in DISC catalytic activity that determines CD95 signalling for death or survival. Here, we will discuss the implications of our findings, not only in terms of death receptor activation per se, but also in the context of human disease particularly with evidence now emerging that death receptors like CD95 and TRAIL-Rs can in some contexts activate pro-survival pathways and promote tumour growth.

Secondly, we have investigated the therapeutic potential of targeting the TRAIL:TRAIL-R axis using relevant pre-clinical tumour models. Although the majority of transformed cell lines are sensitive to the TRAIL, most primary tumours are TRAIL-resistant. However, using receptor-selective ligands specific for the death receptors, TRAIL-R1/TRAIL-R2, we have previously shown that primary Chronic Lymphocytic Leukaemia (CLL) cells can be selectively sensitized to apoptosis by combining an HDACi with a TRAIL-R1-specific form of TRAIL/TRAIL-R1 targeting mAb. To examine the relative potency of TRAIL-R1/TRAIL-R2-selective ligands in breast cancer, a panel of breast cancer cell lines, as well as primary breast tumour explants cultured ex-vivo to maintain their 3-dimensional-architecture were employed, thus providing a clinically-relevant primary tumour model. Despite expressing both TRAIL-R1 and TRAIL-R2, the majority of breast cancer cell lines were highly selective towards apoptosis induced by a TRAIL-R1-selective ligand. Moreover, while the majority of primary breast tumour explants were resistant to TRAIL, these could be sensitized by combining TRAIL with the widely used breast cancer chemotherapeutic, doxorubicin. Importantly, in this ex-vivo tumour model, TRAIL combined with doxorubicin again induced significantly more apoptosis via TRAIL-R1 than TRAIL-R2, while having no effect on normal breast tissue. Here, we will highlight the potential importance of combining a TRAIL-R1-selective agonist with an appropriate sensitizer and discuss the mechanisms underlying these observations.

L-11 Spermidine for a long life

Tobias Eisenberg, Nektarios Tavernarakis, Guido Kroemer, Frank Madeo

University of Graz

Spermidine is a ubiquitous polycation that is synthesized from putrescine and serves as a precursor of spermine. Putrescine, spermidine and spermine all are polyamines that participate in multiple known and unknown biological processes. Exogenous supply of spermidine prolongs the life span of several model organisms including yeast (*Saccharomyces cerevisiae*), nematodes (*Caenorhabditis elegans*) and flies (*Drosophila melanogaster*) and significantly reduces age-related oxidative protein damage in mice, indicating that this agent may act as a universal anti-aging drug. Spermidine induces autophagy in cultured yeast and mammalian cells, as well as in nematodes and flies. Genetic inactivation of genes essential for autophagy abolishes the life span-prolonging effect of spermidine in yeast, nematodes and flies. These findings complement expanding evidence that autophagy mediates cytoprotection against a variety of noxious agents and can confer longevity when induced at the whole-organism level. We hypothesize that increased autophagic turnover of cytoplasmic organelles or long-lived proteins is involved in most if not all life span-prolonging therapies and present new insights into Spermidines mode of action.

L-12 Ubiquitin-mediated regulation of apoptosis

Meike Broemer, Tencho Tenev, Katiuscia Bianchi, Maurice Darding and Pascal Meier

The Breakthrough Toby Robins Breast Cancer Research Centre, Institute of Cancer Research, Mary-Jean Mitchell Green Building, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, UK

The intimate relationship between mediators of the ubiquitin (Ub)-signalling system and human diseases has sparked profound interest in how Ub influences cellular processes such as NF-κB signalling and apoptosis. Inhibitor of Apoptosis (IAP) proteins are among the most prominent Ub-E3-ligases modulating cell survival and oncogenesis. While the consequence of Ub-attachment is intensely studied, little is known with regards to the effects of other Ub-like proteins (UBL), and deconjugating enzymes that remove the Ub- or UBL-adduct. Systematic in vivo RNAi analysis identified three NEDD8-specific isopeptidases that, when knocked-down, suppress apoptosis in vivo. Consistent with the notion that attachment of NEDD8 prevents cell death, genetic ablation of deneddylase 1 (DEN1) suppresses apoptosis. Unexpectedly we find that Drosophila and human IAPs can function as E3-ligases of the NEDD8-conjugation pathway, targeting effector-caspases for NEDD8ylation and inactivation. Finally, we demonstrate that DEN1 reverses this effect by removing the NEDD8-modification. Altogether, our findings indicate that IAPs not only modulate cellular processes via ubiquitylation but also through attachment of NEDD8, thereby extending the complexity of IAP-mediated signalling.

L-13 RIP3-dependent necrosis in viral infection and mammalian development

William J. Kaiser(1), Jason W. Upton(1), Andrew Oberst(2), Christopher P. Dillon(2), Alyssa B. Long(3), Lisa P. Daley(1), Devon Livingston-Rosanoff(1), Tamara Caspary(3), Douglas R. Green(2) and Edward S. Mocarski(1)

(1) Department of Microbiology and Immunology, Emory Vaccine Center, Emory University School of Medicine, Atlanta GA 30322
(2) Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105
(3) Department of Human Genetics, Emory University, Atlanta, GA 30322

Viral pathogenesis is associated with the manipulation of host cytokine and cell death pathways. Murine cytomegalovirus (MCMV) encodes inhibitors of apoptosis as well as receptor interacting protein (RIP)3-dependent necrosis, implicating both types of death pathways in host defense. RIP3 kinase activity and RIP homotypic interaction motif (RHIM)-dependent interactions control virus-induced necrosis as well as death receptor-induced necroptosis; however, unlike necroptosis, the viral death pathway is RIP1-independent. Viral inhibitor of RIP activation (vIRA, encoded by the MCMV M45 gene) mediates a RHIM-dependent inhibition of TNF-induced necroptosis by interfering with the requisite RIP3-RIP1 interaction. RIP3 is the critical target through which vIRA naturally functions to suppress virus-induced death, and DNA-dependent activator of interferon regulatory factors (DAI/ZBP-1), a RHIM-containing pathogen sensor or adaptor, has been implicated as the RIP3 partner in virus-induced necrosis. Sensitivity of cells to virus-induced necrosis requires DAI-expression and a RIP3-DAI complex is detected in cells undergoing virus-induced necrosis. vIRA-deficient MCMV replication is normalized in DAI-deficient cells and mice, although pathogenesis is not completely normalized as occurs in RIP3-deficient mice. vIRA function validates necrosis as central to the elimination of infected cells in host defense, indicates that DAI is the RIP3 partner in virus-induced necrosis, and highlights the benefit of multiple virus-encoded cell death suppressors that subvert not only apoptotic, but also necrotic mechanisms of virus clearance.

To investigate the role of RIP3-dependent necrotic death pathways in mammalian development, we examined embryonic lethality of caspase 8-deficient mice, which has not been explained through any of the established activities of this proapototic adaptor. Death receptor-induced apoptosis requires caspase-8; however, this protease is also known to regulate RIP3-dependent necrotic pathways. The pattern of RIP3 expression appeared consistent with RIP3-dependent events underlying premature death in caspase-$8^{-/-}$RIP$3^{+/+}$ embryos. Mice deficient in both RIP3 and caspase-8 were derived in order to expose any role of RIP3 activity in embryonic death. Double knock-out mice exhibit normal development, viability and fertility, and exhibit resistance to proapoptotic as well as pronecrotic cell death stimuli. Although myeloid and lymphoid cells display novel phenotypic characteristics, the mice do not exhibit major immune deficit. This evidence implicates RIP3-dependent premature necrosis driving embryonic lethality in the absence of caspase-8, and predicts that the embryonic lethality of FADD-deficient and FLIP-deficient mice is likewise a result of RIP3 dysregulation around E10.5. Surprisingly, combined elimination of caspase-8-dependent and RIP3-dependent pathways is compatible with mammalian development and reproduction.

L-14 Clearance of Dead Cells

Shigekazu Nagata

Department of Medical Chemistry, Graduate School of Medicine, Kyoto University, Yoshida-Konoe, Sakyo-ku, Kyoto 606-8501

To maintain homeostasis in animals, many cells die and are engulfed by phagocytes such as macrophages and immature dendritic cells. In phagocytes, the dead cells are transferred to lysosomes, where their cellular components are degraded for re-use as building units. Phagocytes recognize phosphatidylserine exposed specifically on the dead cell surface as an “eat me” signal. If apoptotic cells are not efficiently engulfed, they undergo secondary necrosis, and their intracellular contents are released. These materials activate the immune system, causing systemic lupus erythematosus-type autoimmune diseases. On the other hand, if the DNA of dead cells is not properly degraded in the phagocytes, it accumulates in the lysosomes, and activates the innate immunity to produce various cytokines such as interferon (IFN)beta and tumor necrosis factor (TNF)α, leading to severe anemia and chronic arthritis. Here, I discuss how endogenous components of dead cells activate the immune system through extracellularly- or intracellularly-triggered pathways. This system seems to be similar to that used for the virus- or pathogen-induced inflammation.

L-15 Inflammatory and apoptotic signalling pathways in the regulation of epithelial homeostasis

Manolis Pasparakis

L-16 Coupling of dead cell recognition to adaptive immunity by dendritic cells

Caetano Reis e Sousa

Immunobiology Laboratory,Cancer Research UK, London Research Institute,London WC2A 3PX, United Kingdom

Cell death can be sensed by dendritic cells (DC) and lead to adaptive immunity against cell-associated antigens. We have been studying recognition pathways that allow DC to sense dead cells and present cell-associated antigens to T cells. One pathway involves the C-type lectin DNGR-1, also known as CLEC9A, which signals via Syk kinase. DNGR-1 is selectively expressed by $CD8\alpha^+$ DC in mouse and their equivalents in human, a sub-group of DC that possesses specialised properties including a unique propensity to phagocytose dead cell debris and to crosspresent exogenous antigens to $CD8^+$ T cells. DNGR-1 signalling via Syk in $CD8\alpha^+$ mouse DC appears to regulate the retrieval and crosspresentation of dead cell-associated antigens and may also be involved in the activation of DC by dead cells. The study of DNGR-1 helps build a picture of the receptors and signalling pathways that regulate DC responses to self alterations and has applications in immunotherapy of cancer and infectious diseases.

L-17 Mitochondria, calcium and cell death

Rosario Rizzuto (1), Paolo Pinton (2), Diego De Stefani (1), Carlotta Giorgi (2), Cristina Mammucari (1), Saverio Marchi (2), Alessandro Rimessi (2), Anna Romagnoli (1), Roberta Siviero (1) and Erika Zecchini (1)

(1) Dept. Biomedical Sciences, University of Padua, Italy
(2) Dept. Experimental and Diagnostic Medicine, University of Ferrara, Italy
Corresponding author e-mail: rosario.rizzuto@unipd.it

Mitochondria rapidly accumulate Ca2+ through a low-affinity uptake system (the mitochondrial Ca2+ uniporter, MCU) because they are exposed to high [Ca2+] microdomains generated by the opening of ER Ca2+ channels. These rapid [Ca2+] changes stimulate Ca2+-sensitive dehydrogenases of the mitochondrial matrix, and hence rapidly upregulate ATP production in stimulated cells. Ca2+ also sensitizes to cell death mediators, e.g. ceramide. Accordingly, we demonstrated that Bcl-2 reduces the state of filling of ER Ca2+ stores, and this alteration is effective in reducing the sensitivity to apoptotic challenges. I present data on: 1) The effect on mitochondrial Ca2+ homeostasis of other signalling pathways involved in autophagy and apoptosis (Akt, sirt3). 2) The signalling route that links oxidative stress to the activation of p66shc, an isoform of a growth factor adapter acting as apoptotic inducer. PKCβ, activated by the oxidative challenge, induces p66shc phosphorylation, with ensuing alteration of mitochondrial structure and function. We also showed that this route is involved also in adipose differentiation of muscle-derived precursors, highlighting a novel process of utmost interest in pathophysiological conditions. 3) The molecular elements of the mitochondria-ER Ca2+ connection. I will discuss the role of VDAC in rapidly channelling Ca2+ through the outer mitochondrial membrane and the specific functions of VDAC isoforms in autophagy and apoptosis.

L-18 Caspase activation, specificity and substrates

Guy Salvesen

Sanford-Burnham Medical Research Institute, La Jolla, California, USA

Apoptosis is initiated by ligation of death receptors (extrinsic pathway), or developmental cues, stress and genomic damage (intrinsic pathway). These events result in the activation of apical initiator caspases, and converge on the direct activation of executioner caspases. Thus, a minimal two step activation cascade is at the heart of apoptosis. Understanding how caspase activation is controlled affords the opportunity to formulate the underlying principles that regulate apoptotic signaling pathways. The fundamental shared property of caspases is their ability to execute specific limited proteolysis of cellular proteins, to drive forward apoptotic events. This talk will concentrate on recent advances in determining the activation mechanism of apical caspases, and their in vivo targets.

L-19 Appointment with death - and other cell death mysteries

Nufail Khan, Lynn Wong, Ueli Nachbur, John Silke

La Trobe University, Victoria, Australia

One of the most loved characters of crime author Agatha Christie was the fictional Belgian detective, Hercule Poirot and the title of my talk is reference to one of his assignments. Like him we have to use "the little grey cells" to work out the mysteries of cell death. First there was apoptosis but recently another "killer" has emerged upon the scene; necroptosis. In my talk I will describe new data describing how IAPs are key regulators of both forms of cellular execution. Probably, like Hercule, we need to get into the mind of the killer before we can understand exactly why there appear to be such a surfeit of ways for a cell to die, but in the meantime I will propose models for how the pathways of death are executed.

L-20 A role of the PIDDosome in tumor suppression?

Claudia Manzl, Lukas Peintner, Gerhard Krumschnabel and Andreas Villunger

Division of Developmental Immunology, Innsbruck Medical University

Apoptosis in response to DNA damage is considered a critical event in tumor suppression. The PIDDosome, a multiprotein complex constituted of the p53-induced protein with a death domain (PIDD), receptor-interacting protein (RIP)-associated ICH-1/CED-3 homologous protein with a death domain (RAIDD) and pro-caspase-2 has been defined as an activating platform for this apoptosis-related protease. Notably, both PIDD and Caspase-2 have been implicated in p53-mediated cell death induction in response to DNA-damaging agents but also in DNA-repair responses. In addition, PIDD can promote NF-kB activation upon genotoxic stress, together with RIP and Nemo/IKKg, independently of caspase-2. Since all these cellular responses are considered critical for tumor suppression, we investigated the role of the PIDDosome in oncogenesis induced by either DNA-damage or oncogenic stress in gene-ablated mice. We observed that both PIDD and caspase-2 fail to suppress tumor formation triggered by repeated low-dose g-irradiation. Surprisingly, PIDD is also dispensable for the tumor suppressive capacity of caspase-2 elicited in response to aberrant c-myc expression. Furthermore, the tumor suppressive capacity of caspase-2 appears to be unrelated to apoptosis-induction but linked to cell cycle regulation. Together, out data suggest a PIDDosome independent tumor suppressor function of caspase-2 in connection with the oncogenic stress response machinery.

L-21 The 'Apoptotic' Caspases as Regulators of Inflammation: New Lessons from the Study of Caspase-8 Function

David Wallach, Akhil Rajput, Tae-Bong Kang, Jin-Chul Kim, Konstantin Bogdanov, Seung-Hoon Yang and Andrew Kovalenko

Department of Biological Chemistry and The Weizmann Institute of Science, Rehovot, Israel

While the 'inflammatory' caspases promote inflammation by mediating the generation of cytokines such as IL1-beta, the apoptotic caspases serve to prevent inflammation by safeguarding against release of alarmins from the dying cells. Findings will be presented to demonstrate that caspase-8, which is mainly known for its key role in immune-mediated induction of apoptotic death, also serves to restrict inflammation in other, more direct, manners. One of the ways in which it does so is by associating with the RIG-I helicase complex and suppressing its signaling for activation of IRF3, a transcriptional factor dictating the expression of interferon. As in the case of death induction by caspase-8, its effect in the RIG-I complex depends on the proteolytic activity of this caspase. However, rather than prompting massive cleavage of a large set of cellular substrates through activation of a cascade of other caspases this function of caspase-8 leads to disassembly of the specific protein complex with which caspase-8 associates and thus to arrest of its signaling activity. The action of caspase-8 within the RIG-I complex is temporally restricted. It is coordinated with RIG-I signaling in a way that links maximal activation of the signaling to its pursuant termination. Specific deletion of caspase-8 in the epidermis — a tissue where massive cell death constantly occurs — results in chronic skin inflammation associated with IRF3 activation. Detailed characterization of this process suggests that, besides restricting the activation of this transcription factor by viral stimulators of the RIG-I pathway, caspase-8 also serves to withhold the activation of this pathway by nucleic acids released from epidermal cells that disintegrate during cornification.

Lecture Abstracts

Short Oral Communications

Abstracts are in **alphabetical** order by the name of the presenting speaker.

List of Short Oral Communications

SO-1 Spatiotemporal characterization of caspase activity in single cells

Joel Beaudouin (1), Sabine Aschenbrenner (1) and Roland Eils (1,2)

(1) Division of Theoretical Bioinformatics, German Cancer Research Center (DKFZ), BioQuant, Heidelberg, Germany
(2) Department for Bioinformatics and Functional Genomics, Institute for Pharmacy and Molecular Biotechnology (IPMB) and Bioquant, Heidelberg University, Heidelberg, Germany

Objectives: death receptors induce apoptosis through a cascade of caspase activation. This starts with activation of initiator caspases like caspase-8 that can cleave and activate effector caspases like caspase-3. Effector caspases are tightly regulated so that they are significantly active only when the cellular decision for apoptosis is made, preventing damage in case of survival. As initiator and effector caspases have overlapping activity, initiator caspases could induce damage even before cellular decision. Our aim is to understand how this can be prevented. We hypothesized that compartmentalization of activity can perform such a regulation.

Methods: we visualized activity localization by expressing fluorescent probes based on fusion of fluorescent proteins to localization domains through a linker that can be cleaved by specific caspases. Once activity appears probes are cleaved and the distribution of the fluorescent protein switches from the one of the localization domain to a diffuse one. We designed three categories of probes: cytosolic with and without access to the plasma membrane, and nuclear. Activity was visualized in different cell lines, including HeLa, MCF-7, T98G and MDA-MB-231, induced by CD95 ligand and TRAIL.

Results: caspase-3 activity appears in the whole cell, including the nucleus, abruptly just before formation of apoptotic bodies. In contrast probes specific to caspase-8 are gradually cleaved after CD95 and TRAIL induction, with the exception of nuclear probes that remain intact until caspase-3 becomes active. Cytosolic probes that can access the plasma membrane were more efficiently cleaved that probes with no access to the plasma membrane, showing that caspase-8 activity is mostly concentrated on the plasma membrane. We could induce caspase-3 activity at the same time as caspase-8 by artificially recruiting the protein to the plasma membrane. Using this method we could show that caspase-3 cannot significantly cleave itself or caspase-8 but can efficiently cleave BID, suggesting a positive feedback loop from downstream to upstream of the mitochondrial pathway that bypass caspase-8.

Keywords: caspase-8, single cell, cellular compartmentalization

SO-2 Looking Cell Death in the Eye

TG Cotter, C. O'Mahony, R. Naughton, C. Quinney & G. Groeger.

Dept. of Biochemistry, University College Cork, Ireland.

The cell death pathway in many degenerative diseases is via the process of apoptosis and this is usually driven by some kind of molecular stress. Oxidative stress seems to be one of such stresses in many systems. However, recent work from several laboratories, including our own, suggests that hydrogen peroxide may also have pro-survival properties. While this may appear at first glance to be almost counter intuitive, (it depends on H2O2 concentration) there is increasing evidence to support these 'Janus like' survival properties of this molecule, (see Cell 2010, 140, 454-6).

The eye is a particularly useful model as it has a layered cell structured architecture, is easy accessible for in vivo studies and there are several degenerative diseases that affect this organ where cell death is via apoptosis. So using the eye as a model system we explored in vivo and ex vivo how the specific generation of hydrogen peroxide via members of the NADPH oxidase (NOX) family of enzymes leads to the activation of the PI3k/Akt pathway and increases cell survival. siRNA demonstrated that Nox2 and Nox4 both produce hydrogen peroxide following both ex vivo and in vivo stress and this in turn activates the PI3k/Akt pathway. This increase in PI3k signalling appears to be due to hydrogen peroxide mediated activation of Src which in turn phosphorylates and inhibits PP2a, a serine threonine phosphatase which is a known negative regulator of the Akt pathway. Using both confocal and two-photon microscopy, we can demonstrate, in real time, production of hydrogen peroxide in retinal organ explant cultures as part of a cell survival response.

We also demonstrate in vivo that FGF protects the retina from apoptosis by up-regulating the PI3k/Akt pathway and this is in part due to FGF induced hydrogen production. Thus, we can present mechanistic evidence that cells under stress or as the result of the action of growth factors like FGF, produce hydrogen peroxide at controlled low levels that drives cell survival.

Keywords: apoptosis, redox survival, in vivo, degeneration, mouse models

SO-3 TNFR-induced activation of MAP3K14/NIK enhances TNFR-1-induced cell death

Layla Boutaffala#,1,2, Mathieu Bertrand4, Caroline Remouchamps#,1,2, Corinne Ganeff#,1,2, Sandrine Marchetti3, Jean-Ehrland Ricci3, Jacques Piette#,2, Jorge Caamano5, Peter Vandenabeele4, Mathias Heikenwälder6 and Emmanuel Dejardin#,1,2,*

 # GIGA-Research, University of Liège, Belgium, 1 Unit of Molecular Immunology and Signal Transduction, 2Laboratory of Virology & Immunology, 3C3M, France, 4Department for Molecular Biomedical Research, VIB Gent, Belgium, 5University of Birmingham, UK, 6University Hospital Zürich (USZ), Switzerland.

NIK is a master kinase involved in the activation of the so-called alternative NF-κB pathway by non-death TNFR family members. We observed that non-death TNFR- or Smac mimetic-mediated c-IAP1/2 depletion does not fully synthesize cells to TNFα-induced cell death. We found that NIK displays a pro-death function in TNFR1-induced cell death. First, activation of NIK participates to TNFα synthesis, which kills sensitive cells in an autocrine/paracrine fashion. More importantly, activated NIK acts downstream of TNFR1 by promoting and accelerating the formation of the DISC. We will present new mechanistic insights for this unexpected cross-talk between the alternative pathway and TNFR1 and the in vivo relevance of our findings for lymphoid organ development and cell survival in mice.

Keywords: NIK, TNFR1, cell death

SO-4 Cancer cells undergoing immunogenic death under photo-oxidative ER-stress, surface expose calreticulin via a "non-canonical" pathway

Abhishek D. Garg(1) Tom Verfaillie(1), Noemi Rubio(1), Gabriela B. Ferreira(2), Chantal Mathieu(2), Peter Vandenabeele(3, 4) Dmitri V. Krysko(3, 4) and Patrizia Agostinis(1)

(1)Cell Death Research and Therapy (CDRT) Lab, Dept. Molecular Cell Biology, K. U. Leuven, Leuven, Belgium
(2) Laboratory for Experimental Medicine and Endocrinology (LEGENDO), K.U. Leuven, Leuven, Belgium;
(3)Molecular Signalling and Cell Death Unit, Dept. Molecular Biomedical Research, VIB, Belgium
(4)Dept. Molecular Biology, Ghent University, Belgium

Immunogenic cell death is an emerging concept, which in recent times has shown the potential of amalgamating tumour cell kill with "effective revival" of anti-tumour immunity, within a single paradigm. Research done into this concept has led to establishment of the "immunogenic apoptosis" modality [induced mainly through ER stress pathways and reactive oxygen species (ROS)], which differs from normal apoptosis (tolerogenic) in immunological terms and exhibits three unique properties i.e. (1) Activation of Innate Immune Cells e.g. Dendritic cells (DCs), (2) Induction of 'anti-cancer vaccine effect', in vivo and (3) Surface exposure/secretion of "crucial" Damage-associated Molecular Patterns (DAMPs). However, the chemotherapeutic/apoptosis-inducing agents used to induce immunogenic cell death in previous studies (apart from suffering with various dose-limiting side effects) induced a relatively "less characterized and presumably mild" oxidative ER stress thereby raising the demand for further characterizing this phenomenon through a stronger and well-characterized oxidative ER-stress inducer. To this end, we have been investigating the potential of photo-oxidative ER stress induced by an anticancer modality called Photodynamic Therapy (PDT). In this paradigm we have utilized an ER-localizing photosensitiser (Hypericin) which generates cytotoxic ROS upon visible-light irradiation, wherein these ROS bring about tumour cell-killing (Hyp-PDT).

Studies done at our laboratory showed that photo-oxidative ER stress induced at least two main properties of immunogenic cell death such that treated cancer cells were – (i) capable of causing increased phenotypic maturation of human-immature DCs (hu-iDCs) and (ii) capable of surface exposing/secreting crucial DAMPs like ecto-calreticulin/CRT (pre-apoptotically), exo-ATP (pre-apoptotically and apoptotically) and HSP70/90 (late in apoptosis). Hyp-PDT treated cancer cells were also "preferably" phagocytosed by DCs such that preliminary evidence showed "partial" dependence of phagocytosis on ecto-CRT. Since ecto-CRT was being surface exposed 'actively', we went onto analyze the molecular mechanisms underlying this translocation. We observed that, photo-oxidative ER stress mediated 'pre-apoptotic' ecto-CRT exposure was dependent on PERK and BAX/BAK but, contrary to what was reported for anthracyclines, was neither accompanied by ERp57 co-translocation nor was dependent upon eIF2α; Ser51 phosphorylation or partial caspase 8 (or any other caspases) activation. Moreover, this ecto-CRT followed the "classical" secretory pathway to reach the plasma membrane, with its KDEL ER-retrieval signal intact and did not show strong adherence to cholesterol-rich lipid rafts even when present as 'patches'.

Thus, Hyp-PDT promises to be an effective anti-cancer therapy in terms of induction of immunogenic cell death and DAMPs release/exposure.

Keywords: Immunogenic cell death, apoptosis, ROS, calreticulin

SO-5 Positional proteomics in cell death research

Kris Gevaert (1,2), Petra Van Damme (1,2), Kim Plasman (1,2), Francis Impens (1,2) and Joël Vandekerckhove (1,2)

(1) Department of Medical Protein Research, VIB, B-9000 Ghent, Belgium
(2) Department of Biochemistry, Ghent University, B-9000 Ghent, Belgium

Mass spectrometry-driven proteomics is the main technology to identify those substrates that are processed during cell death ("forward degradomics"), and to catalogue substrates of a selected cell death protease ("reverse degradomics"). Our lab developed a targeted or positional proteomics technology for protease degradomics research which was already applied to different cell death related studies (e.g., (1,2)). We here describe recent expansions to our proteomics platform and show its high potential for protease research in the cell death field.

First, mass spectrometry-driven protease degradomics often produces lists of tens to hundreds of potentially important protease substrates. Key is here to be able to distinguish physiologically relevant substrates from bystanders. By combining metabolic proteome labeling with targeted proteomics in a time-kinetic setup, we have identified 18 highly efficiently cleaved human granzyme B substrates in a background of over 100 different cleavage events in a Jurkat cell lysate. Amongst them the well-known human granzyme B substrate Bid was confirmed next to several other, potential novel regulators of granzyme B induced apoptosis such as Bnip-2 and Akap-8.

Second, comparative analysis by targeted proteomics of living and apoptotic cells, or cell lysates incubated with a selected protease always yields highly regulated or even singleton peptides (here, neo-N-terminal peptides generated upon protease cleavage). Such peptides are inefficiently recognized by data analysis algorithms and further need time-consuming validation. We bypassed these problems by introducing differential isotopic proteome labeling such that substrate reporter peptides are readily distinguished. We validated our approach using the canonical human caspase-3 protease, and applied it to mouse cathepsin D and E substrate processing in a mouse dendritic cell proteome, identifying the largest set of protein protease substrates ever reported and gaining novel insight into unknown substrate specificity differences of these related cathepsins, which was verified using synthetic peptides as substrates.

Third, we developed a novel, complementary positional proteomics approach to simultaneously analyze (neo-)N- and C-terminal peptides (3) and we used it to screen for human protein substrates of granzyme B and carboxypeptidase A4 in human cell lysates. This approach allows for more comprehensive protease degradomics studies, and we here report on the identification of 334 neo-C-termini resulting from granzyme B processing and 16 from carboxypeptidase A4 processing.

Keywords: proteomics, positional proteomics, caspases, granzymes

SO-6 Pursuing Redox-balance modification: PON2 as a new putative anti-tumor target

Ines Witte, Julianna Amort, Petra Wilgenbus, Ulrich Förstermann, Sven Horke

Institute of Pharmacology, University Medicine Mainz, Obere Zahlbacher Str. 67, 55131 Mainz, Germany

Cancer therapies are often hampered as tumor cells acquire apoptosis resistance. Because many malignant cells are under constant pro-oxidant stress, pharmacological interference of redox-balance in cancer cells recently gained increasing attention. Here we propose a new candidate relevant to this concept, the enzyme paraoxonase-2 (PON2), yet known for anti-atherosclerotic properties. PON2 localizes to both endoplasmic reticulum (ER) and mitochondria; its overexpression diminishes oxidative stress and ER stress-induced apoptosis – effects reversed after its knock-down. PON2's cytoprotective role is established; but mechanism and relevance remained elusive. We demonstrate that PON2 modifies two pathways critical for tumor cells, i.e. ER stress and mitochondrial signaling. In response to ER stress, PON2 overexpression considerably reduced expression of pro-apoptotic CHOP via the JNK pathway, which prevented mitochondrial cell death signaling and caspase activation. Further, independent from CHOP and ER stress, PON2 diminished intrinsic apoptosis, because it prevented superoxide formation at and thus cytochrome C release from the mitochondria. This implies that PON2 could contribute to apoptosis evasion and tumor formation, however its role in cancer has never been tested. Here we present three lines of evidence suggesting a vital role for PON2: (1.) PON2 is frequently found overexpressed in various tumors; (2.) its overexpression renders tumor cells more tolerant to chemotherapeutics and cytotoxic compounds, and (3.) some tumor cells undergo redox destabilization and apoptosis simply in response to PON2 knock-down. Collectively, this proposes PON2 as new putative anti-tumor candidate and demonstrates the efficacy of interventions targeting cellular redox-balance.

Keywords: mitochondria, oxidative stress, cancer, endoplasmic reticulum, superoxide

SO-7 An acidic extracellular pH switches TRAIL-induced apoptosis to a RIPK1/RIPK3/PARP-1 dependent programmed necrosis in human colon cancer cells

Sandrine Jouan-Lanhouet (1), Gwénaëlle Le Moigne-Muller (1), Corinne Martin-Chouly (1), Alicia Torriglia (2), Odile Sergent (1), Dominique Lagadic-Gossmann (1) and Marie-Thérèse Dimanche-Boitrel (1)

(1) IRSET, EA 4427 SeRAIC, Faculté de Pharmacie, 2, av. du Pr Léon Bernard, 35043 RENNES CEDEX, FRANCE, Web: http://www.seraic.univ-rennes1.fr
(2) Inserm U872, Centre de Recherches des Cordeliers, 15, rue de l'Ecole de Médecine, 75006 PARIS, FRANCE

Tumoral environment, caracterized by hypoxia, high lactate concentration and acidic extracellular pH (pHe) due to glycolysis, could modify the sensitivity of human cancer cells to treatments. TRAIL (TNF-Related Apoptosis Inducing Ligand), a member of TNF-α superfamily, is a potent anticancer agent that induces cell death in many cancer cells, essentially through apoptosis, but not in most normal cells. The aim of our team is to better caracterize molecular mechanisms of TRAIL-induced cell death under acidic pHe conditions (6.5) in HT-29 human colon cancer cells.

We previously showed that HT-29 cells, resistant to TRAIL-induced apoptosis at physiological pHe (7.4), were sensitized at acidic pHe to a TRAIL-induced programmed necrosis. By Hoechst/Propidium Iodide co-staining and intracellular [ATP] measurement, we showed that TRAIL-induced necrosis depends on both TRAIL-R1 and TRAIL-R2 receptors, on caspases activation following DISC (Death Inducing Signaling Complex) formation, and more interestingly, on RIP1 (Receptor Interacting Protein 1) kinase activity and RIP3. In contrast, under physiological pHe conditions, TRAIL induces a typical apoptotic cell death.

Moreover, we identified by co-immunoprecipitation TRAF-2 (TNF Receptor-Associated Factor 2) and RIPK1 proteins in a secondary cytoplasmic complex formed in parallel to the DISC. These two proteins are involved, at acidic pHe, in high NF-κB activation and early pro-inflammatory cytokines production (IL-8, CCL2), that could be beneficial for the stimulation of an antitumor immune response.

Finally, following our investigations on molecular mechanisms involved in the switch from apoptosis to necrosis, our recent data suggest that PARP-1, which is not cleaved under acidic pHe, plays a major role in intracellular ATP depletion leading to programmed necrosis. Mechanisms leading to PARP-1 activation are still under investigation. However, the two kinases RIP1 and RIP3 seem to be implicated.

This new TRAIL-induced necrotic pathway may account for TRAIL-induced cell death in solid tumors with acidic extracellular pH. These datas may also suggest a better immune response against dying necrotic cancer cells producing inflammatory cytokines.

Keywords: cancer, pH, necrosis, TRAIL, RIP1, RIP3, PARP-1

SO-8 (-)-gossypol targets Mcl-1 for degradation and induces autophagic cell death in glioma cells

Valerie Voss (1), Stefanie Rakel(1), Christian Senft (2), Volker Seifert (2) and Donat Kögel (1)

(1)Experimental Neurosurgery,
(2)Dept. of Neurosurgery, Goethe University Hospital, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany

Anti-apoptotic Bcl-2 family members suppress both apoptosis and autophagy and are of major importance for therapy resistance of malignant gliomas. In order to target these molecules, we employed a panel of BH3 mimetics with different binding profiles to pro-survival Bcl-2 family members. In contrast to other BH3 mimetics failing to target Mcl-1, the pan-Bcl-2 inhibitor (-)-gossypol efficiently induced cell death in glioma cells, but not in primary astrocytes. We also established stable Mcl-1 knockdown cell lines and transiently overexpressed Mcl-1 in U343 cells. U343 control cells and Mcl-1 overexpessing cells were treated with (-)-gossypol and ABT-737 for 48 hours after which whole cell lysates were analyzed for expression levels of Mcl-1. Western blot analysis indicated a pronounced decrease of Mcl-1 protein levels after (-)-gossypol treatment, suggesting that (-)-gossypol had targeted Mcl-1 for degradation. (-)-gossypol triggered translocation of mRFP-LC3-GFP to autophagosomes and flux of LC3 to lysosomes, as well as cytochrome c release, but cell death occurred in the absence of lysosomal damage and effector caspase activation. Lentiviral knockdown of Beclin1 and Atg5 in U87, U343 and MZ-54 cells strongly diminished the extent of cell death induced by (-)-gossypol and combined treatment with temozolomide (TMZ), indicating that autophagy contributed to this type of cell death. In contrast, stable knockdown of the endogenous autophagy inhibitor mTOR increased autophagic cell death. Our data suggest that pan-Bcl-2 inhibitors are promising drugs that induce a caspase-independent, autophagic cell death in apoptosis-resistant, malignant glioma cells and augment the action of temozolomide. Furthermore they indicate that efficient killing of glioma cells requires neutralization of Mcl-1.

Keywords: glioma, autophagy, Bcl-2 family, type II cell death

SO-9 Bim indirectly activates Bax/Bak in ABT-737 induced cell death

Delphine Merino, Seong Lin Khaw, David Huang and Philippe Bouillet

The Walter and Eliza Hall Institute, 3052 Parkville, Melbourne, Australia

Over-expression of pro-survival members of the Bcl-2 family renders cells resistant to numerous death stimuli and favours the acquisition of a malignant phenotype. High expression levels of these proteins is a common occurrence in cancers, and their inhibition with BH3 mimetic drugs is a promising therapeutic approach. In particular, ABT-737 (and its orally form ABT-263) antagonizes Bcl-2, Bcl-xL and Bcl-w [1,2], and is presently being tested in several clinical trials. Early phase human trials indicate that ABT-263 has a significant clinical activity in Bcl-2 over-expressing tumors, particularly those of lymphoid origin [3].

In the present study, we use human or murine cancer models and several strains of transgenic mice to show that the over-expression of Bcl-2 does not protect, but rather sensitizes lymphoid cells to ABT-737. Consistent with previous reports [4,5], we show that the level of pro-apoptotic BH3-only protein Bim is the major determinant of the sensitivity of Bcl-2-over-expressing lymphoid cells to ABT-737. However, we also demonstrate that the activity of Bim relies on its ability to bind Mcl-1 rather than Bax. Our findings may have implications for the identification of new therapeutic targets and the design of new compounds.

Keywords: Cancer, ABT-737, Bcl-2

SO-10 Inflammation-associated autophagy-related programmed necrotic neutrophil death characterized by organelle fusion events

Cristina C. Mihalache, Sébastien Conus, Shida Yousefi and Hans-Uwe Simon

Institute of Pharmacology, University of Bern, Bern, Switzerland.

The most common form of neutrophil death, under both physiologic and inflammatory conditions, is apoptosis. Here, we report a novel form of programmed necrotic cell death, associated with cytoplasmic organelle fusion events, that occurs in neutrophils exposed to inflammatory cytokines upon ligation of CD44. Strikingly, this type of neutrophil death requires both class I and III phosphatidylinositol 3'-kinase (PI3K) activation, signalling events usually required for survival pathways and autophagy induction, respectively. In the death pathway reported here, PI3K is required for the generation of reactive oxygen species (ROS), which somehow trigger the generation of large cytoplasmic vacuoles, likely generated by the fusion of CD44-containing endosomes with autophagosomes and secondary, but not primary, granules. Neutrophils demonstrating vacuolization undergo rapid cell death that depends on receptor-interacting protein 1 (RIP1) kinase activity and papain family protease(s), but not caspases, that are most likely activated and released, respectively, during or as a consequence of organelle fusion. Vacuolized neutrophils are present in infectious and autoimmune diseases under in vivo conditions. Moreover, isolated neutrophils from such patients are highly sensitive toward CD44-mediated PI3K activation, ROS production, and cell death, suggesting that the newly described autophagy-related form of programmed neutrophil necrosis plays an important role in inflammatory responses.

Keywords: apoptosis, autophagy, primary (azurophilic) granules, caspases, cystic fibrosis, inflammation, neutrophils, non-caspase proteases, reactive oxygen species, rheumatoid arthritis, phosphatidylinositol 3 kinase, RIP1 kinase, secondary (specific) granules, sep

SO-11 A caspase- and RasGAP fragment N-activated survivin-dependent protective response in the skin

Nieves Peltzer, Christian Widmann

Department of Phisiology, University of Lausanne. 1005 Lausanne Switzerland

RasGAP, a regulator of Ras GTPase family members, is cleaved under low stress conditions by caspase-3 into fragment N and fragment C. We previously reported that fragment N protects cells in vitro in a Ras/PI3K/Akt-dependent manner and since then efforts to reveal the downstream effectors of Akt mediating fragment-N dependent protection has been done. Preliminary results showed that fragment-N induces survivin expression and that this activation is important for its anti-apoptotic effect.

Survivin, besides being a chromosomal passenger, is a member of the inhibitor of apoptosis protein (IAP) family. It has been shown to be transiently up-regulated in keratinocytes in UV-B illuminated skin.

In this work we provide evidence, using a knock-in mouse model that bears an uncleavable form of RasGAP and that is thereby unable to produce fragment N, that the antiapoptotic effect of fragment N in the skin might be mediated by survivin.

We observed that keratinocytes from knock-in skin are more sensitive to apoptosis after UV-B illumination than wild type skin. We also observed that activation of survivin in the skin by UV-B only takes place if fragment N is produced. More interestingly, we observed that survivin induction after UV-B exposure is abrogated when an inhibitor of caspases is injected prior to illumination, suggesting that survivin induction in the skin of UV-B stressed skin is dependant on casapase-3 activation and the consequent RasGAP cleavage.

Altogether, these results suggest the existence of a protective pathway operating in stressed skin initiated by the cleavage of RasGAP by caspase-3 and culminating in the expression of survivin.

Keywords: apoptosis, UV-B, caspase-3

SO-12 Oncogenes can sensitise tumour cells to TRAIL induced apoptosis: The paradigm of RAS and BRAF oncogenes in colorectal tumours, from models to the clinic

Alexander Pintzas (1) Eftychia Oikonomou (1), Ladislav Andera (2) , George Zografos (3), and Vivian Kosmidou (1)

(1) Lab Signal Mediated Gene Expression, Inst Biol Res Biotech, Nat Hellenic Res Found, 48, Vas. Constantinou Ave., 11635, Athens, Greece,
(2) Lab Cell Signalling and Apoptosis, Inst of Mol Genetics, Czech Acad Scie, Videnska 1083, CZ-14220 Prague 4, Czech Republic
(3) General Hospital of Athens G. Gennimatas, 154 Mesogion Ave., 11527, Athens, Greece

Most data on the therapeutic potential and expression of TRAIL in colorectal cancer have shown increased sensitisation of tumour cells as compared to premalignant or physiological cells or tissues. Thus, particular oncogenes like MYC and RAS can be sensitisers for TRAIL induced apoptosis. We have previously shown that RAS oncogenes can sensitise colon cells to TRAIL induced apoptosis (1). We have presented evidence in cell models that this effect is usually mediated by TRAIL receptor DR4 and DR5 overexpression and/or redistribution (2).

In this study, colorectal cell lines bearing RAS and BRAF mutant oncogenes or colorectal clinical samples were either treated with recombinant TRAIL or analysed for the presence of RAS and BRAF oncogenic mutations and DR4, DR5 expression. We also present evidence that BRAF oncogenes can sensitise colon cancer cells to TRAIL induced apoptosis via TRAIL receptor DR5. The underline mechanisms for this sensitization involve RAF/MAPK and PI3K pathways, depending on the genetic alteration profile of the individual tumour, as shown by co-treatment of TRAIL with specific kinase inhibitors. Other components of apoptotic pathways deregulated by RAS and/or BRAF have been identified through global gene expression analysis and siRNA approaches and their role in TRAIL sensitization will be presented (4). We have shown that DR5 is the most frequently upregulated DR in clinical samples of colon cancer. Furthermore, the presence of K-RAS and BRAF mutations in the tumour may directly or indirectly enhance DR expression (3). Mutations on K-RAS and BRAF oncogenes have been shown in many studies to be associated with resistance to EGFR targeted therapeutics and combinations. TRAIL-based therapeutics, other as mono- or combination therapy could provide a promising alternative for K-RAS/BRAF bearing colorectal tumours. The implications of these findings on personalized therapeutics will be discussed.

Keywords: RAS/BRAF oncogenes, TRAIL, MSI, kinase inhibitors

SO-13 HAMLET kills carcinoma cells but spares healthy, differentiated cells. Molecular basis of the tumoricidal effect

Petter Storm(1), Sonja Aits(1), Maria Trulsson(1), Manoj Kumar Puthja(2), Yinxia Chao(2), Tomas Westergren(1), Ann-Kristin Mossberg(1), James Ho(2), Henry Yang(2) and Catharina Svanborg(1)

*(1) Department of Microbiology, Immunology and Glycobiology, Institute of Laboratory Medicine, Lund, Sweden, (2) Singapore Immunology Network, A*STAR, Singapore*

HAMLET is a potent cell-death inducing complex consisting of partially unfolded α-lactalabumin and oleic acid. Structural studies have shown that partial unfolding is essential for the potent cytotoxic activity of HAMLET, illustrating that partial unfolding of a previously native protein is a mechanism to generate functional diversity; in this case inflammation and cell death. The complex is produced by using human milk or recombinant protein, which after partial unfolding is subjected to ion-exchange chromatography on a matrix preconditioned with oleic acid. HAMLET exists mainly as a monomer, holding an average of 5 oleic acid molecules. The gain of new, beneficial function upon partial protein unfolding and fatty acid binding is remarkable, and may reflect a significant generic route of functional diversification of proteins.

HAMLET kills tumor cells and immature cells but healthy, differentiated cells mount an inflammatory response and survive in the presence of HAMLET. In vivo studies in animal models of glioblastoma and bladder cancer have confirmed that HAMLET is tumor selective and has therapeutic efficacy. In clinical studies, HAMLET showed therapeutic efficacy against skin papillomas and HAMLET killed tumor cells in patients with bladder cancer.

HAMLET shows broad anti-tumor activity (>40 different lymphoma and carcinoma cell lines are sensitive in vitro), suggesting that very basic cell death pathways are identified and activated in tumor cells. Our studies have shown that HAMLET invades tumor cells and interacts with several organelles, including mitochondria, creating a weak apoptosis response. In addition, HAMLET perturbs the structure and function of 20s proteasomes, acting as a proteasome inhibitor. After translocating to the nuclei of tumor cells, HAMLET binds histones and perturbs chromatin structure through high affinity interactions.

To identify mechanisms of HAMLET uptake and important lethal HAMLET-activated signaling pathways we have performed extensive transcriptome and proteome analysis. We detected an activation of p38 signaling pathway members exclusively in tumor cells, in part, due to an ER stress response. Relevance for the tumor specificity of HAMLET was suggested by a comparison with healthy differentiated kidney cells, which showed rapid upregulation of innate immunity but failed to activate p38 and survived HAMLET challenge. Thus, HAMLET identifies different targets in carcinoma cells and healthy, differentiated cells and therefore triggers cellular signalling pathways with different biological effects.

To identify such targets, we have used an shRNA library (collaboration with J Watson and S Powers, Cold Spring Harbor Laboratories). Further data will be provided on the mechanism of tumor cell death in response to HAMLET.

Keywords: HAMLET / tumor specific cell death / apoptosis

SO-14 A lung cancer model linking apoptotic resistance and tumorigenesis via defects in mitochondrial dynamics

Kelly Jean Thomas (1) and Marty R. Jacobson (1)

(1) Saccomanno Research Institute, St. Mary's Hospital and Regional Medical Center, Grand Junction, CO 81502 USA

Inhibition of apoptosis contributes to the tumorigenic conversion of normal cells by extending their viability, favoring the accumulation of transforming mutations. Resistance to apoptosis is linked to increased invasive and metastatic potential in cancers cells. Defects in the apoptotic pathway have been linked to mitochondrial dysfunction. This study investigated mitochondrial membrane potential and its downstream effects on mitochondrial fission and mitophagy in relation to apoptotic resistance in normal and tumorigenic lung cell lines.

Loss of mitochondrial membrane potential is an early initiating event during intrinsic apoptosis. The apoptotic responses of four lung epithelial cell lines treated with the mitochondrial uncoupler CCCP were examined. Cell lines with greater capacity for tumorigenesis had less change in mitochondrial membrane potential following CCCP treatment. All cell lines demonstrated complete mitochondrial depolarization within 24h, however, only the non-tumorigenic cell line underwent apoptosis. Tumorigenic cell lines did not demonstrate cytochrome c release despite significant CCCP-induced depolarization, while non-tumorigenic cells initiated apoptosis and cytochrome c release after treatment. These lung cancer cell lines exhibit defects in mitochondrial membrane depolarization-induced apoptosis, as well as impaired release of cytochrome c.

To further evaluate mitochondrial dysfunction in lung cancer, we used morphometrics to analyze mitochondrial phenotypes. Mitochondrial length and mass correlated with tumorigenicity; mitochondria in the least tumorigenic cell lines had the shortest mean length and increased mass, whereas mitochondria in the most tumorigenic cell lines had the longest mean length and decreased mass. Cells undergoing mitochondrial fission have shorter mitochondrial length when compared to cells that are fusing. Our results suggest that tumorigenic cells are deficient in mitochondrial fission, which correlates with the delayed cytochrome c release. Western blot analysis was used to examine the basal expression levels of mitochondrial fission protein Drp1 and fusion protein Opa1. Drp1 levels were decreased in tumorigenic cells compared to non-tumorigenic cells. Conversely, Opa1 levels were decreased in non-tumorigenic cells when compared to tumorigenic cells. Defects in mitochondrial fission may contribute to the apoptotic resistance observed in tumorigenic lung epithelial cells.

Inhibition of mitochondrial fission results in attenuation of mitophagy, the selective delivery of damaged or dysfunctional mitochondria to lysosomes for autophagic degradation. Mitophagy was induced in normal and tumorigenic lung cell lines by serum starvation and assessed by immunostaining and autophagosomes were noted by LC3 punctae. The percentage of tumorigenic cells with LC3 punctae did not significantly change with treatment, demonstrating resistance to serum starvation-induced mitophagy. Increased inhibition of apoptosis in lung cancer cell lines is correlated with impeded mitochondrial fission and mitophagy. We suggest a model in non-small cell lung cancer that links apoptosis with tumorigenesis, mediated through mitochondrial fission defects.

Keywords: NSCLC, mitochondrial fission, mitochondrial membrane potential, mitophagy

SO-15 A20/tnfaip3 : a master brake on apoptosis

Lars Vereecke (1), Mozes Sze (1), Louis Libbrecht (2), Yuanyuan Chu (3), Marc Scmidt-Supprian (3), Manolis Pasparakis (4), Rudi Beyaert (1) and Geert van Loo (1)

(1)Department for Molecular Biomedical Research, Unit of Molecular Signal Transduction in Inflammation, VIB, and Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
(2)Department of Gastroenterology & Hepatology, University Hospital, Ghent, Belgium
(3)Max Planck Institute of Biochemistry, Martinsried, Germany.
(4)Institute for Genetics, University of Cologne, Cologne, Germany

The ubiquitin-editing enzyme A20 is a critical negative regulator of NF-κB-dependent inflammation and apoptosis. A20 deficient mice spontaneously develop multiorgan inflammation, cachexia and premature lethality, and cells derived hereof are hypersensitive to TNF-induced NF-κB activation and apoptosis, demonstrating the strong anti-inflammatory and anti-apoptotic properties of this protein. A20 may also play important roles in human autoimmune and inflammatory diseases since genetic studies suggest that polymorphisms in or near the human A20/TNFAIP3 gene are associated with Crohn's disease, rheumatoid arthritis, psoriasis, multiple sclerosis and systemic lupus erythematosus.

As A20-deficient mice die early after birth, we generated conditional knockout mice for A20. Conditional gene inactivation was achieved through use of the Cre/LoxP recombination system in which the targeted gene is flanked by LoxP consensus sites, allowing cell-specific deletion of A20 by crossing with transgenic mice expressing Cre recombinase in a tissue specific pattern. We will present new data identifying A20 as an important brake on apoptosis in liver and intestine.

Keywords: knockout, intestine, liver

SO-16 c-IAP1 and TAK1, modulators of TNF-induced necroptosis

Vanlangenakker Nele (1,2) , Vanden Berghe Tom (1,2), Bertrand Mathieu (1,2), Bogaert Pieter (1,2), Laukens Bram (3), Zobel Kerry (4), Deshayes Kurt (4), Vucic Domagoj (4), Fulda Simone (3) and Vandenabeele Peter (1,2)

(1) Dept. for Molecular Biomedical Research, VIB, B-9052 Ghent, Belgium
(2) Dept. of Biomedical Molecular Biology, Ghent University, B-9052 Ghent, Belgium
(3) University Ulm, Klinik fur Kinder und Jugendmedizin, Lab for Apoptosis and Cancer Therapy, D-89075, Ulm, Germany
(4) Genentech, Inc., San Francisco, CA 94080, USA

Three members of the IAP family (XIAP, cIAP1 and cIAP2) are potent suppressors of apoptosis. Recent studies have shown that cIAP1 and cIAP2, unlike XIAP, are not direct caspase inhibitors but block apoptosis by functioning as E3 ligases for effector caspases and RIP1. cIAP-mediated polyubiquination of RIP1 allows it to bind to the pro-survival kinase TAK1 and prevents it from activating caspase-8 dependent death, a process reverted by the de-ubiquitinase CYLD. RIP1 is also a regulator of necrosis, a caspase-independent type of cell death. Here, we show that cells depleted of the IAPs by treatment with the IAP antagonist BV6 are greatly sensitized to TNF-induced necrosis but not to necrotic cell death induced by Fas, poly(I:C) or by oxidative stress. Specific targeting of the IAPs by RNAi revealed that repression of cIAP1 is responsible for the sensitization. Similarly, lowering TAK1 levels or inhibiting its kinase activity sensitized cells to TNF-induced necrosis whereas repressing CYLD had opposite effects. We show that this sensitization to cell death is due to enhanced RIP1 kinase activity and to RIP1 kinase-dependent accumulation of reactive oxygen species (ROS). In conclusion, our data indicate that cIAP1 protects cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent ROS production.

Keywords: necrosis, cIAP1, TAK1

SO-17 A physiological role for linear ubiquitination in innate and adaptive immune signalling

Björn Gerlach (1,5), Stefanie M. Cordier (1,5), Anna C. Schmukle (1,5), Christoph H. Emmerich (1,5), Eva Rieser (1), Tobias L. Haas (2), Holly Anderton (3), Wendy W.-L. Wong (3), Ueli Nachbur (3), Uwe Warnken (4), John Silke (3), Henning Walczak (1)

(1) Tumour Immunology Unit, Department of Medicine, Imperial College London, London, UK
(2) Department of Experimental Oncology, Mediterranean Institute of Oncology, Viagrande, Italy
(3) Department of Biochemistry, La Trobe University, Melbourne, Australia
(4) Protein Analysis Core Facility, German Cancer Research Center (DKFZ), Heidelberg, Germany
(5) These authors contributed equally to this work.

CD40 and Tumour Necrosis Factor Receptor 1 (TNFR1) belong to the TNFR Superfamily (SF) and have important functions in innate and adaptive immunity. Here we identify Sharpin as a novel component of the CD40 and TNF Receptor Signalling Complexes (RSC). Sharpin recruitment to both RSCs requires cellular Inhibitor of Apoptosis Proteins (cIAP). Together with HOIL-1 and HOIP, the previously identified components of the Linear Ubiquitin chain Assembly Complex (LUBAC), Sharpin forms a trimeric complex that is recruited to RSCs by HOIP and is capable of generating linear ubiquitin chains in a HOIP-dependent manner. Thus, HOIP is the central structural and enzymatic component of the trimeric LUBAC. Mutation of the Sharpin gene causes chronic proliferative dermatitis (cpdm) in mice characterised by severe skin lesions. Cpdm mice also have a perturbed immune system, develop multi-organ inflammatory disease, fail to breed and die at an early age. Cpdm-derived murine embryonic fibroblasts (MEFs) exhibited significantly reduced expression of HOIL-1/HOIP and TNF-induced gene activation and were sensitised to TNF-induced apoptosis. Remarkably, even partial Tnf gene deficiency prevented skin lesions in cpdm mice, dramatically extended life span and allowed them to reproduce. However, lymphoid structural and functional abnormalities remained even in completely TNF-deficient cpdm mice. Failure of cpdm B cells to properly activate NF-κB and c-Jun N-terminal kinase (JNK) in response to CD40L links defective IgG isotype switching in cpdm mice to impaired CD40 signalling. Thus, Sharpin is a new, third component of LUBAC and LUBAC plays a physiological role in regulating multiple TNFRSF signalling pathways. Hence, we here identify linear ubiquitylation as crucial for innate and adaptive immunity.

Keywords: NF-κB, ubiquitin, inflammation, cell death, TNF

Poster Abstracts

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

List of Posters

Buqué, Aitziber P-18
Secondary targets of multitarget antifolate in human melanoma cell lines
Cardozo, Alessandra K P-19
CHOP contributes to cytokine/ER-stress induced beta cell apoptosis via decreased Bcl-2 and Mcl-1 expression
Catalan Muñoz, Maria Elena P-20
Mitochondrial DNA depletion sensitizes L929 cells to death receptor-mediated cell death
Cavallucci, Virve P-21
Reelin and sex hormones interactions in the pathological apoptosis during cerebellum development
Cerella, Claudia P-22
Anti-apoptotic effects of synthetic COX-2 inhibitors
Clark, Clay P-23
Substitutions in the Dimer Interface of (Pro)caspase-3 Affect Protein Structure and Function
Conte, Mariarosaria P-24
Role of the silencing of genes that encode for HDAC1 and HDAC2 in the cell line of acute myeloid leukaemia U937
Conus, Sébastien P-25
Cathepsin D induces proteolysis and activation of caspase-8 during neutrophil apoptosis
Corazza, Nadia P-26
Role of the Bcl-2 homolog Bim in T cell-mediated liver immunopathology.
Cornmark, Louise P-27
PKC delta associates with XIAP and Smac in breast cancer cells
De Meyer, Inge P-28
Inhibition of inositol monophosphatase by lithium chloride induces selective macrophage apoptosis in atherosclerotic plaques
de Poot, Stefanie P-29
Human and mouse granzyme M display divergent and species-specific substrate specificities
De Saint-Hubert, Marijke P-30
Apoptosis imaging with 68Ga-labelled AnnexinA5 derivatives for early evaluation of cancer therapy
De Stefani, Diego P-31
VDAC1 selectively transfers apoptotic Ca2+ signals to mitochondria
Declercq, Wim P-32
Caspase-14 is required for filaggrin degradation to natural moisturizing factors in the skin
Decuypere, Jean-Paul P-33
Regulation of inositol 1,4,5-trisphosphate receptors and ER Ca2+ stores during nutrient starvation-induced autophagy
Dell'aversana, Lara P-34
Role of miRNA in leukemogenesis and in epigenetic regulation
Diemert, Sebastian P-35
Implications of CYLD in neuronal cell death
Diez-Fraile, Araceli P-36
Calcium-sensing receptor-mediated survival in granulosa cells.

Doro, Zoltan P-37
Phagocytosis of and cytokine release in response to differently dying cells by murine macrophages
Dubrez-Daloz, Laurence P-38
cIAP1 is a new activator of E2F-1 transcription factor
Duprez, Linde P-39
Role of RIP1/RIP3 in TNF-induced necrosis and Lethal Systemic Inflammatory Response Syndrome (SIRS)
Eberle, Juergen P-40
Sensitization of melanoma cells for death ligand-induced apoptosis by kinase inhibitors correlates with characteristic changes at the level of mitochondria, reactive oxygen species (ROS) and death receptors
Ergene, Emel P-41
Cytotoxıc and apoptotic effects of some boron compounds on the mouse neuroblastoma (NS20Y) cell lines
Fares, Fuad P-42
Natuaral Factors Induce Apoptosis in Human Cancer Cells: development of new strategies for prevention and treatment of cancer
Fecker, Lothar Friedrich P-43
Efficient melanoma cell apoptosis and reduced tumor growth in nude mice in a gene therapy approach with selective replication-competent adenoviral vectors armed with death ligands TRAIL and CD95L
Feoktistova, Maria P-44
cIAPs negatively regulate TLR3-mediated cell death by limiting RIP-1 recruitment to a TRIF-containing signalling complex
Ferraris, Elisabetta Saima P-45
The stress response of nucleolar AATF regulates apoptosis in a c-Jun-dependent and JNK-independent manner
Fourquet, Simon P-46
comprehensive map of cell death and energy metabolism regulation
Fuellgrabe, Jens P-47
Opposing effects of hMOF and SIRT1 on H4K16 acetylation and the sensitivity to the topoisomerase II inhibitor etoposide
Geering, Barbara P-48
A novel TNFR1-triggered apoptosis pathway involving p38 MAPK, class IA PI3Ks, and ROS in neutrophils
Grieco, Luca P-49
Transcriptional regulation is still at work during the execution phase of apoptosis.
Gurzeler, Ursina P-50
Regulation of FasL and TNFα-induced cell death in murine neutrophils
Herrero, Carmen P-51
Development of Apaf-1 inhibitors as therapeutic tools to decrease unwanted pathological apoptosis
Hong, Jiann R P-52
Betanodavirus infection-induced oxidative stress can involve in mitochondria-mediated cell death in fish cells
Impens, Francis P-53
A quantitative proteomics design for systematic identification of protease cleavage events

Irobi-Devolder, Joy P-54
Mutant HSPB8 causes motor neuron specific neurite degeneration
Jacquemin, Guillaume P-55
Sensitization of aggressive B-lymphomas to TRAIL-induced apoptosis by the plant flavonoid quercetin
Jelinkova, Iva P-56
Platinum(IV) derivate LA-12 increases TRAIL-induced apoptosis by upregulation of DR5 and stimulation of mitochondrial events in colon cancer cells
Jennewein, Claudia P-57
Identification of a novel pro-apoptotic role of NF-κB in the regulation of death receptor-mediated apoptosis of glioblastoma cells
Joeckel, Lars P-58
Identifying ER stress induced apoptotic proteases with "click-chemistry" based mass spectrometry
Kaczmarek, Agnieszka P-59
TLR-2 and -9 are sensors of apoptosis in a mouse model of doxorubicin-induced acute inflammation
Kaminskyy, Vitaliy P-60
Inhibition of autophagy sensitizes cells to drug-mediated caspase-dependent and -independent apoptosis via increasing of ROS
Kavanagh, Edel P-61
p57KIP2 enhancement of staurosporine-induced apoptosis requires LIMK1-mediated actin stabilisation
Khalphin, Boris P-62
Protective effect of elastase inhibitors against necrotic cell death
Kim, Do-Sung P-63
Sulforaphane inhibits doxorubicin cardiotoxicity through mitochondrial stabilization and inhibition of NF-κB
Kis, Viktor P-64
Loss of CG6783, a novel lipid-carrier protein, disrupts autophagy and endocytosis in Drosophila
Kiss, Beata P-65
Retinoids induce apoptosis in mouse thymocytes via inducing Nur77 expression
Koci, Lenka P-66
Changes of cytokinetic parameters and adhesive properties in colon cancer cell populations with different sensitivity/resistance to TRAIL
Kovalenko, Elena P-67
Use of a panel of anti-HSP70 monoclonal antibodies for detection of HSP70 translocated to cell surface during lymphocyte apoptosis
Kovar, Jan P-68
Inhibitory effect of unsaturated fatty acids on cell death induced by saturated fatty acids in human pacreatic beta-cells
Kramer, Daniela P-69
Molecular mechanisms of apoptosis induction by the p53 tumour suppressor family
Krautwald, Stefan P-70
TAT-crmA blocks both apoptosis and necroptosis
Kulesza, Dorota P-71
Effects of STAT3 silencing on fate of melanoma and lung cancer cells.

Lavrik, Inna P-72
Towards understanding of life and death regulation at CD95/Fas
Lepore, Ilaria P-73
Characterization of BARD1 expression and function in leukaemia
Liesche, Clarissa P-74
Oligomerization and activity of soluble CD95L
Limami, Youness P-75
COX-2 overexpression confers resistance to ursolic acid-induced apoptosis in human HT-29 colorectal cancer cells
Linkermannn, Andreas P-76
Fas Ligand Mediates Fratricide of Tubular Cells in vivo
López, Marcos P-77
XIAP interaction with FAF1 prevents apoptotic effects in TNFα and FASL mediated pathways
MacKenzie, Sarah P-78
An Allosteric Network of Interactions in Caspase-3 Provides a Novel Strategy for Inhibitor Design
Maes, Hannelore P-79
The Role of Hypoxia-induced Macroautophagy in Melanomagenesis
Marivin, Arthur P-80
Interaction between cIAP1 and RhoGTPases
Martin, Dale P-81
Tandem Reporter Assay for Myristoylated Proteins Post-translationally (TRAMPP) identifies caspase-cleaved Huntingtin as a novel substrate.
Martín Pérez, Rosa P-82
FLIP down-regulation and CHOP-independent up-regulation of TRAIL-R2 induced by ER stress are key events in the sensitization of tumor cells to TRAIL
Masoumi, Katarzyna P-83
Identification of a novel protein kinase C delta – SMAC complex that is dissociated during apoptosis
Mateva, Rada P-84
The influence of Vitamin C and Tempol on cytotoxic and apoptogenic action of myosmine
Melzer, Inga Maria P-85
Functional analysis of the antiapoptotic oncoprotein Aven
Meshkini, Azadeh P-86
Intracellular GTP level determines cell fate by differentiation and apoptosis
Mizuguchi, Junichiro P-87
Requirement for Arginine Methylation in FOXO3a-mediated G1 Arrest of WEHI-231 B Lymphoma Cells upon Engagement of B Cell Receptor for Antigen
Momoi, Takashi P-88
Characterization of Active Caspase-14 From Human Epidermis and Development of the Cleavage
Monaco, Giovanni P-89
BH4 domains of Bcl-2 and Bcl-Xl differentially regulate apoptosis by modulating inositol 1,4,5-trisphosphate receptors activity.
Moquin, David P-90
Regulation of Programmed Necrosis by Cylindromatosis (CYLD)

Morbidoni, Valeria P-91

Role of apoptosis in the pathogenesis of collagen VI myopathies: studies with primary muscle cultures from Ullrich Congenital Muscular Dystrophy patients and Col6a1 null mice

Moret Tatay, Ines P-92

Crohn's disease blood T-cells are resistant to Fas-apoptosis independently of bax/bcl-2 ratio

Morizot, Alexandre P-93

Enhanced caspase-8 activation within the DISC primes over the mitochondrial pathway to sensitize DcR2 expressing cells to TRAIL induced cell death after chemotherapy.

Moserova, Irena P-94

Photokilling of cancer cells by ethylene glycol porphyrin derivatives involves endoplasmic reticulum stress response

Naumann, Ivonne P-95

Bortezomib primes neuroblastoma cells for death receptor-induced apoptosis by activation of the mitochondria

Nys, Kris P-96

UVB-induced Noxa-dependent sunburn cell formation requires p38MAPK/HIF-1

Obexer, Petra P-97

Activation of the transcription factor FOXO3/FKHRL1 by doxorubicin and etoposide induces reactive oxygen species production and programmed cell death in neuroblastoma cells

Ollinger, Karin P-98

Attenuation of the lysosomal death pathway by lysosomal cholesterol accumulation

Orzaez, Mar P-99

Inhibition of cyclin A /CDK2 protein/protein interaction: Re-sensitizing tumor cell lines resistant to Tarceva (erlotinib).

Pardo, Julian P-100

Multiple pathways of granzyme B-induced cell death: result of evolutionary pressure from pathogens (tumors) to evade cytotoxic cells.

Perinpanayagam, Maneka P-101

The regulation of co- and post-translational myristoylation of proteins during apoptosis: roles of N-myristoyltransferases and caspases

Quax, Wim P-102

Designed receptor DR5-specific rhTRAIL variants accelerate induction of apoptosis in cancer cells

Raulf, Nina P-103

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

Reis, Carlos P-104

Rapid and efficient cancer cell killing mediated by high affinity death receptor selective TRAIL variants

Remijsen, Quinten P-105

Neutrophil extracellular trap cell death requires both autophagy and superoxide generation

Repnik, Urska P-106
LeuLeuOMe-induced apoptosis is associated with early autophagosome accumulation and critically depends on cytosolic cathepsin activity
Rezvani, Hamid reza P-107
XPC silencing in normal human keratinocytes increases reactive oxygen species and triggers metabolic alterations that drive the formation of squamous cell carcinomas
Rooswinkel, Rogier P-108
Finding the prerequisites for synergy between ABT-737 and conventional therapy in T-cell malignancies
Rupprecht, Sina P-109
Targeting of XIAP combined with systemic Mesenchymal Stem Cell-mediated delivery of sTRAIL inhibits metastatic growth of pancreatic carcinoma cells
Salim, Hogir P-110
MicroRNA profiling in small and non-small cell lung carcinomas with different response to radio- and chemotherapy
Sánchez, Diego P-111
In vitro-expanded and ex vivo-derived human NK cells as a tool to study cell death induced by cytotoxic cells on hematologic neoplasias
Sapozhnikov, Alexander P-112
Use of 10-N-nonyl acridine orange for flow cytometric analysis of apoptosis
Schroeder, Thilo P-113
Specific targeting of human Caspases with DARPin binders
Schutters, Kristof P-114
Annexin A5 based strategy to improve phagocytosis of apoptotic cells
Seervi, Mahendra P-115
Real Time Analysis of Mitochondrial Events and Caspase Activation by Procaspase Activator : An Essential Role of Mitochondria for Effective Cell Death
Sepe, Sara P-116
Transglutaminase type 2 and autophagy: its involvement in pexophagy
Severin, Fedor P-117
Two mechanisms of antioxidant activity of SkQs
Sgorbissa, Andrea P-118
USP18 modulates the responsiveness to Interferon-α (IFN-a) induced apoptosis in glioblastoma cells: identification of the critical players.
Shirley, Sarah P-119
Divergences in the TRAIL signaling pathway – differences between mice and men.
Silva, Carolina P-120
Serine protease inhibitor-2 blocks inflammatory and cell cytotoxicity responses in mousepox infection
Simovart, Helle-Evi P-121
Apoptosis during luteolysis and its associations with the expressions of Bax and NF-kB in pseudopregnant rats
Sobolewski, Cyril P-122
Determinants of the inhibitory effects of COX-2 inhibitors on cell proliferation in hematopoietic malignancies

Soeur, Jeremie P-123
Selective cytotoxicity of epidermoid cancer cells through apoptosis induction by RW002 essential oil
Sokolov, Sviatoslav P-124
Role of mitochondria in cell senescence-induced death of yeast Saccharomyces cerevisiae
Spes, Ales P-125
Lysosomes and lysosomal proteases are only amplifiers of apoptosis in death receptor pathway
Steinebrunner, Niels P-126
Interaction of Hepatitis B virus (HBV) with apoptosis signaling pathways is involved in viral clearance
Storm, Petter P-127
Why does HAMLET preferentially kill tumour cells? p38- and Ras-dependent death in carcinoma but upregulation of innate immunity in healthy, differentiated cells
Szondy, Zsuzsanna Julianna P-128
Retinoids enhance glucocorticoid-induced apoptosis of T cells by facilitating glucocorticoid receptor-mediated transcription
Tan, Kevin P-129
Novel Insights into Cell Death of Malaria Parasites: Opportunities for Therapeutic Exploit
Tassi, Elena P-130
Rescuing melanoma susceptibility to apoptosis by inhibiting IAPs expression
Triviai, Ioanna P-131
Differentiation-Dependent Apoptosis (DDA) accompanies induced erythroid differentiation of Murine Erythroleukemia (MEL) cells
Trulsson, Maria P-132
HAMLET binding to α-actinin disrupts focal adhesions and facilitates tumor cell detachment
van de Kooij, Bert P-133
Unconventional ubiquitination regulates Bid apoptotic activity
van Domselaar, Robert P-134
Heterogenous nuclear ribonucleoprotein K is a pan-granzyme substrate and is essential for cell survival
Vanden Berghe, Tom P-135
Susceptibility for E. coli LPS-induced lethality is differentially regulated by caspase-1 and -3
Varecha, Miroslav P-136
Alteration in mitochondrial superoxide production by shRNA gene silencing of AIF in various cell lines
Verbeke, Stephanie P-137
Signaling of p75 neurotrophin receptor in breast cancer cells
Verfaillie, Tom P-138
PERK deficiency distorts the ER – mitochondrial interface and protects against ROS-induced intrinsic apoptosis
Verschooten, Lien P-139
Induction of autophagy and caspase dependent cell death by the flavonoid Luteolin specifically in malignant skin cells.

Viola, Giampietro P-140
MG-2477, a new tubulin inhibitor with potent antitumor activity in vitro and in vivo induce autophagy and delayed apoptosis in A549 cells

Vissers, Margreet P-141
The Regulation of HIF-1 by Vitamin C and its Impact on Cell Survival in Inflammation and Cancer

Vurusaner, Beyza P-142
Apoptosis induced by lipid peroxidation end products: cellular and molecular basis

Wahl, Kristin P-143
Serum biomarkers of cell death for monitoring therapy response of gastrointestinal carcinomas

Wejda, Magdalena P-144
Proteome-wide analysis reveals that caspase-2 shares the cleavage and substrate specificity with caspase-3

Widmann, Christian P-145
Improving the survival of pancreatic beta cells

Witte, Ines P-146
The role of PON3 in reduction of oxidative stress and cell death

Wu, Jen-Leih P-147
Stage-specific expression of TNFα to regulate sequential apoptotic and necrotic cell death in birnavirus-infected fish cells

Yazdanparast, Razieh P-148
Intracellular GTP level determines cell fate by differentiation and apoptosis

Zarzynska, Joanna P-149
TGF-β1 regulates apoptosis and autophagy in bovine mammary epithelial cells during involution

Zecchini, Erika P-150
Beyond cell death: role of p66shc and redox stress on autophagy, adipogenic differentiation and metabolism.

Zhivotovsky, Boris P-151
Decitabine synergistically with valproic acid restores caspase-8 expression and sensitizes small cell lung carcinoma cells to TRAIL

P-1 Abnormal IP3R-induced Ca2+ release correlates with anti-apoptotic signaling in Diffuse large B-cell lymphoma and B-cell chronic lymphocytic leukemia

Haidar Akl(1), Heleen Van Melckebeke(2), Giovanni Monaco(1), Barbara Vanhoecke (2), Ludwig Missiaen(1), Jan B. Parys(1), Fritz Offner(2), Humbert De Smedt(1), and, Geert Bultynck(1)

(1) K. U. Leuven Campus Gasthuisberg, Laboratory of Molecular and Cellular Signalling, Department of Molecular and Cellular Biology, O/N1, B-3000 Leuven, Belgium
(2) Ghent University Hospital, Department of Hematology, De Pintelaan 185, 1P7, B-9000 Ghent, Belgium.

Deregulation of the inositol 1,4,5-trisphosphate receptor (IP3R) activity affects the endoplasmic reticulum (ER) Ca2+ homeostasis and its role in apoptosis. However, the exact roles of IP3Rs and their contributions to Ca2+ signals in malignant B-cell behavior remain largely unknown. It has been shown that B-cell receptor (BCR) signaling is chronically active in Diffuse large B-cell lymphoma (DLBCL) and that the Ca2+ response is defective in B-cell chronic lymphocytic leukemia (CLL).

Prosurvival Bcl-2 over-expression is a hallmark of B-cell malignancies. Recently, it has been shown in our laboratory that the BH4 domain of Bcl-2 inhibits ER Ca2+ release by binding to the IP3R. Using a collection of DLBCL cell lines (Profesor A. Letai, Dana Farber Cancer Centre, Harvard Medical School) and primary CLL cells (Professor F. Offner, Ghent University Hospital), we observe differences in IP3R protein levels, Ca2+ fluxes and apoptosis levels. Moreover, cells with a low response to anti-BCR antibody exhibit a decreased Ca2+ response and low IP3R protein expression. This suggests that an altered IP3R Ca2+ signaling could be involved in the reported chronic active B-cell receptor signaling and defective Ca2+ response in malignant B cells.

Keywords: IP3R, Ca2+, CLL, DLBCL

P-2 Homoharringtonine and Manumycin A accelerate TRAIL-induced apoptosis of human colorectal cancer cells via a different mechanism.

Lenka Beranova (1), Ivana Borovickova (3), Antonio Pombinho (2), Petr Bartunek (2), Marcela Simsova (3) and Ladislav Andera (1)

Departments of (1)Cell Signaling & Apoptosis and (2)Cell Differentiation, Institute of Molecular Genetics, Prague, Czech Republic and (3)Apronex s.r.o.3, Vestec U Prahy, Czech Republic

TRAIL, the ligand from TNFa family became well-known for its rather specific and effective induction of apoptosis of a number of cancer cells of various cell type origins, and the recombinant TRAIL or agonistic anti-TRAIL receptor antibodies are being evaluated in clinical trials as potentially new anti-tumor bio-drugs. As the majority of primary tumors is rather resistant to sole action of TRAIL pathway-activating apoptogens, a number of drugs, natural compounds or other biomolecules has been identified as sensitizers or enhancers of TRAIL-induced apoptosis. Our screening for these novel TRAIL sensitizers uncovered two natural compounds - manumycin A (ManA), a farnesyl transferase inhibitor originally produced by Streptomyces parvulus and homoharringtonine (HHT), an alkaloid isolated from evergreen tree Cephalotaxus harringtonia. HHT has been for quite some time enrolled in clinical test as a potential novel anti-myeloid leukemia drug. Though ManA or HHT did not enhance cell surface expression of TRAIL receptors, both they sensitized at individually sub-toxic doses a number of TRAIL-resistant colon cancer-derived cells to TRAIL-induced apoptosis and thus they might be potentially exploited for combinatory therapy of colorectal tumors.

Keywords: TRAIL, colorectal cancer, enhancers, natural compounds

P-3 Synergistic anti-cancer effect of immunotoxins and triphenylmethyl-derivates involves decreased stearoyl-CoA desaturase protein levels.

Karianne Risberg(1), Ingrid Jenny Guldvik(1), Rahul Palchaudhuri(2), Øystein Fodstad(1), Paul J Hergenrother(2), Yvonne Andersson(1)

(1)Department of Tumor Biology, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet and Faculty Division the Norwegian Radium Hospital, University of Oslo, N-0310 Oslo, Norway
(2)Department of Chemistry, Roger Adams Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

The main objective of this work was to evaluate the in vitro and in vivo effect of our in-house immunotoxins (ITs) 425.3PE and 9.2.27PE with two different triphenylmethyl-derivates TPMP-I-2 and 4BI in cancer cell from various origins.

We have previously shown that our ITs inhibits protein synthesis and induces apoptosis in cancer cells in vitro and in vivo. The triphenylmethyl-derivates are novel anti-cancer molecules which have been shown to induce apoptosis in malignant melanoma cells.

By combining our ITs with TPMP-I-2 or 4BI, increased inhibition of protein synthesis and synergistic decreased cell viability were observed in the MA-11 (breast cancer cells), HeLa* (ovarian cancer cells) and FEMX (malignant melanoma cells), compared with IT, TPMP-I-2 or 4BI as mono-treatments. In the MA-11 cells, activation of caspase-3 and DNA fragmentation was induced upon treatment with 425.3PE IT in combination with TPMP-I-2 or 4BI. Interestingly, treatment with IT +/- TPMP-I-2 or 4BI resulted in a strong decreased level of SCD (stearoyl-CoA desaturase) in all three cell lines. SCD is the rate limiting enzyme for converting saturated fatty acids into monounsaturated fatty acids needed for membrane genesis. We suggest that the observed decreased levels of SCD is an important contributor to the decreased cell viability and the increased cell death observed in the three cell lines upon IT +/- TPMP-I-2 or 4BI treatment. Furthermore, a combination of 425.3PE and 4BI was more effective in reducing tumor growth in a breast cancer model in nude mice compared with either agent alone, and a combination of 425.3PE and TPMP-I-2 significantly prolonged the survival time in nude rat models simulating micrometastatic ovarian carcinoma disease.

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Keywords: cancer, stearoyl-CoA desaturase, cell death, immunotoxin

P-4 Deciphering how a RasGAP-derived peptide sensitizes cancer cell to genotoxins

Alessandro Annibaldi and Christian Widmann

Dept. of Physiology, Lausanne University, Rue du Bugnon 7, 1005 Lausanne, Switzerland

Chemoresistance is a major problem in oncology. Strategies to restore the sensitivity of tumor cells to genotoxins would improve the efficacy of current anti-cancer regimens and the survival of cancer patients.

We have already reported that a peptide corresponding to amino acids 317 to 326 of RasGAP, fused to the TAT cell permeable sequence, called TAT-RasGAP317-326, is able to sensitize cancer cells, but not normal cells, to genotoxins both in vitro and in vivo. The molecular mechanisms underlying this TAT-RasGAP317-326 mediated sensitization are still to be fully understood. We have already determined however that the p53/PUMA-induced MOMP (mithochondrial outer membrane permeabilization) is involved in the process of TAT-RasGAP317-326-mediated sensitization.

However, proteins of the p53/PUMA pathway were not modulated or affected by the peptide.

Here we show that TAT-RasGAP317-326 is also able, in most cases, to favour apoptosis towards UV illumination and starvation but not to TNF α stimulation. In order to understand how TAT-RasGAP317-326 favours apoptosis in cancer cells we assessed whether TAT-RasGAP317-326 modulates the levels of specific Bcl2 family members. None of the protein tested was found to have its expression levels affected by the peptide in three different cancer cell lines. On the other hand, we observed that TAT-RasGAP317-326 mediated Bax activation. These results suggest that TAT-RasGAP317-326 sensitizes tumor cells by stimulating Bax activity downstream of PUMA, either by enhancing the activity of pro-apoptotic players, or by hampering the capacity of the anti-apoptotic members to neutralize the pro-apoptotic Bcl-2 proteins.

Keywords: Cancer, Chemotherapy, Bcl-2

P-5 FOXO3/FKHRL1 is activated in high-risk neuroblastoma and contributes to chemotherapy-resistance and angiogenesis

Michael Ausserlechner (1,2), Kathrin Geiger (1), Judith Hagenbuchner (1,3), Martina Rupp (1,2), Christina Salvador (2), Bernhard Meister (2), Consolato Sergi (4) and Petra Obexer (1,3)

(1)Tyrolean Cancer Research Institute, Innsbruck, Austria; Department of Pediatrics (2) II and (3)IV, Medical University Innsbruck, Austria, (4) Department of Laboratory Medicine and Pathology, University of Alberta

Background: FOXO transcription factors control programmed cell death, stress resistance and longevity in normal and malignant cells. We investigated the expression, subcellular localization and phosphorylation of FOXO3/FKHRL1 in tumor sections of post chemotherapy neuroblastoma (NB) patients and analyzed its effect in cultured neuroblastoma cells.

Methods: Paraffin-embedded sections from 27 NB patients were analyzed for FOXO3 expression, localization and phosphorylation. Effects of chemotherapeutic agents on FOXO3 subcellular shuttling were assessed by live cell fluorescence imaging in ECFP-FOXO3 transgenic NB cells. To study how FOXO3 modulates survival of NB cells we generated cell lines expressing a conditional PKB-independent FOXO3 allele (FOXO3(A3)ERtm) that can be activated by 4OH-tamoxifen and studied the effects of FOXO3 activation in vitro and in vivo by xenograft transplantation into nude mice.

Results: We found that FOXO3 was localized in the nucleus and phosphorylated at the protein kinase B (PKB) phosphorylation site T-32 in tumor sections from high-risk NB patients. FOXO3 nuclear localization and phosphorylation significantly correlated with reduced patient survival. The chemotherapeutics etoposide and doxorubicin led to rapid nuclear accumulation and increased phosphorylation of FOXO3 at the PKB site T-32 in NB cell lines as measured by live cell fluorescence imaging and immunoblot, respectively. NB1/FOXO cells expressing the conditional FOXO3(A3)ERtm allele became resistant to chemotherapy-induced cell death whereas NB15/FOXO cells underwent spontaneous apoptosis upon FOXO3 activation. However, when transplanting NB15/FOXO cells into nude mice, low-level activation of FOXO3 strongly induced angiogenesis of NB tumors in vivo whereas full activation led to tumor regression.

Conclusions: The combined data suggest that FOXO3 is activated in high risk NB tumors and contributes to chemotherapy resistance and tumor angiogenesis.

Keywords: FOXO3, neuroblastoma, tumor therapy, angiogenesis

P-6 Clearance of dying autophagic cells induces the inflammasome pathway in primed mouse macrophages

Gizem Ayna (1), Goran Petrovski (1) and László Fésüs (1)

(1) Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, Hungary

Autophagy (AU) is recently recognized as a possible inducer of a distinct cell death mechanism. We previously pointed out that the clearance of dying autophagic (AU) MCF7 cells, but not living or apoptotic ones, can lead to pro-inflammatory response in human macrophages (Ms). Then, we established a mouse model and used the mouse Ba/F3 cell line (IL-3 dependent BM derived pro-B cells) as a possible AU cell clearance model. IL3 depleted Ba/F3 cells are mostly living and a part of these cells are dying during IL3 depletion for 6 hours with signs of AU. We stained cells with either MDC (for autophagolysosomes), acridine orange (for lysosomes) and LC-3 cleavage to observe the AU activity levels with/without IL3 withdrawal. Both western blot and microscopy results represented the increased amount of autophagolysosomes inside the cell after 6 hours IL3 depletion. Our results from several independent assays showed that dying AU Ba/F3 cells but not living cells can induce the IL-1beta release from LPS primed mouse peritoneal MΦs whereas LPS non-primed MΦs can not be activated with dying AU cells.

We also hypothesized that AU dying Ba/F3 cells could release factors which in conditioned medium (CM) could affect the inflammasome pathway in mouse Ms. Indeed, CM elicited peritoneal MΦs released higher amount of IL1-beta upon co-incubation with 6 hours starved dying AU Ba/F3 cells.

These results can open the way of further knock-down and knock-out experiments in order to clarify the exact pathway behind the pro-inflammatory response of Ms after induction of dying autophagic (AU) cells and/or the factors they secrete while IL3 depletion.

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

P-7 Sensitization of glioblastoma cells to TRAIL-induced apoptosis by histone deacetylase inhibitors

Annette Bangert, Klaus-Michael Debatin and Simone Fulda

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

Histone deacetylase inhibitors (HDACI) are anticancer agents targeting aberrant gene transcription and protein activity in cancer cells and can drive these into apoptosis and growth arrest. Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor. Current treatment approaches often fail due to defective apoptotic signaling processes. In this study, we investigated whether combination of HDACI with death receptor ligands is a feasible strategy to overcome this resistance. Pre-treatment with different HDACI sensitized various GBM cell lines for death receptor-induced apoptosis. Experiments addressing the mechanism of sensitization to TRAIL by the HDACI MS275 indicated a caspase-dependent process. No increased cell surface expression of death receptors was induced by MS275. However, decreased recruitment of the caspase-8 inhibitor FLICE-like inhibitory protein (FLIP) as well as elevated levels of processed caspase-8 were detected at the TRAIL death-inducing signaling complex upon combination treatment. Correspondingly, we found enhanced cleavage of caspase-8 and -3 as well as processed caspase-9 and Bid. An involvement of the intrinsic apoptotis pathway in the sensitization process was further evidenced by activation of Bax, loss of the mitochondrial membrane potential and release of cytochrome c from the mitochondria. Examining various apoptosis-modulating proteins, we found that MS275 significantly reduced anti-apoptotic FLIP. Overexpression of both FLIP isoforms (FLIP-L and FLIP-S) reduced apoptosis, pointing to a possible role of FLIP in the sensitization process. FLIP downregulation was not due to proteasome- or caspase-mediated degradation but rather occurred at the mRNA level. We found that MS275 upregulates c-myc which is described as a transcriptional repressor of FLIP. Knockdown of c-myc partially rescued FLIP-L from MS275-induced downregulation and diminished apoptosis by our combination treatment. Considering a possible clinical application, it is interesting to note that MS275 also sensitized primary cultured glioblastoma cells as well as glioblastoma stem cells to TRAIL-induced apoptosis and that this combination was capable of reducing long-term survival of GBM cell lines. Taken together, our data suggest that combining HDACI with TRAIL might be a promising approach to overcome apoptosis resistance in glioblastoma.

Keywords: glioblastoma, HDAC inhibitor, TRAIL

P-8 In vitro and in vivo comparison between two PET tracers for apoptosis: 68Ga-AnxA5 and 18F-ML10

Matthias Bauwens (1), Marijke De Saint-Hubert (2), Jan Cleynhens (1), Ellen Devos (2), Niko Deckers (3), Chris Reutelingsperger (3), Alfons Verbruggen (1)

(1) Radiopharmacy, KULeuven, Herestraat 49, 3000 Leuven, Belgium
(2) Nuclear Medicine, KULeuven, Herestraat 49, 3000 Leuven, Belgium
(3) Biochemistry, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands

Radiolabelled annexin A5 (anxA5) is known to bind to apoptotic cells and has been proposed as an agent to visualise apoptosis in vivo. A 99mTc-labelled anxA5 derivative has been evaluated in a number of clinical trials, but the biodistribution of radiolabelled anxA5 is suboptimal as it shows a high physiological uptake in kidneys and liver, complicating and compromising apoptosis imaging. Recently 18F-ML10, a small molecule taken up by apoptotic cells, has been proposed as an alternative with superior biodistribution characteristics and potentially more favourable imaging qualities.

We compared 68Ga-Cys2-AnxA5, an in-house developed anxA5 analogue, with 18F-ML10 in a series of in vitro and in vivo experiments. In vitro cell binding of both tracer agents was studied in control and anti-Fas mAb treated Jurkat cells, as well as in a model for plasma membrane depolarisation (PMD). Biodistribution and pharmacokinetics were studied with µPET up to 60 min p.i. in healthy mice, in a hepatic apoptosis model (anti-Fas mAb treated mice) and in a model for muscular apoptosis. Tracer uptake was measured and imaged ex vivo using autoradiography and correlated to histological evidence of apoptosis (TUNEL).

In vitro binding of 68Ga-Cys2-AnxA5 and 18F-ML10 was respectively 4 and 1.5 times higher in anti-Fas mAb treated tumour cells as compared to normal cells. In the PMD model, the uptake of 68Ga-Cys2-AnxA5 did not increase, while 18F-ML10 showed a 6-fold increase as compared to normal cells. However, it must be mentioned that the absolute uptake values of 18F-ML10 were very low. In vivo, 68Ga-Cys2-AnxA5 was shown to have a very slow clearance from blood and a high uptake in the kidneys and the liver (respectively 75% I.D. and 6.8% I.D. at 60 min p.i.). Upon treatment of mice with anti-Fas mAb, the uptake of 68Ga-Cys2-AnxA5 in the liver increased to 51% I.D., proving uptake in apoptotic tissue. 18F-ML10 on the other hand was cleared very rapidly from blood with only minor uptake in the rest of the body. In anti-Fas mAb treated animals, its uptake in all body parts increased strongly, with the liver showing a 100-fold higher uptake as compared to the behaviour in healthy mice. In a muscular apoptosis model (ethanol induced apoptosis), both 68Ga-Cys2-AnxA5 and 18F-ML10 showed a 3-4 fold higher uptake in apoptotic muscle tissue as compared to healthy muscle tissue.

In conclusion, both 68Ga-Cys2-AnxA5 and 18F-ML10 show a clear uptake in apoptotic cells. 18F-ML10 is inferior to 68Ga-Cys2-AnxA5 in vitro. The mechanism of 18F-ML10-uptake by apoptotic cells is unclear but due to the favourable physiological biodistribution (rapid excretion into the urine) 18F-ML-10 might be more promising for in vivo imaging of apoptosis.

Keywords: PET imaging, apoptosis, annexinA5, ML10

P-9 Development of a Laser based Technology to study the epigenetic de-regulation of leukaemia cell death

Rosaria Benedetti (1), Vincenzo Carafa (2), Mariarosaria Conte (2), Angela Nebbioso (2), Raffaele Velotta (3), Carlo Altucci (3) & Lucia Altucci (2)

(1) Dipartimento di Chimica Organica e Biochimica, Università Federico II di Napoli, Complesso Universitario di Monte Sant'Angelo via Cynthia 4 I-80126 Napoli, IT
(2)Dipartimento di Patologia generale, Seconda Università di Napoli, Vico L. De Crecchio 7, 80138, Napoli, IT
(3) Dipartimento di Scienze Fisiche,Università Federico II di Napoli, Complesso Universitario di Monte Sant' Angelo, Via Cynthia 80126 Napoli, IT

Deciphering the complexity of chromatin-encoded information is the prerequisite for understanding the regulatory circuits governing development and (patho)physiology.

Transcription factors and epigenetic modulators translate chromatin-embedded information in a dynamic and cell/gene context specific manner to orchestrate homeostasis, growth and differentiation. To date, the most powerful and commonly used approach is immunoprecipitation of chemically cross-linked chromatin (XChIP) coupled with single gene or global analysis using DNA tiling arrays (ChIP-chip) or parallel single molecule sequencing (ChIP-seq). At present, serious limitations of the XChIP technology preclude factor-DNA interaction studies at dynamic range below minutes. Moreover, conventional XChIP cannot be used to study samples at very low cellular concentration within complex biological samples. Some of these problems can be avoided using high powered femtosecond (fs) UV tunable lasers, to induce highly efficient DNA-protein crosslinking for ChIP analysis (L-ChIP) with unprecedented precision and reproducibility, and extending the present dynamic time range by order of magnitude. So, based on established proof-of principles experiments, crosslinking by short pulsed UV lasers is a potentially powerful tool to investigate DNA–protein interactions, especially transient interactions and binding kinetics, because the number of photons required for covalent complex formation can be delivered very rapidly, in nano-, pico- or even femtosecond intervals, and the high energy of the pulses should result in efficient crosslinking.

After setting of L-ChIP, this innovative technique of crosslink induction will be applied to the study of the function and regulatory mechanisms of the gene coding for TRAIL (TNF related apoptosis inducing ligand) in leukaemic cells. These data will be further discussed.

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Keywords: Laser, ChIP, leukaemia cells, TRAIL, apoptosis

P-10 E3 ubiquitin ligase and de-ubiquitinase, cIAP1 and TRABID, regulate RIP4-dependent NF-kB activation

Mathieu JM Bertrand1,2, Saskia Lippens1,2, Wim Declercq1,2 and Peter Vandenabeele1,2

(1)Molecular Signaling and Cell Death Unit, Department for Molecular Biomedical Research, VIB
(2)Department of Biomedical Molecular Biology, Ghent University, 9052 Ghent, Belgium

The RIP kinases have emerged as essential sensors of cellular stress, integrating both extracellular stimuli emanating from various cell-surface receptors and signals coming from intracellular pattern recognition receptors. The molecular mechanisms regulating the ability of the RIP proteins to transduce the stress signals remain poorly understood, but seem to rely only partially on their kinase activities. On the contrary, recent studies on RIP1 and RIP2 have highlighted the importance of ubiquitination as a key process regulating their capacity to activate downstream signaling pathways. In this study, we found that XIAP, cIAP1 and cIAP2 not only directly bind to RIP1 and RIP2 but also to RIP3 and RIP4. We found that cIAP1 acts as a direct E3 ubiquitin ligase capable of conjugating diverse types of ubiquitin chains to all four RIP proteins, and that repressing cIAP levels affects RIP1-4-dependent NF-kB activation. Interestingly, we observed that ectopic expression of the de-ubiquitinases CYLD and A20 partially impairs RIP1-3-dependent NF-kB activation but had not impact on RIP4, while expression of the de-ubiquitinase TRABID completely inhibits all RIP-dependent NF-kB activation. In addition, we found that, contrary to RIP1-3, RIP4-mediated NF-kB activation does not require TAK1 kinase activity, suggesting an alternative mode of activation of the IKK complex. Finally, we identified lysines K51 and K145 of RIP4 as two critical acceptor sites for cIAP1-mediated ubiquitination and NF-kB activation.

Keywords: RIP kinase, XIAP, cIAP1, cIAP2, ubiquitin, NF-kB, A20, CYLD, TRABID

P-11 Poly(ADP-ribose)glycohydrolase is an upstream regulator of Ca2+ fluxes in oxidative cell death

Christian Blenn, Philippe Wyrsch, Jessica Bader, Maja Bollhalder and Felix R. Althaus

Institute of Pharmacology and Toxicology, University of Zurich-Vetsuisse, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland

Oxidative DNA damage to cells activates poly(ADP-ribose)polymerase-1 (PARP-1) within seconds and the poly(ADP-ribose) formed is rapidly degraded to ADP-ribose by poly(ADP-ribose)glycohydrolase (PARG). Here we show that PARP-1 and PARG control extracellular Ca2+ fluxes through melastatin-like transient receptor potential 2 channels (TRPM2) in a cell death signaling pathway. TRPM2 activation accounts for essentially the entire Ca2+ influx into the cytosol, activating caspases 3, 6 and 9, and causing the translocation of apoptosis inducing factor (AIF) from the inner mitochondrial membrane to the nucleus followed by cell death. Abrogation of PARP-1 or PARG function disrupts these signals and reduces cell death. ADP-ribose-loading of cells induces Ca2+ fluxes in the absence of oxidative damage, suggesting that ADP-ribose is the key metabolite of the PARP-1/PARG system regulating TRPM2. We conclude that PARP-1/PARG control a cell death signal pathway that operates between five different cell compartments and communicates via three types of chemical messengers: a nucleotide, a cation and proteins.

Keywords: ADP-ribose; AIF; H2O2; PARG; PARP; poly(ADP-ribose)

P-12 Investigating the Role of PIDD in the DNA Damage Response

Florian Bock (1), Gerhard Krumschnabel (1), Claudia Manzl (1) and Andreas Villunger (1)

(1) Division of Developmental Immunology, Medical University Innsbruck, Fritz-Pregl. Str. 3, 6020 Innsbruck, Austria.

The p53-induced protein with a death domain (PIDD) is involved in cellular pathways which can induce programmed cell death (apoptosis), survival via the activation of NFkB and/or DNA-repair after DNA-damage. Depending on the duration, type 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 NFkB by promoting sumoylation of NEMO. Most recently, PIDD has also been shown to form complexes with DNA-PK, assisting DNA repair by enabling non-homologous end-joining. While the first complex induces apoptosis, the latter two prevent apoptosis by enabling the cell to repair the damage. PIDD therefore plays a role in the integration of at least two opposing signalling pathways. This dual role is based on the capacity of PIDD to auto-proteolytic 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 (MEF) derived from those mice we performed survival assays and monitored NFkB activity in response to different stimuli.

Treatment with inflammatory cytokines did not reveal any differences in NFkB activation between wild type and PIDD deficient MEF. In contrast, there was a significant delay in activation of NFkB in response to DNA damage. Furthermore, various inhibitors of NFkB could sensitize wild type cells, but not PIDD deficient cells, towards DNA damage induced apoptosis. Unexpectedly, we observed no differences of short and long term survival between wild type and PIDD deficient cells. However, PIDD deficient cells show a defective cell cycle arrest in response to DNA-damage.

In conclusion, our results suggest that PIDD plays a rate-limiting role in NFkB activation following DNA damage. Surprisingly, this defect is not reflected in differences in cell survival.

Keywords: DNA damage, Apoptosis, NFkB, PIDD

P-13 Crosstalk between autophagy and apoptosis in collagen VI muscular dystrophies

Paolo Grumati (1), Luisa Coletto (4), Patrizia Sabatelli (5), Matilde Cescon (1), Alessia Angelin (2), Bert Blaauw (3), Tania Tiepolo (1), Anna Urciuolo (1), Nadir M. Maraldi (5), Paolo Bernardi (2), Marco Sandri (4) and Paolo Bonaldo (1)

(1) Dept. of Histology, Microbiology & Medical Biotechnologies, (2) Dept. of Biomedical Sciences, (3) Dept. of Human Anatomy & Physiology; University of Padova, 35121 Padova, Italy.
(4) Dulbecco Telethon Institute, Venetian Institute of Molecular Medicine, 35129 Padova, Italy.
(5) IGM-CNR, 40136 Bologna, Italy.

Mutations of genes encoding the extracellular matrix protein collagen VI cause different types of muscle diseases, including Bethlem myopathy (BM) and Ullrich congenital muscular dystrophy (UCMD). Collagen VI null (Col6a1–/–) mice display a myopathic phenotype with ultrastructural alterations of organelles, latent mitochondrial dysfunction and spontaneous apoptosis of muscle fibers [1]. Based on the findings obtained in the murine knockout models, similar defects could be revealed in muscle biopsies and cultures of UCMD/BM patients [2]. Although these findings indicate that collagen VI plays a key role in muscle, the molecular mechanisms linking collagen VI deficiency with organelle alterations and cell death remain unknown. Therefore, we carried out a number of studies in Col6a1–/– mice and patients aimed at shedding light on such mechanisms [3,4].

Analysis of pro- and anti-apoptotic Bcl2 proteins did not reveal any obvious difference between Col6a1–/– and wild-type muscles. An important survival mechanism in muscle is the mTOR pathway, which in turn is regulated by Akt and AMPK. Although Akt phosphorylation was apparently normal, AMPK was markedly activated indicating an energy unbalance in Col6a1–/– muscles. Autophagy is an evolutionarily conserved process, which is crucial in the turnover of cell components both in constitutive conditions and in response to energy deprivation. The above findings suggest that autophagy might be activated in Col6a1–/– muscles to allow for removal of altered organelles and compensation of energy deficiency. Surprisingly, and in contrast to this hypothesis, we found that Col6a1–/– muscles display a striking defect of the autophagic machinery, with impaired autophagosome formation.

The persistence of abnormal organelles and the ensuing apoptosis in collagen VI deficient muscles are caused by defective autophagy. Indeed, Col6a1–/– muscles display decreased LC3 lipidation and impaired autophagic flux, which was matched by the lack of autophagosomes after food starvation. This autophagic defect was paralleled by the lower induction of Beclin1 and Bnip3 and by the constitutive activation of the mTOR pathway during starvation. Notably, forced activation of autophagy by genetic, dietary and pharmacological approaches was able to restore myofiber survival and ameliorate the dystrophic phenotype of Col6a1–/– mice. Furthermore, analysis of muscle biopsies showed reduced levels of Beclin1 and Bnip3 in UCMD and BM patients.

These findings indicate that defective activation of the autophagic machinery plays a key pathogenic role in congenital muscular dystrophies linked to collagen VI deficiency.

Keywords: skeletal muscle, apoptosis, autophagy, muscular dystrophy

P-14 Hypothiocyanous acid is a selective inhibitor of caspase activation and apoptosis

Stephanie M. Bozonet (1), Amy Scott-Thomas (1), Péter Nagy (1) and Margreet C. M. Vissers (1)

(1) Free Radical Research Group, Pathology Department, University of Otago, Christchurch, New Zealand

Neutrophil myeloperoxidase (MPO) generates hypochlorous acid (HOCl) and hypobromous acid (HOBr) to combat microbial infection, but their over-production is implicated in the tissue injury associated with inflammation. Recent evidence suggests a major role for MPO-derived oxidants in the development of cardiovascular disease (CVD): MPO-derived oxidants can cause endothelial cell dysfunction and elevated MPO levels correlate negatively with prognosis in post-infarct patients.

The pseudohalide thiocyanate (SCN-) is readily oxidised by MPO to form hypothiocyanous acid (HOSCN), a mild oxidant that is exclusively reactive with thiol groups and considered to be harmless to mammalian cells. However, recent studies have shown HOSCN-mediated cell damage due to the specific targeting of intracellular thiols, as well as involvement in mitogen-activated protein kinase signalling, mediated through protein tyrosine phosphatases. We have investigated the effects of exposure of human umbilical vein endothelial cells (HUVEC) to HOSCN and have shown that low concentrations have dramatic and damaging effects on cell functional responses.

HOSCN reacts with HUVEC in a time- and concentration-dependent manner, and intracellular thiols are selectively targeted. Interestingly, although high concentrations caused cell death, HOSCN did not induce apoptosis at any level of exposure (measured by morphological changes, caspase activation, Annexin-V / Propidium Iodide (PI) uptake, and trypan blue staining). We found that the activity of the apoptotic caspases (3, 8 and 9), as well as the pro-inflammatory caspase 1, was inhibited with 50 µM HOSCN. At this concentration no other effects were seen, suggesting that caspases may be specific targets. This inhibition was observed in both in vivo cell studies and in vitro assays, and we additionally found that HOSCN prevented the induction of apoptosis by the DNA alkylating agent etoposide or by serum deprivation. Our data suggest that the enhanced survival of HUVEC exposed to HOSCN following induction of apoptosis could result in the survival of potentially damaged cells which would otherwise be removed.

We have demonstrated that HOSCN, although considered a relatively benign oxidant, can have detrimental effects on endothelial cell function, and may play an important role in the onset and/or development of CVD. In addition, the survival of HOSCN-exposed endothelial cells in the presence of apoptosis inducers suggests that HOSCN differs from other oxidants and that it could affect endothelial cell survival pathways to impact on vascular function.

Keywords: Cardiovascular disease, HOSCN, MPO, caspase, apoptosis

P-15 Role of Caspase-7 during early involution of the Escherichia coli- or the Staphylococcus aureus-infected mammary gland

BREYNE Koen (1,Ω), DEMON Dieter (1, Ω), VANDEN BERGHE Tom (2), VANDENABEELE Peter (2), and MEYER Evelyne (1)

(1) Laboratory of Biochemistry, Department of Pharmacology, Toxicology and Biochemistry, Ghent University, 9820 Merelbeke, Belgium, Koen.Breyne@Ugent.be
(2) Molecular Signaling and Cell Death Unit, Department for Molecular Biomedical Research, Technologiepark 927, 9052 Ghent, Belgium
(Ω) These authors contributed equally to this work

The lactating mammary gland plays an important role in providing nutrition and immunity to mammalian neonates. When the offspring is weaned, the lactating mammary gland returns to a compact and non-productive state through a process called involution. Mammary gland infection (mastitis) disturbs the normal post-lactation involutionary process. The pathogenesis of mastitis in humans and animals is insufficiently understood, resulting in excessive use of antibiotics in the dairy industry. Mammary gland involution and infection are both characterized by apoptosis and inflammatory signaling, two processes in which involvement of members of the Caspase family of proteases has been shown. Recent data demonstrates that executioner Caspase-7 not only has a function during apoptosis but is also involved in inflammatory signaling. Therefore, we evaluated the role of Caspase-7 in two acute mouse mastitis models.

Wild type (n=9) and Caspase-7-/- (n=9) mice were inoculated intramammarily with either gram-positive Staphylococcus aureus (S. aureus) (n=5) or gram-negative Escherichia coli (E. coli) (n=4) bacteria. Both pathogens are relevant mastitis isolates and were chosen because E. coli and S. aureus induce a different inflammatory response in mastitis and other infection models. In contrast to phosphate buffered saline-injected contralateral glands, bacterial inoculation with E. coli activates Caspase-7 in mammary glands of wild type mice 18 hours post-inoculation. Interestingly, next to the classical detected p20 fragment of active Caspase-7 we observed an additional fragment around 23 kDa with unknown function. Colony forming units (CFU), and local and systemic pro- and anti-inflammatory cytokines profiles (including IL-6, IL-1beta, TNF-α, MCP-1 and KC protein levels) do not differ significantly between intramammary E. coli-inoculated wild type and Caspase-7-/- mice.

While our data presents clear and specific activation of Caspase-7 upon early involution of the E. coli-infected versus control mammary gland, they suggest no critical role for Caspase-7 either in bacterial growth nor in local and systemic cytokine release during E. coli-induced intramammary infection. Preliminary data also demonstrate activation of the executioner Caspase-3 after intramammary infection of wild type mice with E. coli. Cytokine levels, CFU and Caspase-7 activation after inoculation with E. coli and S. aureus are currently analyzed in Caspase-3-/- mice.

Keywords: mammary gland infection, caspase-7, E. coli, S. aureus, apoptotic and inflammatory signaling

P-16 Thiazolide-induced apoptosis in colon cancer cells: role of GSTP1, Jun kinase and Bim

Daniel Sidler (1), Joachim Müller (2), Pietro Renzulli (3), Nadia Corazza (1), Andrew hemphill (2), Thomas Brunner (1)

(1)Division of Exp. Pathology, Institute of Pathology, University of Bern, Murtenstrasse 31, 3010 Bern, Switzerland
(2)Institute of Veterinary Parasitology, University of Bern, Längassstrasse,3012 Bern, Switzerland
(3) Cantonal Hospital, Münsterlingen, Switzerland

Thiazolides are antibiotics frequently used for the treatment of intestinal helminthes and protozoa infections, with no obvious side effects on host tissue. However, we recently found that thiazolides induce also apoptosis in human colon cancer cells. Using affinity chromatography we identified the glutathione-S-transferase P1 (GSTP1), a well know detoxifying enzyme, as the cellular target of thiazolides. While overexpression of GSTP1 sensitized colon cancer cells to thiazolide-induced apoptosis, downregulation by RNA interference rendered them more resistant. GSTP1 enzyme activity was required for sensitization as a GSTP1 mutant with defective enzymatic activity did not sensitize tumor cells to thiazolides. This finding suggests that thiazolides are prodrugs that require GSTP1 activity for conversion to their apoptosis promoting metabolites. Thiazolides induced a profound activation of all three MAP kinase pathways, but only Jun kinase and p38 activity were required for thiazolide-induced apoptosis. As the relevant downstream target of Jun kinase we identified the BH3-only molecule Bim. Bim expression was induced by thiazolides in a Jun kinase-dependent manner, and downregulation of Bim by siRNA resulted in inhibition of thiazolide-induced apoptosis. Activation of the mitochondrial apoptosis pathway was confirmed by analysis of cytochrome C and SMAC release, and prevention of thiazolide-induced apoptosis by overexpression of Bcl-2 or Mcl-1. Thiazolide also profoundly sensitized colon cancer cells to TRAIL and chemotherapeutic drugs. Analysis of gene expression revealed that GSTP1 is strongly overexpressed in colorectal tumors, compared to normal colonic tissue. Thus, GSTP1 represents an interesting therapeutic target, and thiazolides, in combination with conventional chemotherapy, may represent a novel treatment option for primary colon cancer.

Keywords: colon carcinoma, glutathion-S-transferase, Jun kinase, Bim, TRAIL, chemotherapy, mitochondria

P-17 A C-terminal domain of Bax inhibitor-1 displays Ca2+-channel pore properties

Geert Bultynck (1), Santeri Kiviluoto (1), Tim Vervliet (1), Tomas Luyten (1), Ilya Bezprozvanny (2), Volodomyr Rybalchenko (2), , Nadine Henke (3), Ludwig Missiaen (1), Jan B. Parys (1), Humbert De Smedt (1), Axel Methner (3)

(1) Lab. Molecular and Cellular Signaling, Dept. of Molecular Cell Biology, Campus Gasthuisberg O/N-1 bus 802, K.U. Leuven, Belgium
(2) Dept. of Physiology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390-9040, USA
(3) Research Group Protective Signaling, Department of Neurology, Heinrich Heine Universität Düsseldorf, Moorenstrasse 5, 40225 Düsseldorf, Germany

Bax inhibitor-1 (BI-1) is a highly conserved 6-7 transmembrane protein identified as a suppressor of Bax-induced cell death in yeast (Xu & Reed 1998). It localizes mostly into the ER membrane with both ends facing the cytoplasm. BI-1 is known to interact with the anti apoptotic proteins Bcl-2 and Bcl XL but not with Bax itself. BI-1 inhibits the accumulation of ROS and regulates the UPR by binding to IRE1α; (Lisbona et al 2009). In addition, there is evidence that BI-1-overexpressing cells have lower [Ca2+]ER. The exact mechanism by which BI-1 regulates intracellular Ca2+ homeostasis is still unresolved, but it seems to involve the C-terminus of BI-1 (Westphalen et al 2005).

We developed a set of peptides corresponding to a C-terminal domain of BI-1 (CTP1, CTP2 and CTP3), and analyzed their effects in unidirectional 45Ca2+ fluxes in permeabilized mouse embryonic fibroblasts (MEFs). This approach revealed that CTP2 is able to provoke Ca2+ release from the inositol 1,4,5-trisphosphate (IP3)-sensitive ER-Ca2+ stores with an EC50 of about 30 microM and an EC100 of 80 microM. Similar responses were observed in BI-1-/- MEFs and in DT40 cells lacking all three IP3 receptor (IP3R) isoforms, indicating that the CTP2 induced Ca2+ release is independent of both BI-1 and IP3Rs. Secondary-structure predictions (I-TASSER) suggested that CTP2 forms an α;-helical structure, which can be disrupted by targeted Gly mutations. With these Gly mutants, we were able to abolish the CTP2 induced Ca2+ release. Also, using artificial lipid bilayers, we showed that the CTP2 peptide is able to form Ca2+ pores independently of any other Ca2+-release mechanism. Specific Ala mutants of CTP2 peptide did not provoke Ca2+ release, leading to the identification of critical residues involved in the channel-properties of the peptide. Finally, we investigated the properties of CTP2 peptides derived from the BI-1 of lower organisms. The analysis revealed that CTP2 peptides derived from insects to mammals were able to promote Ca2+ release, whereas peptides from plant and yeast orthologs were not. This suggests that BI-1 has a specific function in Ca2+ signaling that developed during evolution.

To conclude, our results indicate that the C-terminal part of BI-1 has a potency of Ca2+ channel pore formation provoking a Ca2+ leak from the ER.

Keywords: intracellular Ca2+ homeostasis, endoplasmic reticulum, ER stress, Bcl-2-interacting proteins

P-18 Secondary targets of multitarget antifolate in human melanoma cell lines

Buque A., Calvo B., Aresti U., Jangi Sh., Muñoz A., Carrera S., Sancho A., Rubio I., Lopez-Vivanco G.

Medical Oncology Research Unit, Cruces Hospital, Plaza de Cruces s/n, Edif. Anatomia Patologica 2ªplanta, 48903 Barakaldo, Bizkaia, Spain

Introduction: Pemetrexed (MTA) is a potent multitarget antifolate which inhibits de novo synthesis of purines and pyrimidines. Once inside the cell, MTA is polyglutamated and this modified form of MTA cannot leave the cell increasing the half-live of the treatment and allowing accumulation of very high doses of intracellular MTA. Its primary target is thymidylate synthase (TS), but the high intracellular levels of polyglutamated MTA enable the inhibition of other secondary enzymes also involved in de novo synthesis of purines and pyrimidines, such as glycinamide ribonucleotide transformylase (GART) and dihydrofolate reductase (DHFR).

The Aim of this study was to evaluate the effect of MTA on secondary targets on human melanoma cell lines.

Methods: As previously described we assessed the effect of MTA on cell viability after addition to the cell culture medium described enzyme substrates.

Results: Our results showed that addition of thymidine and hypoxanthine clearly inhibited MTA-induced cell death in all employed cell lines, in agreement with previous literature which describes TS as the primary target of MTA. But the determination of aminoimidazolecarboxamide ribonucleotide formyltransferase (AICART) or GARFT as a secondary target was not homogeneous. It is described that addition of AICA and thymidine in human lymphoblastic leukaemia cells did not reverse, even enhanced, the effect of MTA, indicating that the secondary target was AICART. But in our case, there were different responses to the assay, depending on the cell line used. Thus, A375, MeWo and Hs294T showed a significant reduction in the MTA induced cell death, that is, the secondary target is GART but that is not the case of HT144 or Calu-3, where addition of AICA and thymidine did not induce any reversal effect, this is, AICART would be the secondary target.

Conclusion: Regarding those results we hypothesize that MTA secondary targets should change depending on the genetic background of the parental tumor.

Keywords: melanoma, multitarget antifolate, thymidylate synthase

P-19 CHOP contributes to cytokine/ER-stress induced beta cell apoptosis via decreased Bcl-2 and Mcl-1 expression

Cardozo AK (1), Eizirik DL (1), Allagnat F(1)

(1) Laboratory of Experimental Medicine, Université Libre de Bruxelles, Route de Lennik 808, 1070 Brussels, Belgium

Background and Aims: Pro-inflammatory cytokines are early mediators of beta cell death in type 1 diabetes mellitus. Recent studies have shown that cytokines, via nitric oxide production, deplete endoplasmic reticulum (ER) Ca2+ stores, leading to ER stress and beta cell apoptosis. These events correlate with overexpression of the pro-apoptotic C/EBP Homologous Protein (CHOP). However, whether CHOP overexpression is involved in cytokine-induced beta cell apoptosis remains controversial.

Materials and Methods: CHOP was knocked-down using RNA interference in INS-1E cells and FACS purified rat primary beta cells. Beta-cell function, survival, mRNA and protein expression in presence of the pro-inflammatory cytokines IL-1β, IFN-γ and TNF-α, as well as the chemical ER stress inducer cyclopiazonic acid (CPA) were analyzed.

Results: CHOP knock-down using two different siRNAs efficiently decreased CHOP basal expression and fully prevented cytokine- or CPA-induced mRNA and protein overexpression. The expression GADD34, a target gene for CHOP, was abolished in cells transfected with the CHOP siRNA. However, CHOP knock-down did not influence the expression of other ER stress markers such as BiP and spliced XBP-1. Knocking-down CHOP did no affect insulin content and either basal or stimulated insulin secretion. Importantly, CHOP knock-down decreased caspase 9 and 3 activation and partially prevented apoptosis induced by IL-1β+INF-γ, TNF-α+INF-γ and CPA in INS-1E and FACS purified primary beta cells. The expression of the pro-apoptotic proteins Bax and Bak was not modified by CHOP. On the other hand, cytokines and CPA decreased the protein level, but not the mRNA level, of the anti-apoptotic proteins Bcl-2 and Mcl-1 and this was prevented in CHOP knocked-down cells.

Conclusion: Our findings indicate that CHOP is involved in cytokine/ER stress-induced beta cell apoptosis. We propose that CHOP is responsible for the increased degradation of Bcl-2 and Mcl-1 by cytokines and CPA by a mechanism that remains to be unraveled.

Keywords: Diabetes, CHOP, Mcl-1, Bcl-2, pancreatic beta cells, cytokines, endoplasmic reticulum stress

P-20 Mitochondrial DNA depletion sensitizes L929 cells to death receptor-mediated cell death

Elena Catalán1, Juan Ignacio Aguiló1, José Antonio Enríquez1,2, Javier Naval1, Alberto Anel1 and Julián Pardo1,3

1 Department of Biochemistry, Molecular and Cell Biology, Faculty of Sciences, University of Zaragoza, 50009 Zaragoza (Spain).
2, CNIC, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain
3, ARAID, Fundación Aragón I+D, Zaragoza, Spain

Citotoxic T Lymphocytes (CTL) and Natural Killer cells (NK) are key components of the host inmune sistem against viruses and transformed cells. To cause cell death in target cells, both CTL and NK can release citotoxic granules containing granzymes and perforin. Granzyme B (gzmB), released in the target cell cytosol by perforin (perf) action, is able to generate reactives oxigen species (ROS), a hallmark in some pro-apoptotic processes and cell death. We have generated EL4 cells that lack mitochondrial DNA (EL4Rhozero) to find out the intracellular source of ROS during gzmB-mediated cell death. These cells do not perform mitochondrial respiration and are unable to produce ROS in that organelle. By using ex vivo virus specific Tc cells or purified gzmB we showed that gzmB is able to induce cell death in EL4-Rhozero cells and also the production of ROS from an extramitochondrial source involving NADPH oxidases. In an attempt to extrapolate our results to other cell lines, we have generated L929rho0 cells to analyse cell death induced by ex vivo NK cells. Surprisingly, we have found that L929rho0 cells are more susceptible than parental L929 cells to cell death induced by gzmA deficient ex vivo NK cells. Perf or perfxgzmAxB deficient NK cells were still able to kill L929rho0. Further analyses showed that L929rho0 cells were very susceptible to apoptosis induced by a cytotoxic anti-Fas antibody. To elucidate the molecular bases of this phenomenon, we are currently analysing how the absence of mtDNA affects the expression of molecules involved in death receptor-mediated apoptosis. Our results seems to indicate that specific tumor cells lines strictly dependent on glycolysis for their survival maybe more susceptible to death receptor-induced cell death.

Keywords: Citotoxic, mitochondria, granzyme B.

P-21 Reelin and sex hormones interactions in the pathological apoptosis during cerebellum development

Virve Cavallucci (1), Filippo Biamonte (2), Ramona Marino (2), Francesco Cecconi (1), Marcello D'Amelio (1,3) and Flavio Keller (2)

(1) Lab. Molecular Neuroembriology, Neuroscience, IRCCS, Fondazione Santa Lucia, Via del Fosso di Fiorano, 64, 00143 Rome, Italy; (2) Lab. Developmental Neuroscience and Neural Plasticity, University Campus Bio-Medico, Via Alvaro del Portillo 21, 00128 Rome, Italy (3) Lab. Molecular Neuroscience, University Campus Bio-Medico, Via Alvaro del Portillo 21, 00128 Rome, Italy.

Cell death in the developing nervous system has been recognized for almost one hundred years; Its potential significance for physiological brain morphogenesis was proposed approximately 80 years ago. Emerging evidence points to apoptotic mechanisms being involved in certain neuropsychiatric disorders, including autism spectrum disorders (ASD). ASD is a male-prevalent heterogeneous group of developmental disorders characterized by deficits of social communication, language and repetitive behaviours. Due to its highly heterogeneous genetic aetiology, researchers have incorporated intermediate phenotypes (endophenotypes) and environmental factors, into genetic analyses. In association with social, linguistic and perseverative behavioural deficits, several ASD patients display dysfunctional neuroanatomical pathways involving the cerebellum.

The stable ASD male prevalence may stem from gene x environment interactions. Furthermore, prenatal and/or early postnatal exposure to excessive testosterone appears to increase the likelihood of developing autism-like symptoms and to explain the increased male-to-female ratio (4:1).

Neurohistological findings have shown Purkinje cell (PC) depletion and atrophy in the cerebellum of autistic subjects. More recently, it has been described a loss of PC that affects only male heterozygous reeler mice, which recapitulate several neurodevelopmental defects similar to those observed in ASD.

We have evaluated whether physiological apoptosis would be altered specifically in the cerebellum of male heterozygous reeler mice. The results gathered from this study should shed light on the biological mechanism that might affect PC survival, thus improving our understanding of the developmental processes that shape the complex cerebellar circuits.

Keywords: autism, cerebellum, neurodevelopment, sex hormones, cell death

P-22 Anti-apoptotic effects of synthetic COX-2 inhibitors

Claudia Cerella (1), Cyril Sobolewski (1), Sebastien Chateauvieux (1), Michael Schnekenburger (1), Mario Dicato (2), and Marc Diederich (1)

(1) Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg, Luxembourg, Luxembourg.
(2) Department of Hematology-Oncology, Centre Hospitalier, Luxembourg, Luxembourg.

Cyclooxygenase-2 (COX-2) is a pro-inflammatory immediate early response gene, found stably expressed in a number of adherent cancers, where a causative role in tumor promotion as well as in determining chemotherapy failure has been suggested. Consequently, the use of preferential/selective COX-2 inhibitors in combination with traditional chemotherapeutic agents has been taken into account as a strategy to potentiate cell death in COX-2 expressing cancer cells. More recently, a stable expression of COX-2 was described in hematological malignancies, where similar pro-carcinogenic effects have been hypothesized.

In this study, we analyze the role of synthetic COX-2 enzyme inhibitors (nimesulide, Ns-398 and celecoxib) in survival/apoptosis on hematological cancer models. We found that COX-2 inhibitors, unexpectedly, strongly prevent apoptosis induced by a panel of chemotherapeutic agents on a selected COX-2-positive acute myeloid leukemia model. The data collected are consistent with a prevention of the apoptotic signaling at very early steps, prior to Bax/Bax activation.

Here, we provide evidence that this modulatory role is due to two converging anti-apoptotic mechanisms, one affecting the correct intracellular accumulation of chemotherapeutic drugs and the other hampering the activation of JNK signaling pathway. Accordingly, COX-2 inhibitors enhance drug efflux, as assessed by rhodamine 1,2,3 and doxorubicin efflux assays, and attenuate chemotherapeutic-induced DNA damage on one side. Concomitantly, they up-regulate the glutathione-S-transferase isoform GST-PI and prevent JNK signaling pathway activation, absolutely required for the cytotoxicity of the chemotherapeutic agents we tested, on the other side. Consequently, COX-2 inhibitors do not exert any anti-apoptotic potential when the cells were challenged with physiological stimuli (i.e., with TNFα and FAS) as well as by the stressing agent hydrogen peroxide, both neither targeted by multidrug channels nor affected by cellular GST levels.

Our findings show a novel paradoxical anti-apoptotic effect of COX-2 inhibitors on hematopoietic COX-2-positive cancer cells and recommend caution in the use of anti-inflammatory agents as chemoadjuvant as well as in counteracting side adverse effects of chemotherapy during the treatment.

Keywords: COX-2, chemoresistance, drug efflux, Jun kinase

P-23 Substitutions in the Dimer Interface of (Pro)caspase-3 Affect Protein Structure and Function

A. Clay Clark (1), Denise Appel (1), Joe Maciag (1)

(1)Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, NC, USA

As the terminal protease in apoptosis, procaspase-3 is a potentially important therapeutic target in the treatment of cancer and autoimmune disorders. The dimer interface of procaspase-3 contains allosteric sites that affect conformational exchange between an active conformation and an inactive conformation. We have shown that a mutation of V266E in the interface activates the zymogen by stabilizing the active conformation. The activated procaspase-3 efficiently induces apoptosis in several cell lines, does not require cleavage of the polypeptide chain, and is not inhibited efficiently by XIAP. The data show that, in principle, one can induce apoptosis by stabilizing an active conformation of procaspase-3. In contrast, a mutation of V266H abolishes activity in (pro)caspase-3, and structural studies demonstrate that the mutation destabilizes an active site loop via a network of amino acids on the protein surface. By replacing V266 with all other amino acids, we show that the activity of (pro)caspase-3 can be modulated between the active and inactive forms. Structural studies of several mutants further establish the allosteric network of amino acids between the dimer interface and the active site. An understanding of the allosteric network may be useful in small molecule drug discovery assays for targeting the conformations of procaspase-3 in order to activate or inhibit the enzyme. This work was supported by NIH grant GM065970.

Keywords: (pro)caspase-3, activation, inhibition, apoptosis

P-24 Role of the silencing of genes that encode for HDAC1 and HDAC2 in the cell line of acute myeloid leukaemia U937

Mariarosaria Conte(1), Dell'Aversana Carmela(1,2), GianLuigi Franci(1), Angela Nebbioso(1) & Lucia Altucci(1)

(1)dep.of general pathology, second university of naples, vico l. de crecchio 7, 80138, naples, it.
(2)dep.of sperimental pathology and microbiology university of messina-consolare valeria, 1 - 98125 messina

Epigenetic alterations are involved in cancer progression. In particular, histone acetylation and deacetylation have a very important role in the modification of chromatin structure and in the regulation of gene expression. Protein acetylation level is regulated by two classes of enzymes: histone acetyl-transferases (HATs) and histone deacetylase (HDACs). Different tumors are associated to alteration between HAT/HDAC activity: this also applies to acute myeloid leukaemias (AML). HDACs are part of multi-subunit complexes involved in the regulation of cell cycle progression and apoptosis. Their function is mainly applied to the repression of transcription. An HDAC aberrant expression is associated to different human tumours. HDACs are also involved in mediating the function of oncogenic traslocation product in specific forms of leukaemia and lymphomas.

For all these reasons HDAC inhibitors (HDACis) are object of biomedical interest given that by inhibiting the HDAC action, silenced genes in tumours can be re-expressed leading to growth arrest and apoptosis.

Our study has verified the effect of the silencing HDAC1 and HDAC2 in haematological cancer cells. Our data, obtained by transcriptome and global miRNA analyses, indicate that silencing of HDAC1 and 2 in acute myeloid leukemias only partially recapitulate HDAC inhibition, suggesting that these targets display also functions different from the enzymatic one. These data will be further analysed and discussed.

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Keywords: Epigenetic, cancer, HDAC, knock-down

P-25 Cathepsin D induces proteolysis and activation of caspase-8 during neutrophil apoptosis

Sébastien Conus (1), Cristina Pop (2), Scott Snipas (2), Hans-Uwe Simon (1), Guy Salvesen (2)

(1)Institute of Pharmacology, University of Bern, Switzerland.
(2) Program in Apoptosis and Cell Death Research, Sanford-Burnham Institute for Medical Research, La Jolla, California, USA.

Although it has already been suggested that cathepsins may act in concert with caspases,

our finding that, in neutrophils, cathepsin D acts proximal to caspases and activates directly the initiator caspase-8 was unexpected (1). Most current data supports the hypothesis that caspase-8 is activated by dimerization, not proteolysis – reviewed in (2, 3), although some authors dispute this – reviewed in (4). Interchain proteolysis, but not enforced dimerization of caspase-8 by Fas-associated protein with death domain (FADD), was proposed for its activation by granzyme B or caspase-6 (5, 6). Therefore, proteolytically processing of caspase-8 by cathepsin D might influence its activation.

We report here a new activation mechanism of caspase-8 in which, during neutrophil apoptosis, cathepsin D induces activation of the initiator caspase-8 by intra-chain proteolysis. At acidic pH, cathepsin D cleaved the recombinant human caspase-8 protein at several sites and significantly increased its activity. This increased activity was completely blocked by the pharmacological inhibitor of cathepsin D, pepstatin A. Under non-dimerizing conditions, the caspase-8 fragment generated by incubation of the recombinant human caspase-8 protein with cathepsin D could be affinity labelled with the biotinylated caspase substrate VAD-fmk arguing that the 21-kD fragment of caspase-8 is enzymatically active. When cathepsin D was incubated together with the recombinant human proteins of the initiator caspase-9 and -10, no significant increase of their activities was detected suggesting that cathepsin D could selectively activate the initiator caspase-8, but not the initiator caspase-9 and -10. In an in vitro cell-free assay using cytosolic extracts of freshly isolated blood neutrophils, we observed that the addition of recombinant human caspase-8 protein incubated with cathepsin D was followed by the activation of caspase-3 and consequently the induction of apoptosis. Thus, we demonstrated that cathepsin D is able to launch neutrophil apoptosis by directly and selectively inducing the intra-chain proteolysis of the initiator caspase-8.

Keywords: Apoptosis, caspase-8, cathepsin, lysosome, neutrophil

P-26 Role of the Bcl-2 homolog Bim in T cell-mediated liver immunopathology.

Christoph Lauer (1), Thomas Kaufmann (2),Thomas Brunner (1), Nadia Corazza (1)

(1) Institute of pathology, University of Bern, Murtenstrasse 31,3010 Bern, Switzerland
(2) Institute of Pharmacology, University of Bern, Friedbuehlstrasse 49,3010 Bern, Switzerland

The liver is known to be a classical immune privileged site with a relatively high resistance against immune cell-mediated damage. However, during liver virus infections this immune privileged situation is broken, and activated lymphocytes are involved not only in the control of the infections and the elimination of virus-infected cells, but also in the induction of tissue injuries. The pro-apoptotic BH-3 only protein Bim has been shown to play a central role in both lymphoid homeostasis after viral infection as well as in the regulation of hepatocyte apoptosis. Nevertheless, the respective roles of Bim in the control of T cell retraction after viral infection on one hand and the induction of tissue damage on the other hand during the pathogenesis of T cell mediated liver diseases are currently far from being understood. In this study we demonstrated that Bim plays a central role in the development of lymphocytic choriomeningitis virus (LCMV-WE) induced liver disease. While Bim-/- mice showed equal numbers of virus specific CD8+ T cells and similar virus titers in their livers compared to wild type animals, the analysis of liver damage after LCMV infection clearly demonstrated that Bim-/- mice are partially protected from the development of T cell mediated liver disease. These results suggest that Bim plays a critical role in T cell mediated liver disease. Still these experiments do not completely prove that the outcome of liver disease depends on the involvement of Bim in hepatocyte apoptosis and not on the role of Bim in T cell activation and/ or retraction phase after virus infection.

To specifically define the role of Bim in hepatocytes apoptosis after T cell activation, we generated bone marrow chimeras. Consequently bone marrow from WT mice was transferred into WT (WT→WT) or Bim-/- (WT→Bim-/-) mice and after 8 weeks the mice were infected with LCMV-WE. WT→Bim-/- mice exhibited reduced liver damage compared to WT→WT mice, demonstrating that Bim expressed in the hepatocytes contributes to hepatocyte cell death and to the severity of liver disease. As expected WT→WT and WT→Bim-/- chimeras showed equal numbers of virus specific CD8+ T cells and similar virus titers in their livers. Taken together these results suggest that Bim expressed in the hepatocytes represents an important apoptosis mediator during T cell induced liver damage.

Keywords: Bim, Liver, Virus infection, T cells

P-27 PKC delta associates with XIAP and Smac in breast cancer cells

Louise Cornmark (1), Katarzyna Chmielarska Masoumi (1), Christer Larsson (1)

(1) Department of Laboratory Medicine, Lund University, Entrance 78, 3rd floor, Malmö University Hospital, SE-205 02 Malmö, Sweden

Background: Protein kinase C (PKC) is a family of serine/threonine kinases consisting of 10 isozymes whose specific functions are not all yet established. The PKC isoforms have for a long time been known to influence cellular functions of importance for malignancy and they can have both pro- and anti-apoptotic effects. Second mitochondria-derived activator of caspases (Smac), released from the mitochondria binding to inhibitors of apoptosis protein (IAP), is a pro-apoptotic protein. The function of Smac and inhibition of IAPs is intensely studied with the aim to modulate cancer cell survival. Unpublished data from our lab show that PKC delta associates with Smac. We show here that PKC delta also associates with X-linked IAP (XIAP) and that XIAP might be necessary for the PKC delta-Smac association.

Aim: To investigate the role of XIAP for the PKC delta-Smac association.

Materials & Methods: Breast cancer cell lines were analyzed for protein interaction by immunoprecipitation followed by Western blot analysis. Proteins were downregulated with siRNA.

Results: Immunoprecipitation of PKC delta demonstrated interactions with both Smac and XIAP. Our results also show that XIAP co-precipitates Smac. Preliminary results indicate that following downregulation of XIAP, the interaction between PKC delta and Smac is suppressed.

Conclusions: Our results indicate that PKC delta interacts with both Smac and XIAP in breast cancer cells and that the interaction with Smac may be XIAP-dependent. These findings can be of importance in designing drugs targeting IAPs for cancer treatment.

Keywords: PKC delta, XIAP, Smac, breast cancer

P-28 Inhibition of inositol monophosphatase by lithium chloride induces selective macrophage apoptosis in atherosclerotic plaques

Inge De Meyer, Wim Martinet, Cor E. Van Hove, Dorien M. Schrijvers, Paul Fransen, Hidde Bult and Guido R.Y. De Meyer

Division of Pharmacology, University of Antwerp, 2610 Wilrijk, Belgium

Macrophages play a key role in atherosclerotic plaque destabilization and rupture, whereas smooth muscle cells (SMCs) contribute to plaque stability. Selective depletion of macrophages from plaques may be a promising strategy to stabilize the structure of the plaque. Lithium chloride (LiCl) has been shown to induce cell death of cultured macrophages. Here, we investigated whether LiCl could induce macrophage death without affecting SMCs. In vitro studies showed that 30 mM LiCl led to cell death of cultured J774A.1 macrophages and primary macrophages, whereas SMCs were highly resistant. Cell death was characterized by externalization of phosphatidylserine, caspase-3 cleaving and DNA fragmentation, all indicative of apoptosis. Although LiCl can exert osmotic effects, this was only seen from 100 mM onward, similar to 100 mM NaCl. We then investigated two possible mechanisms of LiCl-induced macrophage apoptosis: inhibition of glycogen synthase kinase (GSK)-3beta and inositol monophosphatase (IMPase). Neither GSK-3beta inhibitor SB216763 nor GSK-3beta gene silencing induced macrophage death. However, LiCl reduced myo-inositol-1,4,5-triphosphate levels in macrophages but not in SMCs. Moreover, the specific IMPase inhibitor L-690,330 as well as IMPase gene silencing induced macrophage apoptosis, similar to LiCl. These findings strongly indicated that LiCl-induced cell death was mediated by IMPase inhibition and not by GSK-3beta blockage. In vitro treatment of atherosclerotic rabbit carotid artery rings with 30 mM LiCl resulted in induction of macrophage death, whereas SMCs were unaffected. Local in vivo administration of LiCl via osmotic minipumps to collared rabbit atherosclerotic carotid arteries reduced the macrophage content in the plaques through apoptosis, as shown by TUNEL. The contractile and relaxing responses of atherosclerotic carotid arteries were unaltered after LiCl-treatment. In conclusion, LiCl selectively decreased the macrophage load in rabbit atherosclerotic plaques without affecting SMC viability and functionality.

Keywords: atherosclerosis, apoptosis, macrophage

P-29 Human and mouse granzyme M display divergent and species-specific substrate specificities

S.A.H. de Poot(1), M. Westgeest (1), D.R. Hostetter (2), R. Broekhuizen (1), C.S. Craik (2), N. Bovenschen (1)

(1) Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands
(2) Department of Pharmaceutical Chemistry, University of California, San Francisco, USA

An important mechanism of self-defense against tumor and virus-infected cells is formed by the granule-exocytosis pathway, in which cytotoxic lymphocytes release a set of serine proteases known as granzymes. Human granzyme M (hGrM) is a potent inducer of tumor cell death and cleaves at least α-tubulin and nucleophosmin (NPM). Although GrM of human and mice display extensive homology, the substrate specificity of mouse GrM (mGrM) remains unknown. Here, we show that GrM from both species efficiently triggered apoptosis in tumor cells. However, mGrM preferred to cleave after a Met residue at the P1, whereas hGrM displays a higher preference for Leu at this position. Murine GrM was about 4.5-fold less efficient in cleaving the optimal hGrM chromogenic substrate suc-KVPL-pNA. Both mGrM and hGrM cleaved α-tubulin with similar kinetics. Strikingly, whereas both mGrM and hGrM cleaved human NPM, neither could hydrolyze mouse NPM. While the P1-P4 residues of the GrM cleavage site in human NPM are conserved in mouse NPM, the P1'-P2' residues differ. Replacement of the putative P1' and P2' residues in mouse NPM with the corresponding residues of human NPM completely restored cleavage of mouse NPM by both mGrM and hGrM. In a proteomic approach, mGrM and hGrM displayed highly restricted macromolecular substrate specificities that overlapped only partially. These data indicate that human and murine GrM not only exhibit divergent substrate specificities, but may also trigger species-specific cell death pathways.

Keywords: granzymes, cancer, apoptosis

P-30 Apoptosis imaging with 68Ga-labelled AnnexinA5 derivatives for early evaluation of cancer therapy

Marijke De Saint-Hubert (1), Matthias Bauwens (2), Ellen Devos (1), Niko Deckers (3), Chris Reutelingsperger (3), Alfons Verbruggen (2), Luc Mortelmans (1), Felix Mottaghy (1,4)

(1) Nuclear Medicine/MoSAIC, KUleuven, Herestraat 49, 3000 Leuven, Belgium.
(2) Radiopharmacy, KUleuven, Herestraat 49, 3000 Leuven, Belgium.
(3) Biochemistry, Maastricht University, PO Box 616, 6200 Maastricht, The Netherlands
(4) Nuklearmedizin, RWTH Aachen, Pauwelsstr 30, 52057 Aachen, Germany

Introduction: AnnexinA5 (AnxA5) binds with high affinity to phosphatidylserine (PS) which is externalized by apoptotic cells to be recognized and removed by macrophages. As a result AnxA5 has become a standard for in vitro detection of apoptosis and has extensively been studied for in vivo imaging of apoptosis. Due to suboptimal imaging quality of 99mTc labelled AnxA5 we aimed to specifically label AnxA5 with an emerging radioisotope, 68Ga, allowing Positron Emission Tomography (PET).

Methodology: We radiolabelled AnxA5 with 68Ga using two mutated forms of AnxA5 with a single cysteine residue at position 2 or 165 (Cys2-AnxA5 and Cys165-AnxA5). This allowed site-specific coupling with 68Ga-Dota-maleimide at 37°C for 15 minutes. In vitro binding to apoptotic cells was tested in anti-Fas treated Jurkat cells and compared to healthy cells. The distribution throughout the body and pharmacokinetics were studied with µPET and quantified ex vivo following dissection. This was done for healthy mice and mice with hepatic apoptosis (anti-Fas mAb treated) up to 60 min p.i.. Finally a tumour model was used to study imaging of treatment induced apoptosis. Lymphoma xenograft mice (Daudi cells) were scanned before and 1 day after treatment with combined chemotherapy (125 mg/g Cyclophosphamide) and radiotherapy (10 Gy/tumour) using µPET and µMRI. The tracer uptake in the tumour was measured and visualized ex vivo using autoradiography. The uptake was correlated to histological evidence of apoptosis (TUNEL).

Results: 68Ga-Dota-maleimide labelling yield was more than 98% and coupling yield of 68Ga-Dota-maleimide to Cys2-AnxA5 and Cys165-AnxA5 was around 70%. Labelling and purification took about 60 min, with a final radiochemical purity of at least 98%. In vitro binding of 68Ga-Cys2-AnxA5 and 68Ga-Cys165-AnxA5 to anti-Fas treated tumour cells was 5 times higher compared to normal cells. This confirmed that the coupled AnxA5 preserved its ability to bind externalized PS. In vivo we noticed a fast clearance from the blood towards the kidneys for both tracers. There was no significant change in biodistribution from 30 min p.i. on. Dissection data confirmed that the clearance was mainly via the urinary tract. Dynamic PET images following anti-Fas treatment depicted an elevated tracer uptake in the apoptotic liver for both tracers. Compared to normal mice anti-Fas treated livers showed a 7 to 9 times higher uptake (for respectively 68Ga-Cys2-AnxA5 and 68Ga-Cys165-AnxA5) as compared to healthy animals. Autoradiography images confirm the higher uptake in anti-Fas treated livers, corresponding to TUNEL positive cells. MRI-PET fusion images allowed clear delineation of each tumor. Using this technique, we observed that the tumour standardized uptake values (SUVs) post-therapy significantly exceeded those prior to therapy. The absolute tumour uptake of 68Ga-Cys2-AnxA5 and 68Ga-Cys165-AnxA5 was respectively only 0.5 ± 0.1 % ID/g and 1.0 ± 0.3 % ID/g and significantly increased to 1.5 ± 0.2 % ID/g and 1.6 ± 0.1 % ID/g after therapy. Autoradiography showed a heterogeneous tumor uptake of the tracers. Several regions displayed 10-20 times more uptake compared to the tumor uptake prior to treatment while other regions were found relatively unaffected. The same heterogeneous distribution of apoptosis was observed with TUNEL stainings.

Conclusion: 68Ga-Cys2-AnxA5 and 68Ga-Cys165-AnxA5 were prepared with a high yield and within a reasonable time period. Satisfying apoptosis targeting capacity was demonstrated in a model of hepatic apoptosis. PET-MRI fusion images revealed a significantly higher tumor uptake after cancer therapy, indicating that 68Ga-anxA5 may be useful for the early evaluation of tumor therapy.

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Keywords: molecular imaging, AnnexinA5, cancer

P-31 VDAC1 selectively transfers apoptotic Ca2+ signals to mitochondria

Diego De Stefani (1), Anna Romagnoli (1) , Angela Bononi (2), Paolo Pinton (2) and Rosario Rizzuto (1)

(1)Department of Biomedical Sciences, University of Padua, Via G. Colombo 3, 35131 Padua, Italy
(2)Department of Experimental and Diagnostic Medicine, Section of General Pathology, Interdisciplinary Center for the Study of Inflammation (ICSI) and Emilia Romagna Laboratory BioPharmaNet, University of Ferrara, Via Borsari 46, 44100 Ferrara, Italy

Voltage-Dependent Anion Channels (VDACs) are the most abundant proteins of the outer mitochondrial membrane. Despite the high homology in sequence among the three existing mammalian isoforms, several data suggest an isoform-specific role of VDAC in several cellular processes, in particular in determining cellular apoptotic sensitivity. Indeed here we demonstrate that VDAC1 acts as a pro-apoptotic protein, while VDAC2 protects cells from death stimuli and VDAC3 doesn't significantly modify cellular sensitivity to apoptosis triggering. Given the central role of mitochondrial Ca2+ signals in the regulation of apoptosis we first investigated the specific role of the three VDAC isoforms on cellular Ca2+ homeostasis, showing no differences in Ca2+ responses to classical IP3-mobilizing agents, thus indicating that the differential effect on cell death is not due to differences in Ca2+ channeling properties among the isoforms. On the contrary, mitochondrial signals induced by Ca2+-dependent apoptotic agents such as H2O2 are strikingly affected by the selective knockdown of VDAC1 but not of VDAC2 or VDAC3. This is explained by the presence of an isoform-specific coupling of endoplasmic reticulum and mitochondrial Ca2+ channels, with VDAC1 acting as the only isoform interacting with the IP3Rs. Moreover, we demonstrate that apoptotic stimuli strengthen the association of the complex, thus posing the structural basis of the VDAC1 selective role in apoptosis. All these data indicate the existence of a preferential signaling route transferring Ca2+-dependent apoptotic stimuli from one organelle to the other.

Keywords: mitochondria, calcium, apoptosis, VDAC

P-32 Caspase-14 is required for filaggrin degradation to natural moisturizing factors in the skin

Esther Hoste,(1,2), Patrick Kemperman (3), Michael Devos (1,2), Geertrui Denecker (1,2), Sanja Kezic (4), Nico Yau (4), Barbara Gilbert (1,2), Saskia Lippens (1,2), Petra Van Damme (5), Kris Gevaert (5), Richard B. Presland (6), Gerwin Puppels (3,7), Peter Caspers (3,7), Peter Vandenabeele (1,2) and Wim Declercq (1,2)

(1)Molecular Signaling and Cell Death Unit, Department for Molecular Biomedical Research, VIB, Ghent, Belgium.
(2) Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium.
(3) Department of Dermatology and Venereology, Erasmus MC, Rotterdam, The Netherlands.
(4) Coronel Institute of Occupational Health, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands.
(5) Department of Medical Protein Research, VIB-Ghent University, Ghent, Belgium.
(6) Departments of Oral Biology and Medicine (Dermatology), University of Washington, Seattle, WA, USA.
(7) River Diagnostics BV, Rotterdam, The Netherlands.

Caspase-14 is mainly expressed in suprabasal epidermal layers and activated during keratinocyte cornification. Caspase-14 deficient mice display a reduced epidermal barrier function and an increased UVB radiation sensitivity. We found that although profilaggrin, a protein with a pivotal role in skin barrier function, is processed correctly to its functional filaggrin monomeric unit in caspase-14-/- mice, these mice accumulate proteolytic filaggrin fragments in the epidermis. We show here that the accumulation of these filaggrin fragments is due to a defect in filaggrin degradation in the cornified layers of caspase-14-/- skin. Consequently, this lack of normal filaggrin degradation results in a significant reduction of the natural moisturizing factors (NMF) levels, such as urocanic acid and pyrollidone carboxylic acid, and skin hydration in caspase-14 deficient mice as compared to wild-type mice. In addition, we demonstrate that caspase-14 can directly cleave the filaggrin monomer. Taken together, our data identify caspase-14 as a crucial protease in filaggrin catabolism.

Keywords: caspase, skin, knock-out, non-apoptotic funtion

P-33 Regulation of inositol 1,4,5-trisphosphate receptors and ER Ca2+ stores during nutrient starvation-induced autophagy

Decuypere Jean-Paul (1), Luyten Tomas (1), Ponsaerts Raf (1), Missiaen Ludwig (1), De Smedt Humbert (1), Parys Jan B. (1), Bultynck Geert (1)

(1) Dept. of Molecular Cell Biology, Campus Gasthuisberg O&N 1, bus 802, K.U.Leuven, 3000 Leuven, Belgium

Autophagy is a conserved lysosomal degradation pathway. During development and in response to stress conditions, it is involved in the clearing of long-lived proteins and other macromolecules, protein aggregates, damaged organelles and even pathogens. Macroautophagy involves the formation of double-membranous vesicles, autophagosomes, in which the cargo is enclosed. These vesicles subsequently fuse with lysosomes after which the enclosed material is degraded.

Although subject of several studies, the role of intracellular Ca2+ in autophagy remains controversial: the inhibition of Ca2+ channels in the endoplasmic reticulum (ER) or in the plasma membrane (e.g. ref. 1) as well as intracellular [Ca2+] elevations (e.g. ref. 2) stimulate autophagy. From these findings it is clear that there is a regulatory, but still unclear role for the inositol 1,4,5-trisphosphate (IP3) receptor (IP3R), a ubiquitous ER-resident Ca2+-release channel (e.g. ref. 3). All these studies involve the manipulation of the intracellular Ca2+ machinery, but a correlation between intracellular Ca2+ dynamics and autophagy has never been documented.

In the present study, we show that following changes in intracellular Ca2+ signaling occur during the early phase of nutrient starvation-induced autophagy: a) an increase of the intracellular Ca2+ store content (and consequently of agonist-induced Ca2+ release) and b) a sensitization of the IP3R to IP3.

On the one hand, consistent with the increase in Ca2+-store content, we find increased levels of ER Ca2+-binding proteins (BiP, calreticulin) and a reduction in the rate of ER Ca2+ leak. Neither the levels of the SR/ER ATPase Ca2+ pump SERCA were affected nor ER remodeling was activated during this first of phase of autophagy.

On the other hand, the sensitization of the IP3R correlated with an increased interaction of the channel with Beclin-1, a haploinsufficient tumor suppressor gene essential for autophagy. This interaction was demonstrated to occur in vitro via the N-terminal suppressor domain (aa 1-225) of the IP3R. Importantly, siRNA-mediated knockdown of Beclin-1 is able to reduce the sensitization of the IP3R after nutrient starvation. Finally, addition of recombinantly expressed and purified Beclin-1 in unidirectional 45Ca2+-fluxes in permeabilized cell monolayers directly stimulates IP3-induced Ca2+ release, confirming the role of Beclin-1 in this process.

These data suggest that a sensitization of the intracellular Ca2+ release occurs during nutrient starvation-induced autophagy. Both an increase in the Ca2+ store content and a sensitization of the IP3R lead to an increase in agonist-induced Ca2+ release. Further investigation will reveal how these Ca2+ dynamics regulate cell survival, cell death or autophagy.

Keywords: Ca2+ signaling, autophagy

P-34 Role of miRNA in leukemogenesis and in epigenetic regulation

Dell'aversana Carmela1, 2 , Roberta Sarno(1), Annamaria Carissimo(1), Francesco Paolo Tambaro(1),Angela Nebbioso(1), Diana Teti(2) & Lucia Altucci(1)

1)dep.of general pathology, second university of naples, vico l. de crecchio 7, 80138, naples, it.
(2)dep.of sperimental pathology and microbiology university of messina-consolare valeria, 1 - 98125 messina

Epigenetic mechanism and post-translational modification of nucleosomal histone proteins contribute to the correct modulation of gene expression and to the maintenance of tissue- and cell-type specific functions. Deregulation of epigenetic mechanism cooperates with genetic alteration to the establishment and the progression of cancer. Several evidences suggested the relevant relationship between cancer mechanism and expression of the small non coding RNA, microRNA (miRNA). MiRNAs are non coding RNAs 18-25 nucleotides in length that regulate in a variety of biologic processes, including cell cycle, proliferation, differentiation, apoptosis and senescence. MiRNA are a new class of evolutionary conserved small RNA affecting gene expression at the post-transcriptional level. The role of miRNA in different pathological processes, including the cancer such as oncogenes or tumour-suppressors, led to their application in the molecular diagnosis and prognosis.

Epigenetic drugs, such as the histone deacetylation inhibitors (HDACi), are currently used in several anticancer terapies thanks to their antiproliferation, proapoptotic and differentiative activity. They can represent a new frontier in the oncological medicine.

Our study was direct to the comprehension of miRNA expression profiles and roles in different leukemic cell lines after treatment with the known HDACi, Suberoylanilide hydroxamic acid (SAHA), compared these to gene expression profiles in order to identify specific miRNAs and gene targets that could be used in anti-leukaemic therapy.

Here we show that miRNA expression profiles are altered after SAHA treatment in leukemic cell lines and in primary leukemic samples. We focused on a specific miRNA, miR-194, that is over-expressed after SAHA treatment, and we identify its mRNA target. miR-194 directly targets Bclaf1 on its 3'UTR and dowregulates its expression. The role of Bclaf1 is still unclear. It is a proapoptotic gene with other unknown functions. These data will be further discussed.

This work was supported by: EU (Epitron) contract n° 518417; ATLAS n 221952; Associazione Italiana per la Ricerca contro il cancro (AIRC).

Keywords: Microrna; Saha; Microarray, Bclaf1

P-35 Implications of CYLD in neuronal cell death

Sebastian Diemert(1), Sandro Krieg(2), Seong Woong Kim(2), Nikolaus Plesnila(2), Carsten Culmsee(1)

(1) Klinische Pharmazie, Institut für Pharmakologie und Klinische Pharmazie, Philipps–Universitaet Marburg, Deutschland; (2) Department of Neurodegeneration & Department of Physiology, Royal College of Surgeons in Ireland (RCSI), Dublin, Ireland;

The tumor suppressor CYLD is a deubiquitinating enzyme (DUB) involved in inhibitory regulation of the NF-κ B pathway. Dysfunctional CYLD mutations are known to cause skin tumors and possibly stimulate exacerbations of other cancers through enhanced NF-κ B activation. In neurons, NF-κ B may promote survival through increased expression of anti-apoptotic target genes like bcl-2, X-chromosome-linked inhibitor of apoptosis (XIAP) or MnSOD. Here, we investigated the implications of CYLD in models of neuronal cell death, both in vitro and in vivo. We found that depletion of CYLD attenuated glutamate toxicity in immortalized mouse hippocampal neurons (HT-22 cells) and cultured primary neurons. Further, CYLD knockout mice exhibited a significantly reduced infarction area compared to wildtype animals in an in vivo model of focal cerebral ischemia. In the investigated cell types CYLD depletion seemingly leads to alterations in transcriptional activity of Nf-κ B resulting in increased levels of e.g. MnSOD, whereas levels of other antiapoptotic proteins were not affected. In summary, our findings suggest that CYLD may contribute to neuronal cell death through inhibition of NF-κ B transciptional activity. Thus, inhibition of CYLD may serve as an efficient therapeutic strategy to preserve NF-κ B dependent transcription of anti-apoptotic genes and thereby preventing neuronal damage.

This work was supported by a DFG grant (CU43/6-1) to CC and NP.

Keywords: ischemia, CYLD, Nf-kB, neuroprotection

P-36 Calcium-sensing receptor-mediated survival in granulosa cells.

Araceli Diez-Fraile (1), Sylvie Mussche (1), Tom Vanden Berghe (2,3), Marc Espeel (1), Peter Vandenabeele (2,3), Petra De Sutter (4) and Katharina D'Herde (1)

(1) Faculty of Medicine and Health Sciences, Department of Basic Medical Sciences, Ghent University, Ghent, Belgium.
(2) Molecular Signalling and Cell Death Unit, Department for Molecular Biomedical Research, VIB, Ghent, Belgium.
(3) Department of Molecular Biology, Ghent University, Ghent, Belgium.
(4) Faculty of Medicine and Health Sciences, Department of Reproductive Medicine, Ghent University Hospital, Ghent, Belgium.

This study investigated the potential role of the calcium-sensing receptor (CaR) in mediating survival of granulosa cells (GCs) obtained from the Japanese quail (Coturnix coturnix japonica) and from patients enrolled in a program for assisted reproduction or undergoing female to male sex change. Immunoreactivity of CaR was shown in GCs of quail preovulatory follicles as well as in the remnants of the GC layer after ovulation. Conversely, the CaR could not be detected by immunohistochemistry in the granulosa of smaller undifferentiated follicles, frequently undergoing follicular atresia. The CaR in quail follicles destined to ovulate was identified as a protein of 115-125 kDa. Immunoreactivity of CaR was detected in human follicles with a gradient of staining intensity along the GCs, being more pronounced in the large preantral follicles compared to smaller follicles. In addition, human mural and cumulus GCs from antral follicles also express the CaR as assessed by immunofluorescence. Addition of the different CaR agonists to quail granulosa explants obtained from the largest preovulatory follicle of quails in culture for 24 h caused inhibition of apoptosis elicited by gonadotropin withdrawal on its own or in combination with C8-ceramide addition. Furthermore, R-568, a specific, positive allosteric modulator of CaR, not only inhibited apoptosis but also increased GC number per viewing field in cultured quail granulosa explants. This observation could be attributed not to a rise in GC proliferation but to a more compact tissue structure, including a distinct distribution pattern of connexin-43 gap junction proteins. Incubation in the presence of LY294002, a specific phosphatidylinositol-3 kinase inhibitor, increased GC apoptosis, indicating that this pathway is involved in GC survival signalling. However, LY294002-induced apoptosis was considerably attenuated by incubation with R-568, indicating that other pathways might be major contributors to the survival mediated by CaR agonists. We provide direct evidence for the presence of CaR in avian preovulatory granulosa explants and an increasing gradient of staining of CaR from the smallest to the largest follicles in human GCs, suggesting a pivotal regulatory role for CaR in follicle selection.

Keywords: calcium-sensing receptor, folliculogenesis, survival signalling, granulosa cells

P-37 Phagocytosis of and cytokine release in response to differently dying cells by murine macrophages

Zoltán Doró (1), Gyöngyi Buchan (1), András Mádi (1) and László Fésüs (1)

(1)Department of Biochemistry and Molecular Biology, Medical and Health Science Center, University of Debrecen, Hungary

Macrophages can efficiently eliminate dying neutrophil granulocytes preventing development of inflammation. It is well known how macrophages engulf apoptotic or necrotic cells separately, however, it is still unclear how they behave when different types of dead cells are present in their environment simultaneously.

We established models for three different types of cell death. 1. Spontaneous apoptosis, 2. Spontaneous necrosis induced by serum deprivation; 2. Heat-induced necrosis; 3. Necroptosis induced by the pan-caspase inhibitor, Z-VAD.fmk. We characterized the death processes of neutrophils, and performed phagocytosis assays with either bone-marrow derived or peritoneal murine macrophages. We found that cells undergoing spontaneous necrosis are engulfed by macrophages most efficiently compared to other death types. We could demonstrate that different forms of dead cells have no influence on each other's uptake by macrophages when they are applied simultaneously in phagocytosis assays. Our results suggest that the engulfment of several phagocytosis substrates present together is a more complex molecular process than just a simple competition between the substrates.

We have also investigated how production of proinflammatory cytokines (IL-6 and Il-1beta) changed during the phagocytosis processes in co-incubation assays. Our observation is that no IL-6 release occurred during and after phagocytosis of any of the three types of necrotic cells. Suprisingly the three types of necrotic cells could even inhibit LPS induced secretion of proinflammatory cytokines by both mouse peritoneal macrophages and the RAW 264.7 cell line and this could be observed in human system as well. A preliminary observation shows that the inhibitory effect on LPS induced IL-6 production is more pronounced in the case of mouse neutrophil granulocytes than in response to other cell types, and is partially PS dependent.

In macrophages primed with ultra pure LPS each of dying cell types lead to inflammasome activation, and, as a preliminary result, it was observed that this activation is more pronounced in response to dying neutrophil granulocytes then to other cell types..

Our results may contribute to better understanding of the development of inflammatory and autoimmune diseases

Keywords: phagocytosis, necrosis, necroptosis, proinflammatory cytokines

P-38 cIAP1 is a new activator of E2F-1 transcription factor

Jessy Cartier (1,2) Arthur Marivin (1,2) Jean Berthelet (1,2) Valérie Edmond (3,4) Eric Solary (1,2,5) Béatrice Eymin (3,4) and Laurence Dubrez (1,2)

(1) Institut National de la Santé et de la Recherche Médicale (Inserm) UMR866, Dijon, F-21079, France.
(2) University of Burgundy; Faculty of Medicine, Institut Fédératif de recherche (IFR) 100 ; Dijon, F-21079, France.
(3)Inserm U823, Equipe Bases Moléculaires de la Progression des Cancers du Poumon, Institut Albert Bonniot, Grenoble F-38042, France.
(4) Université Joseph Fourier, Grenoble, F-38041, France.
(5) Inserm UMR1009, Institut Gustave Roussy, Villejuif, France

The inhibitor of apoptosis protein cIAP1 (cellular inhibitor of apoptosis protein-1) is an E3 ubiquitin ligase that displays oncogenic properties. In the cytoplasm, the protein is a potent regulator of TNF receptor family and NF-κB signalling pathways. However, cIAP1 is mainly expressed in the nucleus on many cell types which is not in accordance with its cell signalling activity.

The objective of the present work was to investigate the nuclear function of cIAP1.

A screen of partners of cIAP1 among cell cycle regulators revealed a potential interaction of cIAP1 with the transcription factor E2F-1. We confirmed by GST-pull down and immunoprecipitation a direct interaction between both proteins. cIAP1 stimulates the transcriptional activity of E2F-1 in a gene reporter assay on cyclin promoters, in particular cyclin E which is completely abolished by a mutation into E2F binding site. We demonstrate by ChIP experiments that cIAP1 is recruited, along with E2F-1, on E2F binding site of the promoter of cyclin E and A, in a cell cycle dependent manner. The recruitment of E2F-1 on cyclin E and A promoters is prevented by silencing of cIAP1 suggesting that cIAP1 is necessary for the binding of E2F-1 on gene promoters. Lastly, we demonstrated that cIAP1 modulates the transcriptional expression of cyclin E and interferes with the progression of cell into S phase of cell cycle. The ability of cIAP1 to stimulate E2F-1 transcription activity may account for its oncogenic properties.

Keywords: IAP, E2F1, transcription factor, cell differentiation, cell proliferation

P-39 Role of RIP1/RIP3 in TNF-induced necrosis and Lethal Systemic Inflammatory Response Syndrome (SIRS)

Linde Duprez (1,2)*, Nozomi Takahashi (1,2)*, Filip Van Hauwermeiren (3,4), Nele Vanlangenakker (1,2), Claude Libert (3,4), Anje Cauwels (5,6) and Peter Vandenabeele (1,2)

**Equally contributed*
(1) VIB, Department for Molecular Biomedical Research, Unit for Molecular Signaling and Cell Death, Technologiepark 927, B-9052 Ghent, Belgium
(2) Ghent University, Department of Biomedical Molecular Biology, Unit for Molecular Signaling and Cell Death, Technologiepark 927, B-9052 Ghent, Belgium
(3) VIB, Department for Molecular Biomedical Research, Unit for Molecular Mouse Genetics, Technologiepark 927, B-9052 Ghent, Belgium
(4) Ghent University, Department of Biomedical Molecular Biology, Unit for Molecular Mouse Genetics, Technologiepark 927, B-9052 Ghent, Belgium
(5)VIB, Department for Molecular Biomedical Research, Unit for Molecular Pathophysiology and Experimental Therapy, Technologiepark 927, B-9052 Ghent, Belgium
(6) Ghent University, Department of Biomedical Molecular Biology, Unit for Molecular Pathophysiology and Experimental Therapy, Technologiepark 927, B-9052 Ghent, Belgium

Engagement of TNF to its receptor TNFR1 signals to two diametrically opposed pathways: cell death vs. survival / inflammation. The latter pathway is mainly mediated by NF-kB-dependent gene activation. RIP1 is a member of the RIP kinase family and plays a crucial role in determining the cellular fate, death or survival. RIP1 kinase activity is reported to be indispensable for necrotic cell death induced by TNF, while dispensable for NF-kB activation. We explored the role of RIP1 and RIP3, another member of the RIP kinase family, in TNF-induced cell death in the mouse fibrosarcoma cell line L929 and the human colon cancer cell line Caco2 by use of RIP1 kinase inhibitor necrostatin-1 (Nec-1) and siRNA-mediated RIP3 gene knockdown . Nec-1 as well as knockdown of RIP3 protected against TNF-induced cell death.

We extrapolated this observation to a mouse model for systemic inflammatory response syndrome (SIRS) triggered by a bolus injection of high dose TNF. Pretreatment with Nec-1 protected mice against TNF-induced SIRS and its lethal outcome. Likewise, absence of RIP3 by targeted gene deletion conferred total protection.

These results underscore an indispensable role of RIP1 and RIP3 in TNF-induced SIRS and point to the critical involvement of necrotic cell death in the development of SIRS. We conclude that different players of the necrotic cell death pathway are potential therapeutic targets for treatment of sepsis and degenerative diseases.

Keywords: RIP1, RIP3, necrosis, TNF, SIRS

P-40 Sensitization of melanoma cells for death ligand-induced apoptosis by kinase inhibitors correlates with characteristic changes at the level of mitochondria, reactive oxygen species (ROS) and death receptors

Anja Berger, Annika Quast, Antje Kammermeier, Bahtier M. Kurbanov and Jürgen Eberle

Department of Dermatology and Allergy, Skin Cancer Center, University medical center Charitè, Berlin, Germany

The death ligand TRAIL (TNF-related apoptosis-inducing ligand) triggers apoptosis by death receptors DR4/TRAIL-R1 and DR5/TRAIL-R2 in a variety of cancer cells, while normal cells appear as largely protected. Resistance to death ligands (prevalent or induced) however appears as a major limitation for therapeutic approaches. We have previously described three groups of melanoma cells with regard to their TRAIL sensitivity: i) DR4(+)/DR5(+) highly sensitive (A-375, SKM-13) ii) DR4(-)/DR5(+) moderately sensitive (Mel-HO, SKM-19) and iii) DR4(-)/DR5(+) resistant melanoma cell lines (MeWo, Mel-2a). Permanent resistance to TRAIL coincided with resistance to CD95L. In addition to prevalent death ligand resistance, previously sensitive cell lines were selected for induced resistance by long-term incubation with TRAIL or CD95 agonistic antibody (A-375-TRAIL-selected, Mel-HO-TRAIL-selected, A-375-CH11-selected, Mel-HO-CH11-selected).

For overcoming death ligand resistance, melanoma cells with permanent and selected resistance were treated with a panel of selective kinase inhibitors. These were U0126 (MEK), L-779450 (b/c-RAF), indirubin 8-Rha-beta (CDKs), wortmannin (PI3K), BML-257 (PKB/Akt), rapamycin (mTOR) and BMS-345541 (IKK). Different kinase inhibitors resulted in sensitization of melanoma cells for death ligands. The enhanced apoptosis revealed however large differences at the molecular level. Thus, sensitization of melanoma cells for TRAIL or CD95 by MAPK, IKK and CDK inhibitors coincided with enhanced caspase activity and loss of the mitochondrial membrane potential, whereas inhibition of PI3K, PKB/Akt appeared as caspase-independent and was associated with increased ROS levels. In agreement were the effects of caspase inhibitors and antioxidative substances. In addition, and partly explaining the effects, characteristic regulations of p53, pro- and antiapoptotic Bcl-2 proteins, cIAPs and death receptors are reported.

This study illuminates the high complexity of apoptosis regulation in melanoma cells, which may or may not depend on classical pathways as related to caspase activation and cytochrome c release. It further demonstrates the involvement of multiple survival pathways in the sensitivity to TRAIL and CD95L and indicates strategies for overcoming death ligand resistance of tumor cells.

Keywords: melanoma, death ligands, TRAIL, CD95L, kinase inhibitors

P-41 Cytotoxıc and apoptotic effects of some boron compounds on the mouse neuroblastoma (NS20Y) cell lines

Selda A. Arslan (1), Tuğba Tunçay (1), Emel Ergene (1)

(1)Anadolu University, Faculty of Science, Dept. of Biology, Eskisehir, Turkiye

Foods and drinking water contains boron as a trace element and it is found in the blood as boric acid. It is essential to organism for to maintain homeostasis. Boron compounds have been widely used as biologically active agent and drug. Recently, boron has been reported as chemo-preventative agent in human prostat cancers. The effects of boron on activation of the apoptotic mechanism was shown in breast and skin melanom cancer cells.

In this investigation, we studied the effects of the some boron compounds (potassium perborate, sodium perborate and anhydrous borax) both on the activation of the apoptotic pathway and cytotoxicity in the mouse neuroblastoma (NS20Y) cells. We used the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) Cell Proliferation and Viability Assay, Neutral Red (NR) for cytotoxicity. Apoptotic effects was determined by the using Mitochondrial Assay (Mitocapture), Caspase 3/8 Enzyme Activation Test and DNA Fragmentation Method. The NS20Y cells were incubated in the ranges of the 25-0,0312 mM concentrations of potassium perborate, sodium perborate and anhydrous borax. The cell proliferation was measured 24, 48 and 72 hours intervals.

As a result, the cytotoxic effects were indicated for potassium perborate at 2 mM, sodium perborate at 0,025 mM and anhydrous borax at 7,5 mM consantrations. In the Mitochondrial Assay (Mitocapture), the apoptotic mouse neuroblastoma (NS20Y) cells were found 70 percent for potassium perborate, 77 percent for sodium perborate and 76 percent for anhydrous borax. Caspase 3/8 Enzyme Activation Test and DNA Fragmentation Method were confirmed above results.

Keywords: neuroblastoma, apoptosis, cytotoxicity, boron

P-42 Natuaral Factors Induce Apoptosis in Human Cancer Cells: development of new strategies for prevention and treatment of cancer

Fuad Fares (1,2), Rinat Bar-Shalom (1), Lital Sharvit (1) and Naeil Azzam (2)

(1)Department of Biology, Faculty of Natural Sciences, University of Haifa, Haifa,
(2)Department of Molecular Genetics, Carmel Medical Center, Haifa, Israel. ffares@sci.haifa.ac.il; fares@clalit.org.il;

Dysregulation of apoptosis cause signifecant human diseases and promote cancer development. Fucosing on identifying of apoptotic factors and understanding their molecular mechanisms in human cancer cells, may inhance our understanding on the 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. The main goal of the present study is to elucidate the beneficial effect in human cancer prevention and therapy, of natural compounds from edible plants, and extracts from medicinal mushrooms.

Human cancer cell lines of colon, breast pancreatic and prostate were exposed to different dietary indole derivatives (indol-3-carbinol or to 3-3' diindolylmethane), natural antioxidents (NAOs) extracted from the leaves of Spinach (S), Inulla viscosae (IV) and Citrullus Colocynyhis (CC) or extracts from medicinal mushrooms. 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 subcunaneously prostate (TRAMP-C2) or colorectal (MC38) cell lines. This effect 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 ($p<0.01$) 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 factors, apoptosis, cancer, prevention

P-43 Efficient melanoma cell apoptosis and reduced tumor growth in nude mice in a gene therapy approach with selective replication-competent adenoviral vectors armed with death ligands TRAIL and CD95L

Lothar F. Fecker (1), Stefanie Rückert (1), Henry Fechner (2) and Jürgen Eberle (1)

(1)Department of Dermatology and Allergy, Skin Cancer Center, University medical center Charité, Berlin, Germany.
(2)Institute of Biotechnology, University of Technology, Berlin, Germany

High mortality and therapy resistance of melanoma demands the development of new strategies, and overcoming apoptosis deficiency of melanoma cells appears as particularly promising. TNF-related apoptosis inducing ligand (TRAIL) has been shown by us as highly effective for apoptosis induction in melanoma cells and may apply for gene therapy due to its selective impact on tumor cells.

We have constructed two conditional replication-competent adenoviral vectors for inducible expression of TRAIL (AdV-TRAIL) and for CD95L/FasL (AdV-CD95L). A variant viral E1A protein and the deletion of E1B aimed at the general restriction of viral replication to tumor cells. In particular, the replication gene E1A is controlled by a tyrosinase promoter with high selectivity for melanoma cells. The tetracycline/doxycycline-responsive transactivator rtTA and the respective death ligand gene are controlled by a bidirectional tetracycline-inducible promoter.

AdV-TRAIL mediated strong expression of E1A and doxycycline-dependent induction of TRAIL selectively in melanoma cells, which resulted in melanoma cell lysis and induction of apoptosis. In contrast, non-melanoma cells and normal human melanocytes appeared as protected. Comparison of AdV-TRAIL with AdV-CD95L revealed largely similar efficacies of both death ligands for melanoma cells in vitro. In melanoma xenotransplantation models, AdV-TRAIL demonstrated its efficacy by significant growth reduction of established melanomas after intratumoral application. Melanoma cell killing by AdV-TRAIL could be further improved in vitro by combinations with chemotherapeutics.

Thus, we demonstrate that melanoma cells can be efficiently targeted by death ligand-based gene therapies, and possible resistance may be overcome by combined chemotherapy.

The study has been supported by the German Cancer Aid (Deutsche Krebshilfe, grants 107398 and 108008).

Keywords: cancer, gene therapy, apoptosis, death ligands, adenoviral vectors

P-44 cIAPs negatively regulate TLR3-mediated cell death by limiting RIP-1 recruitment to a TRIF-containing signalling complex

Maria Feoktistova (1,2*), Peter Geserick (1,2*), Mike Hupe (1), Georg Haecker (3) and Martin Leverkus (1,2)

(1) Laboratory for Experimental Dermatology, Department of Dermatology and Venereology, Otto-von-Guericke-University Magdeburg, Germany
(2) Section of Molecular Dermatology, Department of Dermatology, Venereology, and Allergology, Medical Faculty Mannheim, University Heidelberg, Germany
(3) Department of Microbiology, University of Freiburg, Germany

Toll-like receptors (TLRs) regulate inflammatory responses, control tumor cell proliferation and have been shown to confer apoptosis resistance. Activation of TLR3 by agonists such as poly (I:C) can also promote apoptosis in tumor cells such as transformed keratinocytes. However many malignant cell lines have been proven to be resistant to TLR3-induced cell death by thus far unknown mechanism. cIAPs were postulated as critical regulators of cell death processes, but understanding of their functional role in apoptotic TLR3-signalling pathways is lacking to date. In this report we demonstrate that loss of cIAPs sensitizes HaCaT keratinocytes to poly (I:C) induced cell death. This cell death requires both caspase and RIP-1 kinase activities. In the absence of cIAPs an intracellular complex containing TRIF, caspase 8, cFLIP, FADD and RIP-1 is formed in a stimulation-dependent manner. In the absence of caspase activity a cryptic necrotic cell death in these cells is unmasked. Loss of RIP-1 protects cells from both types of poly (I:C)/IAP antagonist induced cell death, and this protection correlates with suppressed formation of the intracellular complex. Cells expressing high cFLIP levels are resistant to poly (I:C) induced cell death in the absence of cIAPs. Intriguingly, cFLIPL but not cFLIPS confers substantial protection from IAP antagonist induced formation of the RIP-1 containing intracytoplasmic complex and RIP-1 kinase dependent necrotic cell death. Furthermore, the degradation of IAPs by the TNF family member TWEAK duplicated the findings with IAP antagonist and thus supports a potential physiological role of cIAPs for TLR3-induced cell death. Our results thus indicate that cIAPs negatively regulate cell death signalling after TLR3 ligation in a RIP-1-dependent manner. Our data furthermore indicate that TWEAK signalling may allow for RIP-1 dependent necrotic cell death with further important pathophysiological consequences. Therefore, inhibitors that antagonize IAP function may represent a potential anti-tumor strategy to overcome apoptosis resistance of tumors to TLR3 agonists.

Keywords: TLR3, cIAP, cell death, RIP-1, cFLIP, keratinocytes, SCC

P-45 The stress response of nucleolar AATF regulates apoptosis in a c-Jun-dependent and JNK-independent manner

Saima E. Ferraris (2), Isoniemi Kimmo (1), Elin Torvaldson (2), Sara Van der Bloomen (3), Susumu Imanishi (1), Julius Anckar (2) and John E. Eriksson (1,2).

(1) Turku Centre for Biotechnology, University of Turku, FIN-20521 Turku.
(2) Turku Centre for Biotechnology, Åbo akademi University, FIN-20521 Turku.
(3) University of Surrey, Guildford, GU2 7XH, England

The control of damage-mediated cellular apoptosis involves the engagement of an integrated network of survival and death pathways. Among the best characterized pathways modulating survival and cell death responses is the c-Jun NH2-terminal protein kinase/stress-activated protein kinase (JNK/SAPK), which conveys a multitude of extracellular and intracellular stress stimuli to the the AP-1 transcription factors. Here we identify the apoptosis antagonizing transcription factor (AATF/Che-1) as a novel regulator of the specific AP-1 transcription factor c-Jun.

Overexpression of AATF in mouse embryonic fibroblasts (MEFs) and in 293T human embryonic kidney cells subjected to UV-light irradiation or TNF treatment strongly potentiated the activation of the c-Jun transcription factor. Conversely, downregulation of AATF by siRNA markedly reduced cJun activity. Interestingly, the activation of c-Jun corresponded to a more diffuse localization of AATF from the nucleolus through the nucleus, suggesting that AATF is carefully confined to the nucleolus compartment waiting for specific triggering signals. Using a c-Jun non-phosphorylable construct cJun-Ser63/73Ala, we show that the stimulatory effect of AATF on c-Jun activity is JNK-independent. Importantly, AATF1 overexpression markedly promoted commitment of cells to c-Jun-dependent apoptosis upon UV-light exposure, while its depletion rendered cells resistant to the same apoptotic signal, as indicated by a number of established markers. Together these findings demonstrate that AATF is a key regulatory factor of the c-Jun transcriptional activation. The presence and magnitude of the AATF response in different cells will turn c-Jun activation into a context-dependent response, the final outcome of which is determined by the AATF attendance.

Keywords: UV-stress, cJun activation, nucleolus, apoptosis

P-46 comprehensive map of cell death and energy metabolism regulation

Simon Fourquet (1,2,3), Laurence Calzone (1,2,3), Emmanuel Barillot (1,2,3), Andrei Zinovyev (1,2,3)

(1) Institut Curie
(2) INSERM U900
(3) Mines ParisTech

A comprehensive review of molecular events that control the regulation of cell death and energy metabolism was assembled in the form of a large network of biochemical reactions. Attention was focused mainly on reactions that occur at the level of mitochondria, and on signalling through the TNF, AKT-mTOR, hypoxia and ROS responsive pathways. This network contains ~1500 species (including ~600 different gene products and ~100 metabolites), and provides links to ~500 pubmed indexed articles. This network was constructed using CellDesigner (v4.0.1), which uses standard system biology formats for the description and graphical representation of biological networks. This allows to use this network for i) the visualisation of high-throughput data from cancer samples, ii) the inference of dynamic models, and iii) the prediction of cellular phenotypes from transcriptomic data.

Keywords: systems biology

P-47 Opposing effects of hMOF and SIRT1 on H4K16 acetylation and the sensitivity to the topoisomerase II inhibitor etoposide

Jens Fuellgrabe (1), Nabil Hajji (2), Karolina Wallenborg (3), Pinelopi Vlachos (1), Ola Hermanson (3), Bertrand Joseph (1)

(1)Department of Oncology Pathology, Cancer Centrum Karolinska, Karolinska Institutet, Stockholm, Sweden
(2)Department of Experimental Medicine and Toxicology,
Division of Investigative Science, Imperial College London, UK
(3)Department of Neuroscience, CoE in Developmental Biology (CEDB/DBRM), Karolinska Institutet, Stockholm, Sweden

Various inhibitors of histone deacetylase (HDAC) activity can sensitize drug resistant cancer cells to chemotherapeutic agents. However, the mechanisms underlying such effects of distinct HDAC inhibitors (HDACi) remain poorly understood. We have shown that both the HDACi trichostatin A and valproic acid induce a sensitization of multidrug-resistant cancer cells to the topoisomerase II inhibitor etoposide/VP16. This effect was associated with increased acetylation of certain lysines on histones H3 and H4, including lysine 16 on histone H4 (H4K16). Overexpression of the histone acetyltransferase hMOF, known to target H4K16, was sufficient to mimic HDACi treatment on sensitization and H4K16 acetylation, and importantly, small interfering RNA (siRNA)-mediated knockdown of hMOF abolished the HDACi-mediated sensitizing effects as well as the increase in H4K16 acetylation. Conversely, siRNA-mediated knockdown of the H4K16 deacetylase SIRT1 mimicked HDACi treatment whereas overexpression of SIRT1 abolished H4K16 acetylation and significantly reduced the sensitizing effects of HDACi. Interestingly, the effects of hMOF on H4K16 acetylation and sensitization to the topoisomerase II inhibitor could be directly counteracted by exogenous expression of increasing amounts of SIRT1 and vice versa. These results suggest that hMOF and SIRT1 activities are critical parameters in HDACi-mediated sensitization of multidrugresistant cancer cells to topoisomerase II inhibitor and increased H4K16 acetylation.

Keywords: epigenetics, cancer, topoisomerase inhibitor, cell death, HDAC inhibitor

P-48 A novel TNFR1-triggered apoptosis pathway involving p38 MAPK, class IA PI3Ks, and ROS in neutrophils

Barbara Geering (1), Ursina Gurzeler (1), Elena Federzoni (1), Thomas Kaufmann (1), and Hans-Uwe Simon (1)

(1) Institute of Pharmacology, University of Bern, Friedbuehlstrasse 49, 3010 Bern, Switzerland

The most common form of neutrophil death is apoptosis, both under physiological and inflammatory conditions. Here, we report that the molecular mechanisms used for caspase activation are surprisingly different between FAS and TNF receptor-stimulated neutrophils. Whereas neutrophil apoptosis induced by FAS ligation was followed by caspase-8 activation and required BID to initiate the mitochondrial amplification loop, TNF-α-induced apoptosis in these cells was independent of the intrinsic pro-apoptotic pathway. Instead, the TNF-α-triggered pathway strikingly involved class IA phosphoinositide 3-kinases (PI3Ks), which were constitutively bound to TNF receptor 1 (TNFR1) and activated by mitogen-activated protein kinase p38 in a caspase-independent manner. TNF-α-induced p38 and subsequent PI3K activation resulted in the generation of reactive oxygen species (ROS), which activated effector caspases, a mechanism that did not operate in neutrophils without functionally active NADPH oxidase. In contrast, FAS-mediated neutrophil apoptosis was largely independent of ROS. Taken together, in neutrophils, proximal pro-apoptotic pathways following TNFR1 stimulation are initiated by p38 and PI3K, but not caspase-8, a finding that should be considered in anti-inflammatory drug development strategies.

Keywords: apoptosis, signaling, caspases, TNF, FAS, inflammation

P-49 Transcriptional regulation is still at work during the execution phase of apoptosis.

L. Grieco (1,2), G. Hua (2), A. Bergon (1,2), N. Boulanger (2), V. Gall (2), D. Thieffry (2,3,4), B. Kahn-Perlès (2)

(1) Université de la Méditerranée, Marseille, France
(2) TAGC (INSERM U928), Marseille, France
(3) IBENS (CNRS UMR 8197 / INSERM U1024), Ecole Normale Supérieure, Paris, France
(4) CONTRAINTES, INRIA Paris-Rocquencourt, Le Chesney, France

When activated human T lymphocytes are exposed to apoptotic stimuli targeting mitochondria, they enter an early caspase-independent commitment phase, followed by a caspase-dependent execution phase. In this study, we define and compare the transcriptional programs supporting this two phases. To do so, a gene expression profiling experiment was performed on Jurkat leukemic T-cells, treated with the HDAC inhibitor TSA. The two apoptotic response phases were operationally distinguished according to phosphatidyl serine exposure on cell surface. Annexin V binding (dying) and non-binding (committed) populations were sorted and, for each of them, mRNA transcripts were hybridised on an Agilent microarray platform. Over-/under- expressed genes were specified relative to untreated control cells. Over-expressed genes were found not only in committed cells but also in dying cells. Strikingly, the two sets of genes only partially overlap suggesting that transcription is still at work during the execution phase. A functional enrichment analysis showed that: a) in both cases, most under-expressed genes belong to specific T-cell activities; b) up-regulated genes from committed cells are involved in cell differentiation and cell cycle inhibition; c) up-regulated genes from cells in execution phase are involved in chromatin remodelling, negative regulation of transcription, and induction of apoptosis. These conclusions have been reinforced by qPCR analyses performed on a small selection of genes. Our results strongly suggest that the execution phase of apoptosis is partly regulated by transcriptional mechanisms.

Keywords: apoptosis, commitment phase, execution phase, T-lymphocytes, HDAC inhibitors, TSA, gene expression profiling

P-50 Regulation of FasL and TNFα-induced cell death in murine neutrophils

Ursina Gurzeler, Nohemy Echeverry, Daniel Bachmann and Thomas Kaufmann

Institute of Pharmacology, University of Bern, Bern, Switzerland

We are interested in how Bcl-2 family members regulate the mitochondrial (stress-induced) apoptotic pathway and the crosstalk between the death receptor-induced and the mitochondrial pathway. For apoptosis induced by the death receptor Fas (CD95/APO-1) it has been shown that this crosstalk is required in some cell types (called type II cells, including hepatocytes, pancreatic β-cells as well as many cancer cells) but not in others (type I cells). The crosstalk is mediated by the BH3-only protein Bid, which is cleaved and activated by caspase-8, the initiator caspase proximal of Fas. To verify whether a Fas sensitive cell type is type I or type II one has to interfere with the initiation phase of the mitochondrial pathway. This can be achieved by either overexpressing anti-apoptotic Bcl-2 family members (such as Bcl-xL or Mcl-1) or by deleting the BH3 only protein Bid.

Here, we investigate whether murine neutrophils, which are sensitive to Fas-induced killing, behave like type I or type II cells. In a first approach we are comparing FasL-induced killing in primary Gr1+ neutrophils isolated from the bone marrow of wildtype and bid-/- mice. Our data suggest that FasL-induced death is indeed delayed in bid-/- neutrophils at early time points. Surprisingly, and in contrast to hepatocytes, addition of the pan-caspase inhibitor Q-VD-oph results in a very moderate protection only. Therefore we also investigated caspase independent events as death receptors, especially TNF-R1, have been described to also induce necroptosis (programmed necrosis) and non-caspase like proteases, such as cathepsin D, have been linked to some forms of neutrophil cell death. Preliminary data indicate that mouse granulocytes can in fact be better protected from cell death when Q-VD-oph is added in combination with the RIP1 kinase inhibitor Necrostatin-1. On the other hand, experiments with TNFα, a death ligand that activates the death receptor TNF-R1 and can induce apoptosis as well as necroptosis, indicate that in mouse neutrophils TNFα-induced death is fully blockable by addition of Q-VD-oph. TNFα-induced apoptosis in neutrophils is however not delayed in the absence of Bid, indicating that either the mitochondrial pathway is not involved or - as seen in hepatocytes - other BH3-only proteins besides Bid (e.g. Bim) are activated downstream of TNF-R1 [1].

In a second approach, we are using a system that allows in vitro differentiation of Hoxb8-immortalized myeloid progenitor cells into mature neutrophils. Using this approach, non-transformed progenitor cell lines can be generated from any desired genetically modified mouse strain and mature neutrophils can be obtained in shear unlimited numbers, which is a major drawback of experiments with primary mouse neutrophils. We have successfully generated several Hoxb8 cell lines and our first characterization of differentiated neutrophils indicate that they resemble primary murine neutrophils both morphologically and functionally.

Keywords: Neutrophil apoptosis, TNF-α, FasL

P-51 Development of Apaf-1 inhibitors as therapeutic tools to decrease unwanted pathological apoptosis

Mónica Sancho(1), Sandra Marchan(3), Rebeca Montava(1), Eliana Sirvent(1), Beatriz Santamaria(2), Adrián Ramos(2), Alberto Ortiz(2), Carmen Herrero(3), Carmen Lagunas(3), Mar Orzáez(1), Enrique Pérez Payá(1,4)

1 Centro de Investigación Príncipe Felipe, Valencia, Spain
2 Fundación Jiménez Díaz, Madrid, Spain
3 Laboratorios Salvat,
4 IBV-CSIC, Valencia, Spain

Drug targets elucidated in studies of cell death have lead to the discovery of potential pharmacological agents that could modulate the increased apoptosis found in some pathological situations such us degenerative diseases, AIDS, septic shock or ischemia-reperfusion damage. Caspases have been the target for the initial drug discovery efforts and although some of these inhibitors have reached clinical studies, they have encountered problems in their pharmacological development. Recent approaches postulate the formation of the apoptosome as an alternative target for the development of new apoptosis inhibitors.

We have identified a new structural class of apoptosome inhibitors that target Apaf-1. These inhibitors bind to the CARD domain of Apaf-1 and preclude the recruitment of procaspase-9 to the apoptosome inhibiting the intrinsic cellular pathway. The anti-apoptotic activity of Apaf-1 inhibitors has been analyzed in cellular models that resemble pathological situations of interest such us apoptosis induced by toxic products and drugs (e.g., Cisplatin) and certain inflammatory conditions that may lead to tissue injury. In these cellular models, we have evaluated the activity of Apaf-1 inhibitors with those designed against other pharmacological targets such as z-VAD or IDN-6556. Apaf-1 inhibition results in inhibition of caspase-3 activity comparable to that of other inhibitors. Unlike inhibition of caspases, the inhibition of Apaf-1 allowed cellular recovery in our experimental models. This different behavior will be discussed in the context of the molecular mechanism of the intrinsic apoptosis pathway. Therefore inhibition of Apaf-1 would provide an additional apoptosis inhibition point which could potentially result in a better performance as a therapeutic strategy.

Keywords: Apaf-1, pathological apoptosis, cell death, cell recovery

P-52 Betanodavirus infection-induced oxidative stress can involve in mitochondria-mediated cell death in fish cells

Chih-Wei Chang1, Jen-Leih Wu2 and Jiann-Ruey Hong1*

1Laboratory of Molecular Virology and Biotechnology, Institute of Biotechnology, National Cheng Kung University, Tainan 701, Taiwan, ROC. 2Laboratory of Marine Molecular Biology and Biotechnology, Institute of Cellular and Organismic Biology, Academia Sinica, Nankang, Taipei 115, Taiwan, ROC. E-mail: jrhong@mail.ncku.edu.tw

The role of oxidative stress in the pathogenesis of RNA nervous necrosis virus infection is still unknown. The present study demonstrated that beta-nodavirus can induce oxidative stress leading to mitochondria-mediated cell death and decreased host anti-oxidant enzymes. Red-spotted grouper nervous necrosis virus (RGNNV) quickly induced ROS production at 24 h post-infection (pi) in fish GF-1 cells, then upregulated expression of the anti-oxidant enzymes Cu/Zn SOD and catalase, and eventually expression of the transcription factor Nrf2 at 48 h and 72 h pi. Moreover, diphenyliodonium or N-acetylcysteine blocked RGNNV-induced ROS signaling. In addition, overexpression of zebrafish catalase in GF-1 cells reduced ROS production and protected cells from death due to RGNNV infection.

Furthermore, localization of ROS production using esterase activity and Mitotracker staining assays found that the ROS generated in mitochondria changes mitochondrial morphology or causes mitochondrial membrane potential (MMP) loss, both of which can be reversed by antioxidant treatment. Taken together, our data suggest that RGNNV-induced oxidative stress either primes the host oxidative stress defense system to upregulate expression of antioxidant enzymes or induces cell death via disrupting the mitochondrial morphology and inducing MMP loss, which can be reversed by anti-oxidants and catalase.

Keywords: nervous necrosis virus; mitochondria; ROS; ATP; anti-oxidants; catalase

P-53 A quantitative proteomics design for systematic identification of protease cleavage events

Francis Impens (1,2), Niklaas Colaert (1,2), Kenny Helsens (1,2), Bart Ghesquière (1,2), Evy Timmerman (1,2), Pieter-Jan De Bock (1,2), Benjamin M. Chain (3), Joel Vandekerckhove (1,2) and Kris Gevaert (1,2)

(1)Department of Medical Protein Research, VIB, B-9000 Ghent, Belgium
(2)Department of Biochemistry, Ghent University, B-9000 Ghent, Belgium
(3)Division of Infection and Immunity, UCL, London, UK

We present a novel proteomics design for systematic identification of protease cleavage events by quantitative N-terminal proteomics, circumventing the need for time-consuming manual validation. We bypass the singleton detection problem of protease-generated neo-N-terminal peptides by introducing differential isotopic proteome labeling such that these substrate reporter peptides are readily distinguished from all other N-terminal peptides. Our approach was validated using the canonical human caspase-3 protease and further applied to mouse cathepsin D and E substrate processing in a mouse dendritic proteome, identifying the largest set of protein protease substrates ever reported and gaining novel insight into substrate specificity differences of these cathepsins.

Keywords: degradomics, quantitative proteomics, cathepsin substrates

P-54 Mutant HSPB8 causes motor neuron specific neurite degeneration

Joy Irobi1,3, Leonardo Almeida-Souza1,3, Bob Asselbergh1,3, Vicky De Winter1,3, Sofie Goethals 1,3, Ines Dierick1,3, Jyothsna Krishnan4, Jean–Pierre Timmermans5, Wim Robberecht4, Peter De Jonghe2,3,6, Ludo Van Den Bosch4, Sophie Janssens1,3, Vincent Timmerman*1,3

(1)Peripheral Neuropathy and (2)Neurogenetics Groups, Department of Molecular Genetics, VIB and University of Antwerp;
(3)Neurogenetics Laboratory, Institute Born-Bunge and University of Antwerp;
(4)Laboratory of Neurobiology and Experimental Neurology, Vesalius Research Center, VIB and University of Leuven;
(5)Laboratory of Cell Biology and Histology, Department of Veterinary Sciences, University of Antwerp; 6Division of Neurology, University Hospital of Antwerp, Belgium.

Missense mutations (K141N and K141E) in the &α;-crystallin domain of the small heat shock protein HSPB8 (HSP22) cause distal hereditary motor neuropathy (distal HMN) or Charcot-Marie-Tooth neuropathy type 2L (CMT2L). The mechanism through which mutant HSPB8 leads to a specific motor neuron disease phenotype is currently unknown. To address this question, we compared the effect of mutant HSPB8 in primary neuronal and glial cell cultures. In motor neurons, expression of both HSPB8 K141N and K141E mutations clearly resulted in neurite degeneration, as manifested by a reduction in number of neurites per cell, as well as in a reduction in average length of the neurites. Furthermore, expression of the K141E (and to a lesser extent, K141N) mutation also induced spheroids in the neurites. We did not detect any signs of apoptosis in motor neurons, showing that mutant HSPB8 resulted in neurite degeneration without inducing neuronal death. While overt in motor neurons, these phenotypes were only very mildly present in sensory neurons and completely absent in cortical neurons. Also glial cells did not show an altered phenotype upon expression of mutant HSPB8. These findings show that despite the ubiquitous presence of HSPB8, only motor neurons appear to be affected by the K141N and K141E mutations which explains the predominant motor neuron phenotype in distal HMN and CMT2L.

Keywords: CMT: Charcot-Marie-Tooth disease dHMN Distal hereditary motor neuropathy HSPB8: Small heat shock proteins 22kDa (HSP22), Motor neurons, Neurite degeneration

P-55 Sensitization of aggressive B-lymphomas to TRAIL-induced apoptosis by the plant flavonoid quercetin

Jacquemin Guillaume, Granci Virginie, Lalaoui Najoua, Iessi Elisabetta And Micheau Olivier

INSERM U866 "Lipids, Nutrition, Cancer", Faculty of Medicine, 7 Bd Jeanne d'Arc, 21000 Dijon, FRANCE

TNF-related apoptosis-inducing ligand (TRAIL) and its receptors are attractive targets for anticancer therapy owing to their ability to trigger apoptosis selectively in cancer cells but not in normal cells. However, a major problem is that some cancer cells are or become resistant to TRAIL-induced cell death. In this light, combined treatments seem to be the best way to by-pass the resistance to TRAIL. Recently, natural compounds from plants have become a popular subject of investigation. Among these phytochemicals, the flavonoid quercetin exhibits promising anti-carcinogenic properties. This polyphenol is present in fruits and vegetables, and has been shown to have anti-proliferative and pro-apoptotic effects on cancer cells, but not on normal cells. In this study, we aimed to sensitize two aggressive and highly resistant non-Hodgkin's B lymphoma cell lines to TRAIL-induced apoptosis, using quercetin. These cancerous cells are resistant to TRAIL because of a chromosomal translocation t(14:18) which leads to the constitutive expression of the anti-apoptotic protein Bcl-2, and in turn blocks the mitochondrial pathway. Our results demonstrate that quercetin can efficiently sensitize these lymphomas in a caspase-dependant manner. Synergistic activation of the apoptotic machinery occurs downstream of the Death Inducing Signalling Complex (DISC), through a restoration of the mitochondrial pathway of apoptosis. Interestingly, Bcl-2 was not regulated by quercetin. However, the inhibition of Mcl-1 and survivin by quercetin could be responsible for the activation of the mitochondria. Finally, a deeper understanding of the mechanisms of this synergy is needed to establish an effective combination therapy using TRAIL and quercetin against aggressive lymphomas.

Keywords: Cancer, apoptosis, TRAIL, polyphenol, quercetin, mitochondria, Mcl-1, survivin

P-56 Platinum(IV) derivate LA-12 increases TRAIL-induced apoptosis by upregulation of DR5 and stimulation of mitochondrial events in colon cancer cells

Jelínková I.(1,2), Vondálová Blanářová O.(1,2), Skender B. (1,2), Souček K. (1), Sova P.(3), Hofmanová J.(1,2), Kozubík A.(1,2), Vaculová A.(1)

(1)Department of Cytokinetics, Institute of Biophysics, AS CR, v.v.i., Brno, Czech Republic
(2) Institute of Experimental Biology, Faculty of Science, Masaryk University Brno, Czech Republic,3R&D, Pliva-Lachema, a.s., Brno, Czech Republic

Platinum(IV) drugs have been introduced as any important approach in the treatment of solid tumours. We demonstrated that novel adamantylamine Pt(IV) complex LA-12 is highly effective in various cancer cells and able to overcome resistance to conventional platinum(II) drug cisplatin. TRAIL (TNF-related apoptosis inducing ligand), a TNF-family cytokine, represents a potent and selective inducer of apoptosis in many cancer cell types. Although some of them are still resistant to its apoptotic effects, it may be possible to overcome this resistance by combination of TRAIL with chemotherapeutic agents such as platinum compounds. We observed that pretreatment of human colon cancer cells with LA-12 increased death receptor DR5 but not DR4 surface expression, which resulted in enhanced apoptosis following incubation with TRAIL. The functional role of DR5 in these effects was confirmed by specific DR5 siRNA. In type II cancer cells, apoptotic signaling from DRs requires amplification via the mitochondrial pathway, and these events are tightly controlled by Bcl-2 family proteins. Using Bax-deficient HCT116 cells, we also demonstrated an important role of mitochondria and pro-apoptotic Bcl-2 family proteins in LA-12-mediated potentiation of TRAIL-induced apoptosis. In summary, we showed that modulation of DR5 level and mitochondrial engagement are important events in sensitization of colon cancer cells to TRAIL-induced apoptosis mediated by LA-12.

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Keywords: colon cancer, TRAIL, LA-12

P-57 Identification of a novel pro-apoptotic role of NF-κB in the regulation of death receptor-mediated apoptosis of glioblastoma cells

Claudia Jennewein (1), Sabine Karl (1), Bernd Baumann (2), Klaus-Michael Debatin (1) and Simone Fulda (1)

(1) University Children's Hospital
(2) Department of Physiological Chemistry, Ulm University, Ulm, Germany

The transcription factor NF-κB is generally considered as anti-apoptotic, thus causing resistance to anti-cancer treatment in several tumor types. However, a growing body of evidence indicates that NF-κB can also promote apoptosis in a cell type- and stimulus-dependent manner. Therefore, a better knowledge of NF-κB-regulated signaling is crucial to exploit the NF-κB pathway for cancer therapy. Glioblastoma is the most aggressive primary tumor of the brain and highly resistant to current anti-cancer treatments. Here, we explore the role of NF-κB in death receptor-mediated apoptosis signaling in glioblastoma by engineering glioblastoma cell lines that stably express the dominant-negative mutant IκBα-superrepressor (IκBα-SR) that cannot be degraded upon NF-κB activation.

Interestingly, we found that in several glioblastoma cell lines inhibition of NF-κB profoundly reduces TRAIL- and CD95-induced apoptosis as determined by DNA fragmentation in a dose- and time-dependent manner. Instead, NF-κB inhibition significantly increases apoptosis upon TNF-α stimulation, in line with the well-established anti-apoptotic function of NF-κB upon TNF-α treatment.

Mechanistic studies revealed that apoptosis markers such as activation of caspases, cleavage of Bid and mitochondrial perturbations were significantly reduced upon NF-κB blockage. The decreased sensitivity towards death receptor-mediated cell death was neither caused by NF κB dependent alterations in the expression of apoptosis-regulating proteins (i.e. c-FLIP, Bcl2 family members and IAPs), nor by alterations in the surface expression of death receptors. Intriguingly, we found a NF-κB dependent modulation at a key apical event in death receptor-induced apoptosis, namely the formation of a death-inducing signaling complex (DISC).

By demonstrating that inhibition of a NF-κB reduces DISC formation and apoptosis upon TRAIL and CD95 treatment, our findings will contribute to unraveling yet unknown regulators of death receptor-induced apoptosis and molecular mechanisms that govern pro- or anti-apoptotic effects of NF-κB.

Keywords: glioblastoma, NF-κB, TRAIL

P-58 Identifying ER stress induced apoptotic proteases with “click-chemistry” based mass spectrometry

Lars Jöckel (1,2,3), Karen Schrader (1), Bernhard Breit (4), Monika Lutterbeck (4) and Christoph Borner (1,3)

(1) Institute of Molecular Medicine and Cell Research, Albert-Ludwigs-University Freiburg, D-79104 Freiburg, Germany
(2) GRK 1104, From Organs to Cells, Albert-Ludwigs-University Freiburg, D-79104 Freiburg, Germany
(3) SGBM, Albert-Ludwigs-University Freiburg, D-79104 Freiburg, Germany
(4) Institute for Organic Chemistry and Biochemistry, Albert-Ludwigs-University Freiburg, D-79104 Freiburg, Germany

Apoptosis induced by Endoplasmic Reticulum (ER) stress has been associated with an increasing number of pathologies like neurodegenerative disorders and diabetes over the last two decades. Nevertheless, not much is known about the signaling pathways and proteins implicated in this type of cell stress.

It is known that apoptosis induced by ER stress proceeds via both caspase-dependent and -independent processes in multiple mammalian cell lines. The caspase-independent step involves a component that acts upstream of mitochondrial membrane permeabilization and which can be blocked by pan-serine protease inhibitors such as AEBSF. In order to unravel this component (or components) we synthesized an alkyne version of AEBSF. This probe was used to “click” a biotin tag onto the modified inhibitor after its cellular uptake and covalent binding to the target protein(s) in order to perform pull down experiments and subsequent mass-spectrometry analysis. This method is termed “click chemistry” a technique, which allows clicking two compounds irreversibly together in a copper-catalysed azide-alkyne reaction in vitro.

Using this method we obtained some promising candidates, which we are currently validating. This will allow us to better understand the signaling pathway from ER stress to mitochondria-mediated apoptosis.

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

P-59 TLR-2 and -9 are sensors of apoptosis in a mouse model of doxorubicin-induced acute inflammation

Agnieszka Kaczmarek,(1,2) Dmitri V. Krysko,(1,2) Jerzy Woznicki,(1,2)Olga Krysko,(3)Stefan Magez,(4,5) Anje Cauwels,(1,2) Pieter Bogaert,(1,2) Nozomi Takahashi,(1,2) Claus Bachert(3), Peter Vandenabeele(1,2)

(1)Department for Molecular Biomedical Research, VIB, 9052 Ghent, Belgium;
(2)Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium;
(3)Upper Airway Research Laboratory, Department of Oto-Rhino-Laryngology, Ghent University Hospital, Ghent, Belgium; (4)Laboratory for Cellular and Molecular Immunology, Vrije Universiteit Brussel, Brussels, Belgium;
(5)Department of Molecular and Cellular Interactions, VIB, Brussels, Belgium

Anthracycline antibiotics are inducers of an immunogenic form of apoptosis that has immunostimulatory properties due to the release of damage associated molecular patterns. To study the mechanisms used by the innate immune system to sense the immunogenic form of cell death, we established an in vivo model of cell death induced by intraperitoneal injection of doxorubicin, a prototype of anthracyclines. The acute sterile inflammation in this model is characterized by a rapid influx of neutrophils and increased levels of IL-6 and MCP-1. We demonstrate that acute inflammation induced by doxorubicin is mediated by apoptosis. The inflammatory response was significantly reduced in MyD88, TLR-2 and TLR-9 deficient mice. Importantly, a TLR-9 antagonist reduced the recruitment of neutrophils induced by doxorubicin. By contrast, the acute inflammatory response was not affected in TRIFLps2 mutant mice and in TLR-3, TLR-4 and caspase-1 knockout mice. The latter finding shows that the inflammasome does not play a major role in doxorubicin-induced acute inflammation. Altogether these data indicate that MyD88 is essential for sensing apoptotic cells killed in situ by an immunogenic chemotherapeutic agent primarily due to its function as an adaptor molecule in the TLR-2 and TLR-9 signaling pathways. Our data indicate that inhibition of the TLR-2/TLR-9-MyD88 pathways in vivo could reduce the acute inflammation-induced tissue damage caused by doxorubicin when it is used in the clinic as an intraperitoneal treatment for carcinomatosis arising from gastrointestinal and gynecologic malignancies.

Keywords: doxorubicin, TLRs, apoptosis, neutrophils, DAMPs, immunogenic cell death

P-60 Inhibition of autophagy sensitizes cells to drug-mediated caspase-dependent and -independent apoptosis via increasing of ROS

Vitaliy Kaminskyy and Boris Zhivotovsky

Institute of Environmental Medicine, Division of Toxicology, Karolinska Institutet, Box 210, SE-171 77 Stockholm, Sweden

Autophagy is a catabolic process involved in turnover of intracellular content. Although autophagy has been described as a mode of cell death, its role in this process remains controversial. Here we show that inhibition of autophagy stimulated ROS formation in cancer cells and the treatment with cisplatin or etoposide had a synergistic effect on formation of ROS. However, autophagy inhibition sensitized non-small cell lung carcinoma cells to cisplatin-induced apoptosis, but diminished the etoposide-induced cell death. Sensitization of cells to cisplatin was in part dependent on caspases, since a pan-caspase inhibitor, zVAD-fmk, only partially prevented the cell death measured by externalization of annexin-V, suggesting the role of caspase-independent apoptosis. In this experimental system the cell death was completely dependent on ROS, because scavenging of ROS prevented the release of cytochrome c and completely abolished the sensitization of the cells to cisplatin-induced apoptosis when autophagy was suppressed. Furthermore, inhibition of ROS prevented the processing and release of Apoptosis Inducing Factor from mitochondria. It is likely that formation of ROS induced by inhibited autophagy does not kill the cells per se. For instance, inhibition of autophagy weakens the effect of etoposide which affect progression through the cell cycle by delay of the cell growth. Indeed, inhibition of autophagy delayed accumulation of cells in S phase upon treatment with etoposide and, therefore, diminished the execution stage of apoptosis induced by this drug. Thus, our data suggest that autophagy inhibition sensitizes cells to caspase-dependent and -independent apoptosis via increasing formation of ROS; however the inhibition of autophagy may attenuate the cell death if the killing efficiency of the drug depends on the cell cycle progression.

Keywords: autophagy, cancer, apoptosis

P-61 p57KIP2 enhancement of staurosporine-induced apoptosis requires LIMK1-mediated actin stabilisation

Edel Kavanagh, Pinelopi Vlachos, Violaine Emourgeon, Johanna Rodhe, Bertrand Joseph

Cancer Centrum Karolinska, Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden

p57KIP2 is rarely mutated in cancers, yet it has been shown to have potential anti-cancer properties. Originally described as a member of the cdk inhibitor family, it has since been shown to play a role in other processes such as the inhibition of cell migration. This is mediated by binding of p57KIP2 to LIMK1, increasing the kinase activity of the latter, resulting in the inactivation of the actin binding protein cofilin, and stabilisation of actin filaments. Furthermore, p57KIP2 has been shown to enhance cell death induced by staurosporine (STS). This study endeavors to show that p57KIP2 enhancement of cell death is through stabilisation of actin filaments/cytoskeleton. Using p57KIP2 Tet-ON inducible HeLa cells, it was shown that overexpression of p57KIP2 enhanced STS-induced apoptosis (22% in p57KIP2 Tet-On cells vs 11% in p57KIP2 Tet-OFF cells).

Use of Jasplakinolide, an actin stabilising agent, increased cell death induced by STS as measured by Hoechst staining (31.3% apoptosis) and increased PARP cleavage, thereby mimicking the cell death-enhancing effect of p57KIP2. Additionally, Jasplakinolide increased the loss in mitochondrial transmembrane potential induced by STS . This indicates that stabilisation of actin can mimic the cell death promoting effect of p57KIP2.

When p57KIP2 overexpressing cells were pretreated with the actin destabilising agent cytochalsin D prior to STS treatment, there was a decrease in cell death when compared with STS -treated cells not expressing p57KIP2 (7.2 % vs 22.2% DEVDase activity). Cytochalsin D pretreatment also prevented p57KIP2 -mediated loss in mitochondrial transmembrane potential, indicating that destabilisation of the actin cytoskeleton reverses p57KIP2 enhancement of STS -induced apoptosis.

p57KIP2 has been shown to stabilise actin filaments by increasing the activity of LIMK1, a kinase which targets cofilin, resulting in cofilin inactivation and actin stabilisation. Use of siRNA against LIMK1 was shown to be required for p57KIP2 enhancement of STS -induced apoptosis. Knockdown of LIMK1 reversed the pro-apoptotic effect of p57KIP2 on cell death as measured by Hoechst staining (5.8 % with LIMK1 siRNA vs 11.3 % with scrambled sequence siRNA).

Together these results show that stabilisation of actin filaments are required for the pro-apoptotic effect of p57KIP2.

Keywords: p57, actin dynamics, cell death

P-62 Protective effect of elastase inhibitors against necrotic cell death

Boris Khalfin (1), Keren Schultz (2), Amnon Albeck (2) and Ilana Nathan (1,3)

(1) Ben-Gurion University of Negev, Beer Sheva, Israel,
(2) Bar Ilan University, Ramat Gan, Israel
(3) Soroka Medical Center, Beer Sheva, Israel

Necrotic cell death has been long considered uncontrollable and unavoidable. However, recent findings challenged this concept, indicating that necrosis may be a regulated process, and new agents against necrotic death are under development. Among them are poly (ADP-ribose) polymerase inhibitors and necrostatins, shown to inhibit Rip-1 kinase activity.

We demonstrate that necrosis triggered by several inducers and in cell lines of different lineage, was abolished by cell permeable peptidic or heterocyclic elastase inhibitors. We have synthesized new peptidyl trifluoromethane inhibitors of elastase-like proteases and their antinecrotic effects were assessed. We observed that induction of necrosis caused an early and significant increase in intracellular elastase-like activity determined by substrate-gel electrophoresis and elastase activity assay. The induced elastase-like activity was inhibited in cells treated with a permeable elastase inhibitor. Due to the treatment necrosis was abrogated. In a model of closed skull injury in rats, we observed that intracerebrospinal administration of an elastase inhibitor reduced the severity of neurological damage and decreased the volume of the necrotic tissue.

In the present study, we show that elastase inhibitors prevented necrotic cell death in both in vitro and in vivo models. Since there is no effective treatment of necrosis, the use of these inhibitors may provide a basis for new strategies in treatment of necrosis associated diseases.

Keywords: Necrosis, Elastase inhibitors

P-63 Sulforaphane inhibits doxorubicin cardiotoxicity through mitochondrial stabilization and inhibition of NF-κB

Do-Sung Kim(1,2), Geum-Hwa Lee(1,2), Li Bo(1,2), Hak-Yong Lee(1,2), Piao cheng shi(1,2), Young-Rae Lee(3), Seoung Ju Park(2,4), Yong Chul Lee(2,4), Wan Hee Yoo(2,5), Soo-Wan Chae(1), Hyung-Ryong Kim(6), and Han-Jung Chae(1,2)

(1) Department of Pharmacology and BK21 MED, Institute of Cardiovascular Research, Chonbuk National University, Jeonju, South Korea.
(2) Research Center for Pulmonary Disorder, Chonbuk National University Hospital, Jeonju, South Korea.
(3) Department of Biochemistry, Chonbuk National University,Jeonjuu, South Korea.
(4) Department of Internal Medicine and Airway Remodeling Laboratory, School of Medicine, Chonbuk National University, Jeonju, South Korea.
(5) Division of Rheumatology, Department of Internal Medicine, Chonbuk National University, Jeonju, South Korea.
(6) Department of Dental Pharmacology, School of Dentistry, Wonkwang University, Iksan, South Korea.

Doxorubicin (DOX) is one of the most effective chemotherapeutic drugs; however, its incidence of cardiotoxicity compromises its therapeutic index. DOX-induced heart failure is thought to be caused by reduction/oxidation cycling of DOX to generate oxidative stress and cardiomyocyte cell death. Sulforaphane is an organosulfur compound that exhibits anticancer, antidiabetic, and antimicrobial properties. It is obtained from cruciferous vegetables such as broccoli. Sulforaphane has been reported to play a cardioprotective role in disease associated with oxidative stress. The objective of this study was to test the ability of sulforaphane to protect against DOX-induced cardiomyocyte death. We hypothesized that sulforaphane protects cardiomyocytes from DOX-induced oxidative stress and subsequent cell death through changes in mitochondrial function. Furthermore, Sulforaphane inhibits DOX-induced cardiomyocytes death through inhibition of NF-κB. DOX induced a rapid increase in reactive oxygen species (ROS) production in cardiac cell mitochondria, which was inhibited by pretreatment of sulforaphane, most likely owing to an increase in Mn-SOD protein expression and Mn-SOD activity. This effect of sulforaphane caused additional polarization of the mitochondria in the absence and presence of DOX to increase mitochondrial function. Sulforaphane pretreatment also prevented DOX-induced cardiomyoctye death. In response to DOX, NF-κB was activated in cardiomyocytes. However, pretreatment of sulforaphane reduced the activation of NF-κB. Our previous data shown that PDTC (NF-κB inhibitor) and IκB α-dominant negative plasmid-overexpression abrogated the cytotoxic effect on ADR-induced cardiomyocyte death. Our data suggest that sulforaphane protects against DOX-induced cariomyocyte death through inhibition of mitochondrial ROS generation and NF-κB activation. Sulforaphane can be a potential candidate agent which protects cardioxicity in DOX-exposed patients.

Keywords: sulforaphane, doxorubicin, apoptosis, oxidative stress, NF-κB

P-64 Loss of CG6783, a novel lipid-carrier protein, disrupts autophagy and endocytosis in Drosophila

Viktor Kis (1), Gyorgy Csikos (1), Monika Lippai (1), Tamas Maruzs (1), Peter Lorincz (1), Tamas Lukacsovich (2), Peter Maroy (3), Miklos Sass(1)

(1) Department of Anatomy, Cell and Developmental Biology, Eotvos Lorand University, Pazmany Peter setany 1/C, 1117 Budapest, Hungary
(2) Department of Developmental and Cell Biology; Developmental Biology Center; University of California Irvine, Irvine, California, United States of America
(3) Hungarian Academy of Sciences, Biological Research Center, Institute of Genetics, Szeged, Hungary

Autophagy is a highly conserved bulk degradative process of eukaryotic cells from yeast to human. It has an essential role in differentiation, development, homeostasis through the elimination of organelles, unnecessary or misfolded proteins. Autophagy protects organisms against diverse pathological processes, including bacterial infections, cancer, myopathies, neurodegenerative diseases, and aging.

To search for the genes involved in the regulation or mechanism of autophagy a genetic screen has been performed on a Drosophila melanogaster mutant collection carrying modified transposable element insertions.

We have identified the gene CG6783 which encodes a novel, yet non-characterized lipid-carrier protein. In the lack of the gene product the mutant larval fat body cells were unable to form autolysosomes and endocytotic protein granules. RT-PCR and Western blot analysis were performed in this mutant strain to confirm the decrease in the expression of the CG6783 gene. In addition CG6783 silenced clone cells showed less mCherryAtg8 (Drosophila homologue of the human LC3-II marker of autophagosomes) positive puncta than the neighboring control cells after starvation. By immunohistochemistry we proved that the silenced fat body cells were unable to take up larval serum proteins and form protein granules, which certifies CG6783's role in endocytosis. The localisation of the protein was analyzed by immunostaining and using transgenic flies carrying GFP-tagged CG6783 protein.

In conclusion the investigations discussed above unambiguously confirm CG6783's role in autophagy and in endocytosis, together with the yet unexplored connection between lipid-carrier proteins and the autophagosomal-lysosomal system.

Keywords: autophagy, endocytosis, lipid-carrier protein, Drosophila

P-65 Retinoids induce apoptosis in mouse thymocytes via inducing Nur77 expression

Beata Kiss (1), Katalin Toth (1),Zsolt Sarang (1), Laszlo Fesüs (1) and Zsuzsa Szondy (1)

(1)Department of Biochemistry and Molecular Biology, University of Debrecen, Egyetem tér 1., H-4010 Debrecen, Hungary

Vitamin A deficiency has been known for a long time to be accompanied by immune deficiency and susceptibility to a wide range of infectious diseases. In vitamin A deficient animals, a marked atrophy of the thymus and the spleen has been observed. Suggestions have been made that the active metabolites of vitamin A that mediate its effects on the immune system are the retinoic acids (RA)—all-trans RA and 9-cis RA—which are ligands for the nuclear retinoic acid receptor family. Previous studies in our laboratory have demonstrated that retinoids can induce apoptosis in mouse thymocytes by ligating RARgamma . In the present study Affymetrix 430Av2 microarray method was used to identify, which genes are regulated by retinoids. We found induction of Nur77 transcription factor, the overexpression of which is known to induce apoptosis in thymocytes. Nur77 knock out thymocytes did not die in the presence of retinoids suggesting a central role for Nur77 in retinoid-induced apoptosis. Retinoids induced the expression of several genes in a Nur77-dependent manner including FasL and TRAIL, ligands for cell surface cell death receptors, NDG1, which was reported to induce apoptosis via activating caspase 8 by unknown mechanisms, and Bid, a protein that is known to trigger Bax dependent apoptosis following cleavage by caspase-8. In addition, we found that Nur77 translocates into the mitochondria, where it can inhibit the antiapoptotic function of Bcl-2. Our data suggest that to initiate apoptosis retinoids reprogram thymocytes from type I cells to type II cells and induce a cell death ligand mediated death via the mitochondrial pathway of apoptosis.

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Keywords: retinoids, Nur77, mitochondrial apoptotic pathway

P-66 Changes of cytokinetic parameters and adhesive properties in colon cancer cell populations with different sensitivity/resistance to TRAIL

Koci Lenka, Hyzdalova Martina, Vaculova Alena, Hofmanova Jirina, Kozubik Alois

Department of Cytokinetics, Institute of Biophysics, Academy of Sciences of the Czech Rep. v.v.i., Kralovopolska 135, Brno 61265, Czech Republic,

Colorectal cancer is one of the most common cancers worldwide. Healthy colonic epithelium is an excellent example of a tissue that maintains strict equilibrium between proliferation, differentiation and apoptosis. The interactions between epithelial cells and extracellular matrix (ECM) are essential for organization of colon crypt tissue and play a role in signaling of important cytokines. TNF-related apoptosis inducing factor (TRAIL), a member of TNF family, is particularly interesting for its unique properties to induce death of cancer cells while sparing most normal cells, which is promising for its application in the anticancer therapy. However, many cancer cells including colon have been reported to be resistant to the apoptotic effects of TRAIL.

We studied cytokinetic parameters and adhesive properties of TRAIL-sensitive and TRAIL-resistant cell populations derived from HCT116 human colon cancer cell line. Moreover, to assess the importance of cell anchorage type, we cultivated isolated cell populations in ECM protein-coated (collagen IV) or non-coated plastic surface. Our data show that TRAIL-resistant populations of HCT-116 cells exert significant changes in the expression of adhesive molecules when compared to sensitive ones, which can further be modulated by cultivation surface type, and have impact on final cell survival. We observed an increase of the integrin α2 and beta1 levels associated with lower apoptotic response in TRAIL-resistant cell population cultivated on collagen-coated compared to non-coated plastic. To conclude, in cancer cells that do not respond to TRAIL-induced apoptosis, incubation with this cytokine may result in significant changes in cell adhesive molecules, which could have impact on final cell response and tumor development.

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Keywords: TRAIL, adhesion, apoptosis

P-67 Use of a panel of anti-HSP70 monoclonal antibodies for detection of HSP70 translocated to cell surface during lymphocyte apoptosis

Elena I. Kovalenko, Anna A. Boyko, Alexander V. Zyablitsin, Sergei S. Vetchinin, Alexander M. Sapozhnikov

Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry RAS

Intracellular 70 kDa heat shock proteins (HSP70) can be also detected on the surface of tumor, virus-infected, stressed and apoptotic cells. The reasons and mechanisms of HSP70 translocation to the cell surface, as well as structural features of HSP70 embedding into plasma membrane are still unknown. It has been shown that surface-associated HSP70 of tumor cells can activate cytotoxic effectors of immune system. In this connection it is interesting to identify the regions of cell surface presented-HSP70 accessible for intercellular interaction. With this purpose we used elaborated panel of six monoclonal antibodies directed to different epitopes of HSP70. Among this series three antibodies interact with C-domain (clones 2E4, 2F3, 2E11), and other three antibodies – with N-domain of HSP70 (clone 6G2, 2E5, 3C5), that was demonstrated by ELISA and Western-blot analysis. The study was carried out using in vitro culture of EL-4 mouse lymphoma cell line. Surface HSP70 were detected by flow cytometry. Our previous data showed that EL-4 cells expressed HSP70 on the cell surface.

It has been revealed that all tested monoclones bound HSP70 exposed on the surface of both live and apoptotic cells. However, the monoclones differed by the level of surface HSP70 binding and by ratio of HSP70 positive and negative cells. Most interesting data have been obtained by analysis of population of apoptotic cells characterized by high density of surface HSP70. It was demonstrated that monoclones specific to C-domain of HSP70 (2E4, 2F3, 2E11) stained the surface of apoptotic cells in 10-20 times lesser in comparison with monoclones specific to N-domain of the protein (6G2, 2E5, 3C5). These results suggest the ordered orientation of HSP70 exposed on the surface of apoptotic cells, connected with embedding of C-domain of the protein into the cell plasma membrane.

Keywords: surface expression, HSP70

P-68 Inhibitory effect of unsaturated fatty acids on cell death induced by saturated fatty acids in human pacreatic beta-cells

Jan Kovar and Vlasta Nemcova-Fürstova

Dept. of Cell and Molecular Biology & Center for Research of Diabetes, Metabolism and Nutrition, Third Faculty of Medicine, Charles University, Ruska 87, 10000 Prague 10, Czech Republic

Chronically elevated levels of fatty acids in blood contribute to pancreatic beta-cell loss in type 2 diabetes. It is known that saturated fatty acids can induce apoptosis in pancreatic beta-cells and that unsaturated fatty acids seem to be able to counteract this effect. However, the precise molecular mechanisms are unclear. In this study we have tested the effect of unsaturated fatty acids on the proapoptotic effects of saturated fatty acids in the human pancreatic beta-cells NES2Y. Experiments were carried out in chemically defined serum-free media containing individual fatty acids bound to bovine serum albumin (BSA). Cell death in nearly all cells was induced by saturated fatty acids (palmitic acid and stearic acid) at a concentration of 1 mM (bound to 2% BSA). We found that unsaturated fatty acids (palmitoleic acid and oleic acid) at a concentration of 0.2 mM and higher concentrations are able to inhibit completely the proapoptotic effect of saturated fatty acids at a concentration of 1 mM. Cell death induced by stearic acid was associated with significant activation (approximately 4-fold) of caspase-2, caspase-8 and caspase-9. Surprisingly, we did not detect the activation of caspase-3. The activation of caspases was blocked by coincubation with 0.2 mM oleic acid. Stearic acid treatment was not associated with a significant change in mitochondrial membrane potential, reactive oxygen species (ROS) level and with cytochrome c release from mitochondria. Furthermore, stearic acid treatment was not associated with changes in p21, PIDD and Fas receptor expression. However, we detected a significant upregulation of endoplasmatic reticulum (ER) stress marker BiP which was inhibited by coincubation with oleic acid. Taken together, we can conclude that cell death induced by stearic acid in human pancreatic beta-cells NES2Y is accompanied by the activation of caspase-2, caspase-8 and caspase-9, but not by caspase-3 activation. Presented data indicate that oleic acid inhibits apoptosis induction by stearic acid in NES2Y cells upstream of caspase activation and ER stress induction. The effect of oleic acid does not involve an interference with the mitochondrial pathway of apoptosis induction and with p53 activation as well as PIDD and Fas receptor expression.

This work was supported by research program MSM0021620814 of the Third Faculty of Medicine, Charles University, Prague.

Keywords: unsaturated fatty acids, saturated fatty acids, cell death, pancreatic beta-cells, caspases, ER stress

P-69 Molecular mechanisms of apoptosis induction by the p53 tumour suppressor family

Max Koeppel (1), Leonie Smeenk (2), Simon van Heeringen (2), Daniela Kramer (1), Bianca Gilbert (1), Hendrik G. Stunnenberg (2), Marion Lohrum (1)

(1) Georg-Speyer-Haus, Paul-Ehrlich-Str. 42-44, 60596 Frankfurt, Germany
(2) Dept. of Molecular Biology, NCMLS, Radboud University Nijmegen, 6500 HB Nijmegen, The Netherlands

The tumour suppressor p53-family plays a crucial role in cellular growth regulation inducing a plethora of cellular response pathways ranging from transient growth arrest to apoptosis. The molecular mechanisms that discriminate between the different p53 family-responses towards stress treatments have remained largely elusive. Here, we have analyzed the p53 and p73-regulated transcriptional pathways under different stress conditions in order to elucidate the molecular basis for the distinction between growth arrest and apoptosis. We found in ChIP-Seq experiments that growth arrest and apoptosis-inducing treatments result in very similar genome-wide p53 binding patterns notwithstanding transcriptional differences causing distinctive cellular responses. To find the molecular mechanism leading to different transcriptional respones, we have analyzed the role of p53 posttranslational modifications in target gene choice, in particular p53-phosphorylation and acetylation. Also the various isoforms of p73 can cause distinct apoptotic responses. Here, we have studied apoptosis induction by the two most widely expressed isoforms of TAp73, TAp73α and beta. We obtained evidence that p73-induced apoptosis is influenced by a crosstalk with c-Jun. This crosstalk is p73-isoform dependent possibly explaining the different apoptosis induction potential of TAp73α and beta. Thus, global genomic analyses of p53 and p73 under various conditions reveal the role of p53-modifications and p73-isoform expression for the distinctive cellular growth arrest and apoptosis responses.

Keywords: p53 tumour suppressor family, apoptosis induction

P-70 TAT-crmA blocks both apoptosis and necroptosis

Stefan Krautwald (1), Andreas Linkermann (1) and Ulrich Kunzendorf (1)

(1) Department for Nephrology and Hypertention, Christian-Albrechts University Kiel, 24105 Kiel, Germany

In search for an inhibitor of apoptosis that provides effective blockage of both the intrinsic and extrinsic apoptotic pathway, we analyzed viral proteins that evolved to protect the infected host cells. In particular, the cowpox virus protein crmA has been demonstrated to be capable of blocking key caspases of both pro-apoptotic pathways. To deliver crmA into eukaryotic cells, we fused the TAT protein transduction domain of HIV to the N-terminus of crmA. In vitro, the TAT-crmA fusion protein was efficiently translocated into target cells and inhibited apoptosis mediated through caspase-8, caspase-9 and caspase-3. Interestingly, in FADD-deficient Jurkat, HT-29 and L929 cells, TAT-crmA additionally blocked necroptosis. In vivo, the extrinsic apoptotic pathway was investigated following anti-Fas stimulation where 90% of TAT-crmA-treated animals survived an otherwise lethal dose of anti-Fas and showed protection from Fas-induced organ failure. To test the ability to comprehensively block both the intrinsic and extrinsic apoptotic pathway and prevent necrosis in a clinically relevant setting, we employed a murine cardiac ischemia-reperfusion model. TAT-crmA reduced infarction size by 40% and preserved left ventricular function. Strikingly, the size of the central necrotic zone of the infarction was reduced by > 70%, whereas the area at risk remained unchanged. Our results highlight the therapeutical potential of TAT-crmA in rescuing ischemia-induced organ failure.

Keywords: crmA, viral cell death blockers, myocardial infarction, ischemia-induced organ failure

P-71 Effects of STAT3 silencing on fate of melanoma and lung cancer cells.

Dorota W. Kulesza (1), Salem Chouaib (2), Bozena Kaminska (1)

(1) The Nencki Institute of Experimental Biology, Warsaw, Poland.
(2) INSERM U753, Institut Gustave Roussy, Villejuif, France.

STAT3 is one of the critical players in human cancer formation and represents a valid target for novel anticancer drugs. This transcription factor is persistently activated due to tyrosine phosphorylation in many cancer cell lines and tumors, where growth factor signaling is frequently dysregulated. Active STAT3 promotes uncontrolled growth and survival through deregulation of gene expression. Moreover, it modulates tumor microenvirenment by participating in regulation of tumor immune evasion, and thereby contributes to oncogenesis.

We sought to determine consequences of STAT3 silencing in human melanoma and non small lung carcinoma cells on their proliferation (measured by BrdU incorporation assay) and cell death (MTT metabolism assay). The effect of STAT3 silencing with plasmid-transcribed shRNA differs in various cancer cell lines and is dependant on the level of constituvely activated STAT3. In transiently transfected 2 melanoma cell lines with a high level of activated STAT3, its depletion affects proliferation and induces apoptosis suggesting its role as a crucial viability factor. In contrast, silencing of STAT3 in A549 cells has no impact on cell survival and cell lines with stably depleted of STAT3 have been developed.However, STAT3 silencing sensitizes A549 and to some extend melanoma cells to doxorubicin treatment suggesting that activated STAT3 contributes to drug resistance in cancer cells. Furthermore, in lung cancer A549 cells STAT3 silencing modifies cellular responses to hypoxia conditions. Silencing of constitutively activated STAT3 makes A549 cells more prone to NK-induced tumor target cell killing mediated by the perforin/granzymes pathway (established using a classical cytotoxicity test).

Together, our data indicate a crucial role of STAT3 in survival of melanoma cells and show participation of constitutively activated and hypoxia induced STAT3 in tumor resistance to drug- as well as immune-mediate toxicity in A549 cells.

Keywords: STAT3, chemoresistance, hypoxia

P-72 Towards understanding of life and death regulation at CD95/Fas

Nicolai Fricker(1,3), Carina Pforr(1), Roland Eils(2,3,4) Peter H. Krammer(1) and Inna N. Lavrik (1, 3)

(1)Division of Immunogenetics, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany
(2)Division of Theoretical Bioinformatics, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany
(3)Bioquant, University of Heidelberg, 69120 Heidelberg, Germany
(4)Department for Bioinformatics and Functional Genomics, Institute for Pharmacy and Molecular Biotechnology, University of Heidelberg, 69120 Heidelberg, Germany

CD95 (APO-1/Fas) is a member of the death receptor family. CD95 stimulation leads to the induction of apoptotic as well as non-apoptotic pathways. CD95 signaling starts with the formation of the death-inducing signaling complex (DISC). The DISC consists of CD95, the adaptor molecule FADD (Fas-Associated Death Domain), procaspase-8, procaspase-10 and c-FLIPL/S/R (the cellular FLICE-inhibitory proteins). The regulation of life and death decisions at the DISC is largely influenced by the ratio between pro- and anti-apoptotic DED (death effector domain)–containing proteins and is poorly understood. We have applied a systems biology approach to understand the regulation of life/death decisions in CD95 signaling pathway in quantitative terms. In our experiments we have used HeLa cells overexpressing various components of the CD95 system. Stimulation of HeLa cells has led to the induction of both apoptotic and non-apoptotic pathways depending on the concentration of the CD95 DISC components. We have quantitatively analyzed apoptotic and non-apoptotic responses applying single-cell and population-based measurements. Based on these experimental data a mathematical model of the CD95 DISC has been constructed. The model takes into account the complex interplay between various DED-containing proteins of the DISC, e.g. procaspase-8 and c-FLIP isoforms as well as their cleavage products. The application of systems biology allowed to identify new mechanisms of regulation of CD95 signaling on the quantitative level and has demonstrated how exact concentrations of c-FLIP and procaspase-8 and their cleavage products at the DISC can determine life/death decisions.

Keywords: CD95/Fas, c-FLIP, caspase-8, DISC, systems biology

P-73 Characterization of BARD1 expression and function in leukaemia

Ilaria Lepore (1), Dell'Aversana Carmela (1,2), Floriana De Bellis (1), Marco Miceli (1), Francesco Paolo Tambaro (1), Angela Nebbioso (1) & Lucia Altucci (1)

(1) Department of General Pathology, Seconda Università degli Studi di Napoli, vico L. De Crecchio 7, 80138, Naples, IT.
(2) Department of Sperimental Pathology and Microbiology, Università di Messina-consolare valeria, 1 - 98125 Messina, IT.

BARD1 (BRCA1-associated RING domain protein 1) was discovered as BRCA1 interactor partner, a well known tumour suppressor muted in most cases of breast and ovarian cancer, as well as BRCA2. The main BARD1 functions are BRCA1-dependent based on the formation of a stable heterodimer through the RING finger domains interaction. In last years, also BRCA1-independent functions are emerging, making BARD1 an interesting and dynamic research target.

BARD1 is expressed in almost all human tissues, in particular in haematological cells, testis and breast, but it is over-expressed in leukaemias, sarcomas and testis cancer, suggesting an hypothetical role of BARD1 in cancer development. In the literature, different BARD1 isoforms are up-regulated in breast, ovarian and uterine cancers but markedly down-regulated or absent in healthy tissues are mentioned. This observation led to suppose that the presence of these isoforms might be considered a risk factor, or vice versa, a causal event in the cancer pathogenesis.

The aim of our work has been to investigate the role of BARD1 isoforms in leukaemia, through the study of the epigenetic mechanisms involved in the regulation of its expression and function.

We could show that a specific BARD1 isoform is expressed in different human leukaemia cell lines and that its expression is affected by the treatment with the well known HDAC (histone deacetylases) inhibitor SAHA (suberoylanilide hydroxamic acid, Vorinostat). We also demonstrated that BARD1 down-regulation is due to the action of two specific miRNAs (micro-RNA), that directly bind its 3'UTR region. These data will be further discussed.

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Keywords: tumour suppressor, epigenetic, miRNA.

P-74 Oligomerization and activity of soluble CD95L

Clarissa Liesche (1), Michael Schwering (2), Dirk-Peter Herten (2) Roland Eils (1,3), Joel Beaudouin (1)

(1)Division of Theoretical Bioinformatics, German Cancer Research Center (DKFZ), Heidelberg, Germany
(2)Department for Physical Chemistry, University of Heidelberg and Bioquant, Germany
(3)Department for Bioinformatics and Functional Genomics, Institute for Pharmacy and Molecular Biotechnology (IPMB) and Bioquant, Heidelberg University, Heidelberg, Germany

CD95 Ligand (CD95L) is a cytokine that exists as a membrane form on Natural killer (NK) cells and Cytotoxic Lymphocytes (CTLs) and as a soluble form (sCD95L) upon proteolytic cleavage. This soluble form harbors the receptor binding domain. Regarding the function of CD95L and sCD95L, they may both induce either apoptosis or pathways related to survival and proliferation in CD95 receptor expressing target cells. The timing and efficiency of pathway activation depends on the one hand on the type of target cell and on the other hand on the oligomerization state and extend of clustering of CD95L, high level of oligomerization helping CD95 activation.

Regarding sCD95L, trimerization was only shown at unphysiological high protein concentrations but the soluble ligand may dissociate into monomers at low concentrations.

The correlation between oligomerization state of sCD95L at physiological concentrations and its activity is still poorly understood. In our study we investigate both the oligomerization state and activity of sCD95L at nM-concentration in order to characterize this correlation. In order to find the oligomerization state, we use a fluorescence based approach called single molecule imaging. In order to study the activity, we use time resolved confocal laser scanning microscopy to image target cells expressing fluorescent reporters for caspase activity or NF-kB induction.

We show for the first time that sCD95L exists as a trimer at physiologically relevant concentrations. Furthermore, we compared the activity of sCD95L with an artificially trimerized form of CD95L by measuring different parameters including the onset of caspase activation and timing of apoptosis. These measurements, combined with the single molecule imaging, indicate that sCD95L exists potentially as a monomer-trimer mix. With the help of kinetic modeling the results are also theoretically described.

Keywords: soluble CD95L, single moleule imaging,

P-75 COX-2 overexpression confers resistance to ursolic acid-induced apoptosis in human HT-29 colorectal cancer cells

Youness Limami, Aline Pinon, David Y. Leger, Jeanne Cook-Moreau, Jean L. Beneytout, Christiane Delage, Alain Simon and Bertrand Liagre

Université de Limoges, EA 4021 "Biomolécules et thérapies anti-tumorales", Faculté de Pharmacie, 87025 Limoges, France

BACKGROUND

Colorectal cancer is a major health concern worldwide. It is one of the most common cancer types and the third leading cause of cancer-related death in the western world. Generally, colorectal cancers are resistant to anticancer drugs. Several lines of evidence support a critical role for cyclooxygenase-2 (COX-2) during colorectal tumorigenesis and its role in chemoresistance. COX-2 is progressively overexpressed in the transition from normalcy to malignancy. Given this, the comprehension of COX-2 role in resistance to apoptosis seems to be important to develop effective anticancer drugs.

AIM

In this study, we focused our interest on the role played by COX-2 in apoptosis induced in HT-29 human colorectal cancer cells by ursolic acid (UA), a triterpenoid found in a large variety of plants and vegetarian foods.

DISCUSSION

In this work, we showed that UA inhibited HT-29 cell proliferation in a dose and time-dependent manner and that inhibition of cell proliferation was correlated with an induction of an apoptotic process. Furthermore, we observed that COX-2 was overexpressed only in apoptotic cells. We, then, demonstrated the role of p38 as a COX-2 transcription factor. Indeed, inhibition of the p38 pathway in UA-treated cells led to COX-2 inhibition but led unexpectedly to an increase in apoptosis. This result suggests a role of COX-2 in resistance to apoptosis. Involvement of COX-2 in resistance to apoptosis was confirmed using COX-2-specific siRNA. In our model, these results demonstrated that COX-2 overexpression in apoptotic cells delays cell death, and support a major role for COX-2 in colorectal cancer chemoresistance.

FUTURE DIRECTIONS

Having shown that COX-2 overexpression in apoptotic cells plays a role in delaying cell death, we wish to determine whether this resistance to apoptosis via COX-2 is dependent on its activity.

Keywords: ursolic acid, colorectal cancer, apoptosis resistance, COX-2

P-76 Fas Ligand Mediates Fratricide of Tubular Cells in vivo

Andreas Linkermann (1), Nina Himmerkus (2), Ulrich Kunzendorf (1) and Stefan Krautwald (1)

(1) Clinic for Nephrology and Hypertension, Christian-Albrechts University, Schittenhelmstr. 12, 24105 Kiel, Germany
(2) Institute for Physiology, Christian-Albrechts University, Olshausenstr. 12, 24118 Kiel, Germany

The apoptosis inducing death factor Fas Ligand (FasL) is best characterized for its function in T-cells and NK-cells regarding the clearance of virally infected cells and the termination of an immune response by activation-induced cell death, a process referred to as fratricide. Recent reports have suggested a physiologically relevant expression of FasL in the kidney. We treated SCID/beige mice with the FasL-neutralizing monoclonal antibody MFL3 that completely restored survival after otherwise lethal CIN, suggesting a functionally relevant source of FasL in renal homeostasis besides immune cells. In order to analyze whether kidney cells, like T-cells, die in a FasL-dependent manner after cisplatin-stimulation, we isolated segments of the thick ascending limb (TALs) from mouse kidneys and determined FasL-mediated apoptosis, which could be blocked with MFL3 in the complete absence of immune cells. Furthermore, cisplatin-stimulated primary tubular cells induced apoptosis in TALs freshly isolated from GFP-transgenic mice. This renal-cell fratricide could be blocked by MFL3. We conclude that CIN is mediated though FasL, which is functionally expressed on fratricide-inducing tubular cells. Our observations reveal an additional mechanism that significantly contributes to organ failure besides the infiltration of FasL-bearing immune cells into the kidney.

Keywords: Fas Ligand, kidney, apoptosis, fratricide

P-77 XIAP interaction with FAF1 prevents apoptotic effects in TNFα and FASL mediated pathways

López-Rodríguez, M., Yunta, M., Navarro-Ruiz, R.M., Nieto-Díaz, M., Reigada, D., Pita-Thomas, D. W., Lindholm D2., Maza, R.M1.

(1) Laboratorio de Neuroprotección Molecular, Dpto. de Neurología Experimental, Hospital Nacional de Parapléjicos, SESCAM, Toledo, Spain.
(2) Minerva Medical Research Institute, Biomedicum Helsinki, Helsinki, Finland

XIAP (X-linked Inhibitor of Apoptosis Protein) –a member of the inhibitory of apoptosis protein (IAP) family– is able to interfere with cell death process by inhibiting caspases cascade and regulating intracellular signalling pathways (AKT, JNK, NF-κB). To further understand the citoprotective role of XIAP and identifying novel interacting proteins that regulate/modulate its cellular function, we perform a yeast two-hybrid screening. We have identified and characterized the pro-apoptotic FAF1 as new XIAP-interacting protein. The Human Fas Associated Factor-1 (hFAF1) is one of the recently described components of the death-inducting signalling complex in Fas-mediated apoptosis. This protein has a great variety of biologic functions, such as, apoptosis regulation or proteaosome dependent degradation process. It has been shown that overexpressed hFAF1induces apoptosis without extrinsic cell-death signals and modulates TNFα-induced NF-κB pathway. Using coimmunoprecipitation assays, our results have clearly shown the interaction of FAF1 with XIAP and the specific domain of interaction. These results were confirmed by colocalization assays with immunocitochemistry and confocal microscopy. We have studied the functional implications of this interaction both cell death regulation and intracellular signalling and we have observed that XIAP is able to block FAF1-mediated cell death and interfere in the NF-KB pathway inhibition by FAF1. Finally, we have shown a degradative polyubiquitination of FAF1 by XIAP. Assessing the balance of both factors XIAP/FAF1 will shed light on their role in degenerative and cancerous processes.

Keywords: apoptosis, XIAP, caspases, NF-KB

P-78 An Allosteric Network of Interactions in Caspase-3 Provides a Novel Strategy for Inhibitor Design

Sarah H. MacKenzie (1) and A. Clay Clark (1)

(1) Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, NC, USA

Caspase-3, the terminal protease in the apoptotic cascade, has potential as a therapeutic target for a wide variety of diseases including neurodegenerative disorders, heart disease and stroke. Mutational studies in the dimer interface of caspase-3 showed that activity was completely abolished by replacing the central valine (V266) with histidine. Structural studies of the V266H mutant and other caspase-3 (V266H) variants revealed a network of interactions that start in the dimer interface and culminate in the active site which disrupt interactions in loop L1 and prevent substrate binding when the interface region is perturbed. Specifically, M61 adopts an alternate rotomer which prevents substrate binding to the S1 site. Recently, our lab has started to design inhibitors that link these two sites (V266 and S1) as a way to perturb the allosteric network with small molecules. The data presented here will highlight the structural details of the allosteric network as well as our progress in inhibitor design.

Keywords: caspase-3, allosteric inhibition,

P-79 The Role of Hypoxia-induced Macroautophagy in Melanomagenesis

Maes H. (1), Nys K. (1), Wouters J. (2), Vankelecom H. (3), Van den Oord J. (2), De Vos R. (2), Carmeliet P. (4), Garmyn M. (5), Agostinis P. (1).

(1) Laboratory of Cell Death Research and Therapy, Dept. of Molecular Cell Biology, Catholic University of Leuven, Belgium;
(2) Morphology and Molecular Pathology Section, Dept. of Pathology, Catholic University of Leuven, Belgium;
(3) Laboratory of Tissue Plasticity, Dep. of Molecular Cell Biology, Catholic University of Leuven, Belgium;
(4) Vesalius Research Center, Dept. Of Molecular and Cellular Medicine, Catholic University Leuven, Belgium;
(5) Laboratory of Dermatology, Dept. Of Experimental Medicine, Catholic University of Leuven, Belgium.

Cutaneous Malignant Melanoma (CMM), the most lethal type of skin cancer, is largely refractory to existing therapies and has a very poor prognosis, urging better understanding and new therapeutic approaches for this devastating disease. Hypoxia is an important micro-environmental factor for melanocytes and primary CMM (residing in the mildly hypoxic basal layers of the epidermis) as well as within the solid CMM tumor (due to insufficient as well as malfunctioning blood supply). Hypoxia has emerged as a synergistic factor in melanocyte transformation (Bedogni et al 2005) and CMM therapy resistance (Sanna et al 1994). Decreased availability of oxygen is a cellular stress known to stimulate autophagy, a conserved lysosomal pathway for the recycling of cytoplasmic materials, including proteins and damaged organelles. The role of this catabolic process in carcinogenesis remains largely unclear and both a tumor suppressor as well as a tumor promoter role have been reported.

In this study, we examined the relevance of skin mild hypoxic status (1.5-5% O2) in autophagy stimulation and the role played by this process in melanomagenesis. We found that skin mild hypoxia confers a growth advantage to both melanocytes and CMM cell lines representing different melanoma progression stages, which was associated with the induction of autophagy. However, the basal autophagic flux was substantially higher in melanoma cells than in the melanocytes, suggesting that cancer cells may depend more pronouncedly on this catabolic process to cope with stress. Dissection of the molecular pathways revealed mitochondrial superoxide generation, PHD2, HIF-1a and BNIP3 as the key molecular players involved in autophagy induction as well as the selective removal of damaged mitochondria through autophagy (mitophagy) under hypoxia. Intriguingly, blockage of autophagy (using pharmacologic inhibitors or siRNA-mediated knock down) under hypoxia inhibited growth and induced apopotosis in CMM cell lines, but not in melanocytes. The induction of apoptosis in the cancer cell lines was preceded by a disruption of the mitochondrial network, mitochondrial swelling and an increase in mitochondrial ROS, leading eventually to mitochondrial membrane permeabilisation. All together, these data suggest that mild hypoxia-driven mitophagy confers stress tolerance and increases CMM fitness under metabolic stress conditions while being dispensable to sustain the survival of normal melanocytes. This notion, which our ongoing in vivo experiments appear to support, may open a window for new therapeutic approaches in CMM.

Keywords: Melanomagenesis, Cancer, Autophagy, Hypoxia, Apoptosis

P-80 Interaction between cIAP1 and RhoGTPases

Arthur Marivin(1), Jessy Cartier(1), Jean Berthelet(1), Alban Dupoux(1), Eric Solary(1,2) & Laurence Dubrez-Daloz (1)

(1)INSERM UMR866, Université de Bourgogne, Dijon, France.
(2)INSERM UMR1009, Institut Gustave Roussy, Villejuif, France

cIAP1 is a cell signaling molecule that displays an E3 ubiquitin activity. Lastly, oncogenic properties have been attributed to cIAP1. We previously showed that cIAP1 regulates cell cycle and cell proliferation through its capacity to control E2F1 transcription factor activity. Along differentiation, cIAP1 is excluded from the nuclei to the cytoplasm where it regulates NF-κB activation signaling pathway.

In order to study cIAP1 activities and its regulation, we initiated a research of new partners and identified an interaction of cIAP1 with members of the Rho Family of small G proteins. RhoGTPases are described as molecular switches able to regulate cytoskeleton organization and cell signaling. Thereby these GTPases regulates many functions (morphology, motility, trafficking, gene expression) by interacting and modulating their numerous target effectors.

In the first part of my work, we confirmed an interaction in vitro and in vivo of cIAP1 with the most studied RhoGTPase members (RhoA, Rac1 and Cdc42) with a higher affinity for cdc42.

In a second part, we analyzed the capacity of cdc42 to regulate cIAP1 activities. A Cdc42 dominant negative form inhibited the cIAP1-mediated NF-κB activation suggesting that cdc42 is important for the cytoplasmic activity of cIAP1. Moreover, inhibition of Cdc42 increased the ubiquitination of cIAP1 and decreased its level of expression suggesting the role of cdc42 in the stability of the protein.

In the last part we studied the influence of cIAP1 on cdc42 activity. Interestingly, we observed that cIAP1 could increase the level of expression and the activity of Cdc42. Cdc42 is a regulator of actin polymerisation, and thereby is involved in the regulation of cytoskeleton-dependent process (morphology, motility and phagocytosis). We observed a modification of the actin network in MEF cIAP1-/- in a similar manner as in MEF Cdc42-/-. Moreover, downregulation of cIAP1 inhibits cell motility and phagocytosis process, suggesting that cIAP1 is a regulator of cdc42 activity.

To conclude, we identified a new interaction between cIAP1 and RhoGTPases in human cells. Cdc42 could be a regulator of cIAP1 localisation, activity and stability. In return, cIAP1 could control the activity of cdc42.

Keywords: IAP, RhoGTPases, Cytoskeleton,

P-81 Tandem Reporter Assay for Myristoylated Proteins Post-translationally (TRAMPP) identifies caspase-cleaved Huntingtin as a novel substrate.

Dale D.O. Martin, Chrisselle Y. Ahpin, Ryan J. Heit, Maneka A. Perinpanayagam, Luc G. Berthiaume

Department of Cell Biology, University of Alberta, Edmonton, Alberta, Canada

Myristoylation involves the addition of a 14-carbon fatty acid to the N-terminal Gly of a protein and is key to protein-protein and protein-membrane interactions. Typically, myristoylation occurs co-translationally on the nascent polypeptide. However, post-translational myristoylation (PTMyr) of caspase cleaved proteins is now emerging as a well-established protein modification and potentially as an important regulator of apoptosis. Indeed, myristoylation potentiated the effects of the caspase-cleaved C-terminal (ct) fragments of Bid, Gelsolin, p21 activated kinase 2 (PAK2) and actin. In order to identify additional PTMyr proteins we generated a caspase cleavable reporter protein named Tandem Reporter Assay for Myristoylation of Proteins Post-translationally (TRAMPP). The reporter protein consists of tdTomato(Red)-DEVD-"a test myr-sequence"-EGFP(Green). This vector allows for the facile insertion of DNA sequences encoding for putative myristoylatable protein sequences downstream of known caspase cleavage sites identified by computational prediction analysis. Following induction of apoptosis, the reporter protein is cleaved by caspases, which frees a new N-terminal Gly that can be myristoylated. We combined this reporter construct with a sensitive novel chemical biology detection technique (Yap et al. 2010, J. Lipid Res) that employs an alkynyl-myristate analogue to label cells followed by Click chemistry with azido-biotin for Neutravidin-HRP/ECL detection. As such, we validated that pTRAMPP-ctPAK2 is specifically cleaved and myristoylated at the newly exposed N-terminal Gly. Furthermore, using pTRAMPP-ctPAK2 and live cell microscopy allowed us to visualize PTMyr and membrane association as it occurs in cells. This new assay confirmed the PTMyr of the putative substrates cytoplasmic dynein intermediate chain 2A (ctCD-IC2A) and protein kinase C epsilon (ctPKCepsilon) previously identified in Martin et al. 2008, FASEB J. In addition, and excitingly, we identified five more caspase-cleaved potential substrates for myristoylation that include the anti-apoptotic regulator of apoptosis, Mcl-1, and the protein responsible for Huntinton's Disease, Huntingtin.

Keywords: Myristoylation, Huntingtin

P-82 FLIP down-regulation and CHOP-independent up-regulation of TRAIL-R2 induced by ER stress are key events in the sensitization of tumor cells to TRAIL

Rosa Martín-Pérez and Abelardo López-Rivas

Centro Andaluz de Biología Molecular y Medicina Regenerativa (CABIMER), Consejo Superior de Investigaciones Científicas, Sevilla, Spain

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potentially useful anticancer agent with selectivity for cancer cells. However, tumor cells of diverse origin are often resistant to TRAIL. In this study we report that inducing endoplasmic reticulum (ER) stress by the N-glycosylation inhibitor tunicamycin or GRP78 knockdown sensitizes tumor cells to TRAIL-induced apoptosis. ER stress treatments alter the cellular levels of different apoptosis-related proteins including a decline in the levels of FLIP and Mcl-1 and the up-regulation of TRAIL-R2. We have also examined the mechanisms underlying the changes observed in apoptotic proteins. Silencing of CAAT/enhancer binding protein homologous protein (CHOP) by RNA interference neither inhibit the up-regulation of TRAIL-R2 protein levels nor prevent sensitization to TRAIL-induced apoptosis by ER stress. Silencing of TRAIL-R2 expression by siRNA or over-expression of FLIPL partially blocks the sensitization to TRAIL-induced apoptosis. Furthermore, knockdown of TRAIL-R2 expression in FLIPL over-expressing cells completely abrogated sensitization to TRAIL. In summary, our results indicate that ER stress by regulating the expression of key proteins in the TRAIL signaling pathway is a potential target for intervention to sensitize tumor cells to TRAIL.

Keywords: FLIP. CHOP, TRAIL, ER STRESS, CANCER, TRAIL-R2

P-83 Identification of a novel protein kinase C delta – SMAC complex that is dissociated during apoptosis

Katarzyna C. Masoumi (1), Gry K. Lonne (1), Louise Cornmark (1), Ulf Hellman (2) and Christer Larsson (1)

(1) Lund University, Department of Laboratory Medicine, Center for Molecular Pathology, Malmo University Hospital, Malmo, Sweden
(2) Ludwig Institute for Cancer Research, Uppsala University, Box 595, SE-751 24 Uppsala, Sweden

Cell death suppression is relevant for tumor development, progression, and resistance to therapy. To treat cancer it would be beneficial to identify agents that induce the death of cancer cells. Our aim is to characterize survival regulating pathway/s in breast cancer cells, which are controlled by protein kinase C delta (PKC delta). PKC delta is a key enzyme in the regulation of apoptosis in various cellular systems. PKC delta can act as a pro- or anti-apoptotic kinase depending on the specific cell type and stimulus. However, the mechanisms underlying its diverse effects are not understood.

We have screened and identified the pro-apoptotic protein Smac as a PKC delta binding partner in MDA-MB-231 breast cancer cells. The association was also found in other breast cancer cell lines. Confocal analysis indicated that Smac and PKC delta co-localize in mitochondria. Upon induction of cell death the PKC delta-Smac interaction is disrupted and prevention of the disassociation is accompanied by suppressed cell death. PKC delta associates with Smac via the C1b motif in the regulatory domain. Full length and Δ55 Smac bind to PKC delta, which indicates that IAP-binding motif at the N terminus of mature Smac is required for binding to PKC delta.

In conclusion, our data unravel a previously unrecognized interaction between PKC delta and Smac and identify PKC delta as a major regulator for Smac mediated apoptosis in breast cancer cells.

Keywords: protein kinase C, Smac, apoptosis, breast cancer cells

P-84 The influence of Vitamin C and Tempol on cytotoxic and apoptogenic action of myosmine

Mateva R., Boteva B., Iliev I., Gugova R., Gorneva G.

Department of Molecular Design and Biochemical Pharmacology, Institute of Molecular Biology, BAS 21, "Acad. Bonchev" Street, 1113 Sofia, Bulgaria

Myosmine was one of the first structural identified tabacco alkaloids besides nicotine. The occurrence of myosmine is not limited to solanaceae like nicotine. Myosmine has been identified in various foods including staple foods like wheat, maize, rice and milk as well as in different fruits and vegetables. Myosmine nitrosation and peroxidation yield the esophageal carcinogen N-nitrosonornicotine, classified as "carcinogenic to humans" by the IARC and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB). Some researchers connect the rapidly increasing incidents of an adenocarcenome of the oesophagus in Europe with the possible cancerogenic activity of the myosmine. The role of myosmine as a dietary risk factor is supported by the studies demonstrating a highly significant positive correlation between HPB-releasing adducts in the mucosa of the lower esophagus and body mass index, a major risk factor for esophageal adenocarcinoma.

Results and discussion. Results of our investigation demonstrated the ability of myosmine to inhibit cell proliferation and change cell cycle kinetics. The mode of cytotoxic action depends on concentration of myosmine: at low concentrations it was growth inhibition, at concentrations 200-300 mkM – cytostatic action with the significant increase the percentage of cells in G2/M phase, at 350-400 mkM – induction of apoptosis and appearance of hipoploide fraction , at 1 mM – induction of necrosis. This activity of myosmine was significantly enhanced, when cell culture was incubated with inductors of oxidative stress. Antioxidants (Vitamine C and TEMPOL) exerts moderate effect on cytotixic and apoptogenic action of myosmine.

Conclusion. The elevated oxidative stress in cells may be an important contribution to the risk assessment of myosmine exposure.

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Keywords: cancerogenic activity, myosmine, oxidative stress, antioxidants

P-85 Functional analysis of the antiapoptotic oncoprotein Aven

Inga Maria Melzer(1), Sebastian Kehrlösser(2), Julia Moser(1), Sara Mateus Fernández(1,3) and Martin Zörnig(1)

(1)Georg-Speyer-Haus, Paul-Ehrlich-Str. 42-44, 60596 Frankfurt, Germany
(2)Institute of Biophysical Biochemistry, Biocenter, Goethe-University Frankfurt a.M., Max-von-Laue-Str. 9, 60438 Frankfurt am Main, Germany
(3) National Center for Biotechnology, CSIC, Madrid, Spain

The protein Aven, which has recently been assigned an additional function in DNA damage repair had originally been described as an antiapoptotic interactor of Apaf-1 and Bcl-XL. Additionally, Aven overexpression has been correlated with bad prognosis in childhood acute lymphoblastic leukemia, placing this protein in focus as a promising target for chemotherapeutic approaches.

In our laboratory, the C-terminal half of Aven (deltaN-Aven) has been identified in a functional yeast survival screen for new anti-apoptotic oncoproteins. We were able to demonstrate that Aven needs to be processed by a yet unknown protease at its N-terminus to inhibit Apaf-1/Casp-9-mediated apoptosis in mammalian cells. Using different approaches, we aimed to identify the corresponding N-terminal protease which unleashes Aven´s anti-apoptotic potential and tried to map the actual cleavage site(s). We were also able to identify an additional cleavage of Aven at its C-terminus by Caspase-3 which inactivates Aven´s antiapoptotic function after removing the last 69 aa. By mapping of interaction sites of Aven with itself, with Apaf-1 and also with Caspase-9 we are currently trying to elucidate the different steps of the mechanism by which Aven prevents apoptosis.

As N-terminal processing (and, as such, antiapoptotic activity) of Aven was pronounced in the human breast cancer cell line MCF-7, we established a knockdown of hAven by stably transducing these cells with hAven shRNA and investigated the tumorigenic properties of the knockdown cells using a mouse xenograft model.

Keywords: breast cancer, apoptosis, protein cleavage, apoptosome, leukemia

P-86 Intracellular GTP level determines cell fate by differentiation and apoptosis

Azadeh Mezshkini (1) Razieh Yazdanparast (1)

(1) Institute of Biochemistry and Biophysics, University of Tehran ,Enghelab Ave. , Tehran , Iran

Since the provision of adequate supply of guanine nucleotides is vital for many aspects of cellular metabolism and signaling pathways, limitation of their synthesis might result in growth inhibition, differentiation or apoptosis. A point for speculation is whether a sensing mechanism exists in cells that monitor guanine nucleotide levels, essential for each particular cellular response. The aim of this study was to disclose the effect of different level of GTP depletion on signaling pathways and consequently cell fate. In that regard, we measured intracellular GTP level among K562 cells exposed to mycophenolic acid (MPA) or 3-hydrogenkwadaphnin (3-HK) as potent inosine monophosphate dehydrogenase inhibitors. Our results revealed that maturation developed when intracellular GTP level reduced 30-40% of the control level. Under this experimental condition, both 3-HK and MPA caused marked up-regulation of PKCα; and PI3K/AKT pathways; however these responses along with differentiation were abrogated by exogenous addition of guanosine and restoration of intracellular GTP. Further, co-treatment of cells with hypoxanthine plus 3-HK or MPA, caused the reduction of intracellular GTP level to less than 60% of the control value which led to occurrence of apoptosis. Under this circumstance, mitochondrial pathway was activated along with inverse regulation of Bcl-2/Bax expression and finally activation of caspase-3. Moreover, our results demonstrated that reduction of GTP level to less than 60% caused the augmentation of intracellular ROS formation and nuclear localization of p21 and subsequently cell death. Collectively, these results suggest that a threshold level of GTP is needed to initiate apoptosis and oxidative stress rather than differentiation.

Keywords: Intracellular GTP level, mycophenolic acid, 3-hydrogenkowadaphnine, K562, differentiation, apoptosis

P-87 Requirement for Arginine Methylation in FOXO3a-mediated G1 Arrest of WEHI-231 B Lymphoma Cells upon Engagement of B Cell Receptor for Antigen

Junichiro Mizuguchi and Kikumi Hata

Department of Immunology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo, 160-8402, Japan

Engagement of B cell receptor for antigen (BCR) on WEHI-231 B lymphoma cells, cell line model representative of primary immature B cells, results in growth arrest and subsequent apoptosis. WEHI-231 cells have been used as a model to analyze antigen-induced B cell unresponsiveness. FOXO3a, a member of Forkhead family proteins, migrated into the nucleus upon BCR engagement. FOXO3a is a transcriptional factor that plays a crucial role in inducing either growth arrest or apoptosis, probably through transcriptional up-regulation of Bim, Btg1, and p27. WEHI-231 were transfected with expression vector containing Btg1, a member of Btg/Tob family proteins, and the cells expressing Btg1 accumulated in G1 phase at higher proportions than controls. Btg1 bound to protein arginine methyltransferase (PRMT)1 through box C region, which is required for Btg1-mediated growth inhibition. We employed active form of FOXO3a, FOXO3a3A:estrogen receptor (ER) fusion construct which allows induction of FOXO3a by 4-hydroxytamoxifen. Btg1-or anti-IgM-induced growth inhibition was partially blocked by arginine methyltransferase inhibitor AdOx or shRNA-mediated knockdown of PRMT1. In contrast, the G1 arrest induced by the constitutively active form of FOXO3a was substantially resistant to PRMT1-knockdown in WEHI-231 cells. Together, BCR-mediated G1 arrest appears to involve FOXO3a translocation into the nucleus probably at least through induction of Btg1 and p27. The maintenance of FOXO3a activity requires PRMT1, probably through arginine methylation. These results may uncover the complexity of regulation of multifunctional transcriptional factor FOXO3a.

Keywords: B cells, FOXO3a, Apoptosis

P-88 Characterization of Active Caspase-14 From Human Epidermis and Development of the Cleavage

Eriko Fujita (1), Toshihiko Hibino (2) and Takashi Momoi (1)

(1) Center for Basic Science, International University of Health and Wealfare, Ohtawara, Tochigi 287-8501, Japan
(2)Shiseido,Life Science Research Center, 2-12-1 Fukuura, Kanazawa-ku, Yokohama 236-8643, Japan

Restricted expression of caspase-14 in differentiating keratinocytes suggests the involvement of caspase-14 in terminal differentiation. We purified active caspase-14 from human cornified cells with sequential chromatographic procedures. Specific activity increased 764-fold witha yield of 9.1%. Purified caspase-14 revealed the highest activity on WEHD-methylcoumaryl-amide (MCA), although YVAD-MCA, another caspase-1 substrate, was poorly hydrolyzed. The purified protein was a heterodimer with 17 and 11 kDa subunits. N-terminal and C-terminal analyses demonstrated that the large subunit consisted of Ser6-Asp146 and N-terminal of small subunit was identified as Lys153. We successfully developed an antiserum (anti-h14D146) directed against the Asp146 cleavage site, which reacted only with active caspase-14 butnot with procaspase-14. Furthermore we confirmed that anti-h14D146 did not show any reactivity to the active forms of other caspases. Immunohistochemical analysis demonstrated that anti-h14D146 staining was mostly restricted to the cornified layer and co-localized with some of the TUNEL positive-granular cells in the normal human epidermis. UV radiation study demonstrated that caspase-3 was activated and co-localized with TUNEL-positive cells in the middle layer of human epidermis. In contrast, we could not detect caspase-14 activation inesponse to UV. Our study revealed tightly regulated action of caspase-14, in which only the terminal differentiation of keratinocytes controls

its activation process.

Keywords: Caspase-14, apoptosis, keratinocytes, skin

P-89 BH4 domains of Bcl-2 and Bcl-Xl differentially regulate apoptosis by modulating inositol 1,4,5-trisphosphate receptors activity.

Giovanni Monaco (1), Elke Decrock (2), Tim Ulens (3), Haidar Akl (1), Ludwig Missiaen (1), Marc De Maeyer (3), Luc Leybaert (2), Clark W. Distelhorst (4), Jan B. Parys (1), Humbert De Smedt (1) & Geert Bultynck (1)

(1)Laboratory of Molecular and Cellular Signaling, Campus Gasthuisberg O&N I, K.U.Leuven, 3000 Leuven,Belgium; (2)Department of Basic Medical Sciences, UGent, 9000 Ghent,Belgium; (3)Biochemistry, Molecular and Structural Biology Section, K.U. Leuven, 3001, Heverlee, Belgium; (4)Departments of Medicine and Pharmacology,Case Western Reserve University,Comprehensive Cancer Center, Cleveland, OH 44106, USA

Anti-apoptotic members of the Bcl-2 family, like Bcl-2 and Bcl-Xl, control cell fate by modulating intracellular Ca2+ signals. Recently, we have found that Bcl-2 via its BH4 domain directly interacts with the central, modulatory domain (aa 923-1581: “domain 3”) of the inositol 1,4,5-trisphosphate receptor (IP3R), thereby suppressing pro-apoptotic Ca2+ signals and protecting cells against pro-apoptotic stimuli (Rong YP et al., 2008, Mol Cell, 31:255-65; Rong YP et al., 2009, Proc Natl Acad Sci U S A, 106:14397-402). Although Bcl-2 and Bcl-Xl show high similarities in sequence and structure, they seem to differentially regulate IP3Rs. Therefore, we examined the role of the BH4 domain of Bcl-2 and Bcl-Xl in the regulation of IP3R-dependent Ca2+ signaling and apoptosis.

First, using GST-pull downs, we found that only the BH4 domain of Bcl-2 is able to strongly interact with the domain 3 of the IP3R, whereas Bcl-Xl displayed a much weaker interaction. Furthermore, both BH4 domains did not significantly interact with the C-terminal tail (domain 6: aa 2590-2749) of the IP3R. Similar results were obtained using surface plasmon resonance experiments, showing that GST-domain 3 displayed the strongest binding to the biotin- coupled BH4 domain of Bcl-2 (Kd: ~ 1 microM).

Second, in unidirectional 45Ca2+ flux experiments in permeabilized mouse embryonic fibroblasts (MEF), the BH4 domain of Bcl-2 potently inhibited IP3-induced Ca2+ release (IICR) (IC50: ~35 microM), whereas the BH4 domain of Bcl-XL did not.

Third, we compared the anti-apoptotic properties of the BH4 domain of Bcl-2 and Bcl-Xl after electroporating C6 glioma cells with purified cytochrome C (CytC). The apoptotic index (AI) of the electroporated cells was determined using the fluorescent CaspACE tm FITC-VAD-FMK In Situ Marker (Promega). Again, the BH4 domain of Bcl-2 was much more effective (AI: ~40 % of control) than the BH4 domain of Bcl-Xl (AI: ~70 % of control) in protecting cells against CytC. Furthermore, the protective effect of the BH4 domain of Bcl-2 was completely abolished (AI: ~ 95 % of control) by co-electroporation of a peptide corresponding to the Bcl-2-binding site of the IP3R (aa 1389-1408), whereas this peptide did not affect the protective effect of the BH4 domain of Bcl-Xl.

Fourth, we mapped the residues that are responsible for the inhibitory effects of the BH4 domain of Bcl-2 and found three amino acids (Lys17, Tyr21 and Arg26) to be essential. Importantly, of these three residues, only Lys17 is not conserved in Bcl-Xl and corresponds there to an aspartate residue (Asp11). Strikingly, changing Lys into Asp in the BH4 domain of Bcl-2 is sufficient to abolish its inhibitory effects on the IP3R, whereas changing Asp into Lys in the BH4 domain of Bcl-Xl is sufficient to obtain inhibitory effects on the IP3R.

In conclusion, this study revealed that although the BH4 domain of Bcl-2 and Bcl-Xl are very similar in sequence and structure, they differentially regulate IP3Rs and inhibit apoptosis by divergent mechanisms.

Keywords: anti-apoptotic Bcl-2 family members, BH4 domain, IP3Rs, ER

P-90 Regulation of Programmed Necrosis by Cylindromatosis (CYLD)

David Moquin (1,2) and Francis Ka-Ming Chan (1,2)

(1)Department of Pathology
(2)Immunology and Virology Program
University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655, USA

It is now clear that when caspases are inhibited, cytokines such as TNF induce programmed necrosis/necroptosis. Receptor interacting protein I (RIP1) was the first protein identified as being critical for programmed necrosis. Recently, the involvement of receptor interacting protein 3 (RIP3) in programmed necrosis was also identified. TNF stimulation causes RIP1 and RIP3 association, which mediates programmed necrosis through the RIP Homotypic Interaction Motifs (RHIMs) and the kinase domains. In order to identify other genes involved in programmed necrosis, a cancer gene siRNA library was screened. We identified the gene Cylindromatosis (CYLD) as another regulator of TNF-induced programmed necrosis. CYLD is a tumor suppressor with deubiquitinase activity. Previous studies indicate that CYLD negatively regulates NFκB activation, JNK activation, Bcl-3 nuclear localization, and RIG-I mediated type I interferon production. Germline mutations that disrupt CYLD's enzymatic activity cause the formation of skin tumors of the head and neck. We validated the requirement of CYLD for TNF-induced programmed necrosis by RNA interference and by genetic ablation of CYLD. These results strongly implicate a role for protein ubiquitination and deubiquitination in the regulation of programmed necrosis. The mechanism by which protein ubiquitination and deubiquitination regulates programmed necrosis will be discussed.

Keywords: Necrosis

P-91 Role of apoptosis in the pathogenesis of collagen VI myopathies: studies with primary muscle cultures from Ullrich Congenital Muscular Dystrophy patients and Col6a1 null mice

Valeria Morbidoni, Anna Urciuolo, Paolo Grumati, Matilde Cescon and Paolo Bonaldo

Dept. of Histology, Microbiology and Medical Biotechnologies,University of Padova, 35121 Padova, Italy

Collagen VI (ColVI) is an extracellular matrix protein composed by three chains (α1, α2 and α3), encoded by separate genes and forming a microfilamentous network in various tissues. ColVI is particularly abundant in skeletal muscles, where it is localized in the endomysium surrounding muscle fibers. Studies with muscle-derived cell cultures demonstrated that ColVI is produced by interstitial fibroblasts, but not by myogenic cells [1,2]. Interestingly, a recent study has shown that transcription of the Col6a1 gene by muscle fibroblasts is dependent on a specific enhancer region, whose activation requires inductive signals from myogenic cells [3]. Mutations in ColVI genes in humans cause several muscle diseases, including Bethlem Myopathy (BM) and the more severe Ullrich Congenital Muscular Dystrophy (UCMD). An established animal model for ColVI disorders is the ColVI null mouse (Col6a1-/-), which displays a myopathic phenotype resembling human BM/UCMD [4]. Studies of Col6a1-/- mice provided valuable insights into the pathomolecular mechanisms of ColVI muscle disorders. Analysis of muscle biopsies and primary cultures of Col6a1-/- mice and UCMD patients revealed mitochondrial dysfunction and spontaneous apoptosis of muscle fibers, which could be normalized by treatment with cyclosporine A or its non-immunosuppressive derivatives [5,6].

Our studies indicate that primary muscle cultures, containing both interstitial fibroblasts and myogenic cells, represent an appropriate in vitro system to analyze the apoptotic/mitochondrial phenotype of ColVI diseases in humans and in the mouse model. Towards this aim, we investigated in detail primary muscle culture derived from UCMD patients and Col6a1-/- mice. To characterize the role of each muscle cell type in determining the apoptotic/mitochondrial phenotype, we have studied different muscle cell populations and at different culture passages. We found that both muscle fibroblasts and myogenic precursors display an increase in spontaneous apoptosis in UCMD and Col6a1-/- cultures. Further analysis of UCMD cultures showed that the major class of cells displaying the apoptotic phenotype is the desmin-negative population. The amount of myoblasts (desmin-positive cells) decreased with culture passages, and when the muscle-derived cultures lose myoblasts the phenotype of desmin-negative cells was also lost.

We are currently investigating the mechanism through which the ColVI survival signal is established between fibroblasts and myogenic cells in skeletal muscle.

Keywords: skeletal muscle, apoptosis, cell culture, muscular dystrophy

P-92 Crohn's disease blood T-cells are resistant to Fas-apoptosis independently of bax/bcl-2 ratio

Belen Beltran (1,3), Ines Moret (2), Francisco Rausell (1), Marisa Iborra (2), Guillermo Bastida (1,3), Mariam Aguas (3), Pilar Nos (1, 3)

(1) Gastroenterology Department. Hospital Universitari la Fe-CIBERehd. Valencia. Spain
(2) Instituto de Investigación Sanitaria la Fe. Valencia. Spain.
(3) Gastroenterology Department. Hospital Universitari la Fe. Valencia. Spain

Background: Crohn's disease (CD) is a chronic inflammatory disease of the lining of the digestive tract, above all the small and large intestine. In CD, the immune system affects the gastrointestinal tract causing the inflammation. In this context, it has been postulated that a defective intestinal lymphocyte death by apoptosis is related to the disease. However, capacity of peripheral lymphocytes from CD patients to undergo apoptosis has not yet been clarified.

Aims: To study the susceptibility of blood CD4 lymphocyte (before reaching intestine) to undergo apoptosis from active-CD patients after addition of an external apoptotic stimulus (agonist Fas antibody).

Materials & Methods: Heparinised blood samples were taken from healthy subjects (n=13) and from patients at onset of CD and yet to begin any specific medication (n=20). Patients were diagnosed according to Leonard-Jones criteria and disease activity was scored based on the Harvey-Bradshaw index. Lymphocytes were isolated by Histopaque gradient centrifugation, followed by negative purification of non-activated T cells (Depletion Dynabeads®). Isolated cells were cultured for 5 days in the presence (to activate) or absence of bounded anti-CD3 and anti-CD28 diluted in X Vivo-15 medium (Lonza). Afterwards, Fas antibody (Fas Ab) was added (1µg/mL) and apoptosis was detected by flow-cytometry (Annexin-V) after overnight (ON) incubation (24h). Bax and Bcl-2 expression were analysed by Western blot using specific antibodies to each protein. Caspase activities (3, 8 and 9) were analysed in cell lysates by colorimetric assay kits (Apopcyto, MBL).

Results: Activation of CD4-lymphocytes by CD3/CD28 antibodies resulted in a significant apoptosis increase in both patients and controls (46.4±18.2 vs 7.8±4.9, and 53.8±17.2 vs 8.3± 2.7, activated cells vs non-activated cells respectively), but with no significant differences between them. The addition of Fas Ab significantly increased the percentage of apoptotic cells showing a significantly less induced apoptosis in active-CD lymphocytes than in controls (56.4±16.7vs 69.6±11.9, respectively). However, neither differences in Bax/Bcl-2 ratio (0,2742 vs 0,3931 densitometry units) nor in caspase activity were obtained.

Conclusions: Resistance to apoptosis in CD may be a feature of peripheral T-lymphocytes before cells reach the intestinal mucosa. This alteration in their apoptotic capacities helps to clarify the relationship between adaptative and innate immune responses in CD and the physiopathology of the disease. Molecular apoptotic mechanisms different from the classical pathways (Bax/Bcl-2 and/or caspase) could be implied in the apoptotic resistance observed.
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Keywords: apoptosis, lymphocytes, Crohn's disease, Fas

P-93 Enhanced caspase-8 activation within the DISC primes over the mitochondrial pathway to sensitize DcR2 expressing cells to TRAIL induced cell death after chemotherapy.

Morizot A. (1,2), Mérino D. (1,2), Lalaoui N. (2), Jacquemin G. (2), Granci V. (2), Iessi E (2), Lanneau D. (2), Bouyer F. (2), Solary E. (2), Chauffert B. (2,3), Saas P. (4), Garrido C. (2), Micheau O. (2,3)

(1) These authors contributed equally to this work.
(2) INSERM, U866, Dijon, F-21079 France ; Faculty of Medicine and Pharmacy, Univ. Bourgogne, Dijon, F-21079 France.
(3) Centre Georges-François Leclerc, Dijon, F-2100 France.
(4) INSERM, U645, Besançon, F-25020, France; EFS Bourgogne Franche Comté, Besançon, Plateforme BioMonitoring, F-25020, France; Univ. Franche-Comté, Besançon, F-25020 France.

Apo2L/TRAIL is a promising molecule for cancer therapy that is now being evaluated in clinical trials in association with conventional anticancer therapeutic drugs. Expression of the TRAIL decoy receptor DcR2 has been shown to impair TRAIL induced cell death, however it remains unknown whether DcR2 might compromise combined therapeutic approaches associating TRAIL and chemotherapy.

We demonstrate here that sequential treatments with pharmacological concentrations of chemotherapeutic drugs restored sensitivity to TRAIL-induced cell death in cells expressing DcR2 in vitro and in vivo. This sensitization primarily involved proper DISC formation including caspase-8 recruitment and activation. Importantly, restoration of cell sensitivity to TRAIL-induced cell death was conserved when mitochondrial pathway was blocked but was severely compromised by the concomitant overexpression of DcR2 and c-FLIP.

Our results uncover the inhibitory potential of DcR2 and c-FLIP regarding chemotherapy-mediated sensitization to TRAIL-induced cell death. Their expression levels should thus be monitored and taken into consideration for future clinical trials aiming at combining TRAIL with chemotherapy.

Keywords: TRAIL, cancer cells, DcR2, CDDP, VP16, 5FU, Bcl-2, FLIP

P-94 Photokilling of cancer cells by ethylene glycol porphyrin derivatives involves endoplasmic reticulum stress response

Irena Moserova and Jarmila Kralova

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Videňská 1083, 142 20 Prague 4, Czech Republic
kralova@img.cas.cz

Photodynamic therapy (PDT) is a treatment modality for cancer. It combines selective accumulation of chemical compounds, called photosensitizers (PS), and light to irreversibly damage cancer cells via oxidative stress. In our study we tested porphyrin derivatives with the ethylene glycol (EG) chain linked to the phenyl ring at meta or para position. Such subtle changes in the structure of porphyrins resulted in their different intracellular localization, so that porphyrin with EG chain at para position (p-TPP(EG)4) was localized mainly in lysosomes, whereas porphyrin with EG chain at meta position (m-TPP(EG)4) in the endoplasmic reticulum (ER). m-TPP(EG)4 displayed superior PDT efficacy leading to permanent ablation of human mammary carcinoma (MDA-MB-231) in immunodeficient mice (Kralova et al., J.Med. Chem. 2008, 51, 5964–5973). The importance of the p38 MAP kinase signalling mechanism for the induction of apoptosis in various cell lines was demonstrated for p-TPP(EG) 4, while the mechanism for m-TPP(EG)4-mediated apoptosis remains to be elucidated. Because of prevalent localization of m-TPP(EG)4 in ER, which is the main cellular store of Ca2+, we evaluated the impact of Ca2+ homeostasis on the cell death pathway. Cells pre-loaded with membrane-permeable intracellular calcium chelator BAPTA-AM displayed a reduced level of m-TPP(EG)4-mediated apoptosis. The effect on cytosolic Ca2+ levels was monitored by loading cells with UV-excitable fluorescent Ca2+ indicator Fluo-4-AM and then measured by FACS. A major rise of (Ca2+)cyt was observed within one minute after the cells were exposed to the laser beam. These experiments indicate that the disturbance of Ca2+ homeostasis substantially contributes to m-TPP(EG)4-mediated apoptosis. Results of further investigation of the exact mechanism and implication of ER stress sensors are presented.

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Keywords: photodynamic therapy, apoptosis, porphyrin, cancer cells

P-95 Bortezomib primes neuroblastoma cells for death receptor-induced apoptosis by activation of the mitochondria

Ivonne Naumann, Klaus-Michael Debatin and Simone Fulda

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

Neuroblastoma is the most common extracranial solid tumor in children. Despite intensive treatment, based on combination chemotherapy, the cure rate of patients with more aggressive neuroblastoma has not improved over the last two decades. Therefore, novel therapeutic approaches are needed to treat neuroblastoma. A strategy, which has recently emerged, is the inhibition of the ubiquitin-proteasome system. Bortezomib is the first proteasome inhibitor used in clinical trials.

In this study we investigated the modulation of apoptosis sensitivity to death receptor ligands using Bortezomib in neuroblastoma cells.

Treatment with Bortezomib in combination with either TNF-related apoptosis inducing ligand (TRAIL), agonistic antibodies against TRAIL receptor 2 (ETR2) or CD95 led to significant enhancement of cell death in various neuroblastoma cell lines as well as primary cultured neuroblastoma cells. Combination treatment also exerted a strong effect on longterm survival.

Experiments exploring the mechanism of sensitization showed that treatment with Bortezomib led to upregulation of surface expression of TRAIL receptor 2. Consistently, combination treatment increased the quantity of death-signaling complex formed. Furthermore, this is associated with enhanced cleavage of caspase-8, -3 and Bid. Additional studies of the apoptotic pathway demonstrated an involvement of the mitochondrial branch of apoptosis regulation, since combination treatment with Bortezomib and TRAIL led to enhanced Bax activation, loss of the mitochondrial membrane potential (MMP) and cytochrome c release. This mitochondrial activation could be partially blocked by overexpression of Bcl-2.

Our data demonstrate that inhibition of the proteasome by Bortezomib sensitizes neuroblastoma cells for death receptor-induced apoptosis by promoting the activation of the mitochondria. Thus, the combination of Bortezomib and death receptor triggering represents a promising therapeutic approach for neuroblastoma treatment.

Keywords: Neuroblastoma, Bortezomib

P-96 UVB-induced Noxa-dependent sunburn cell formation requires p38MAPK/HIF-1

Kris Nys (1), An Van Laethem (2), Carine Michiels (3), Noemi Rubio (1,4), Jacques G. Piette (4), Maria Garmyn (2) and Patrizia Agostinis (1)

(1)Cell Death Research and Therapy Laboratory, Department of Molecular Cell Biology, Catholic University of Leuven, Leuven, Belgium
(2)Laboratory of Dermatology, Faculty of Medicine, Catholic University of Leuven, Leuven, Belgium
(3)Laboratory of Biochemistry and Cellular Biology (URBC), FUNDP-University of Namur, Namur, Belgium
(4)GIGA-Research, Laboratory of Virology and Immunology, University of Liège, Liège, Belgium

The signal transduction pathways leading to apoptosis of human keratinocytes responding to ultraviolet B (UVB) irradiation are complex and not completely understood. Previously, we reported that in UVB-irradiated keratinocytes, p38MAPK instigates Bax activation and mitochondrial apoptosis. However, the molecular mechanism underlying the pro-apoptotic function of p38MAPK remained unclear. Here, we show that in UVB-treated human primary keratinocytes the activation of p38MAPK is necessary to upregulate Noxa, a BH3-only pro-apoptotic dominantly induced by UVB and required for apoptosis. Whereas p53-silencing was marginally cytoprotective and poorly affected Noxa expression, p38MAPK inhibition in p53-silenced keratinocytes or in p53-/- cells could still efficiently prevent Noxa induction and intrinsic apoptosis following UVB, indicating that p38MAPK signals mainly through p53-independent mechanisms. Furthermore, p38MAPK was required for the induction and activation of HIF-1 in response to UVB and HIF-1 knockdown reduced Noxa expression and apoptosis. In UVB-irradiated keratinocytes Noxa targeted the anti-apoptotic Mcl-1 for degradation and siRNA-mediated knockdown of Noxa or p38MAPK inhibition restored levels of Mcl-1 and abolished apoptosis. Thus the pro-apoptotic mechanisms orchestrated by p38MAPK in human keratinocytes in response to UVB involve a HIF-1-Noxa axis, which prompts the downregulation of anti-apoptotic Mcl-1, thereby favouring Bax-mediated mitochondrial apoptosis of UVB-damaged keratinocytes.

Keywords: UVB, apoptosis, p38MAPK, HIF-1, p53, Noxa, epidermis, keratinocytes

P-97 Activation of the transcription factor FOXO3/FKHRL1 by doxorubicin and etoposide induces reactive oxygen species production and programmed cell death in neuroblastoma cells

Petra Obexer (1,2), Judith Hagenbuchner (1,2), Martin Hermann (4), Andrey Kuznetsov (5), Michael Ausserlechner (1,3)

(1) Tyrolean Cancer Research Institute, Innsbruck, Austria; (2) Department of Pediatrics IV, and (3) Department of Pediatrics II, Medical University Innsbruck, Austria; (4) KMT-Laboratory, Department of General and Transplant Surgery, Medical University Innsbruck, Austria; (5) Cardiac Research Laboratory, Heart Surgery, Medical University Innsbruck, Austria

Background: FOXO transcription factors are regulators of cell death, cell cycle progression, and stress resistance in neuronal cells and downstream targets of hyperactive PKB in neuroblastoma (NB). FOXO3/FKHRL1 was shown to protect against reactive oxygen species (ROS) by regulating detoxifying enzymes. We investigated the relationship between FOXO3 activation by chemotherapeutic agents, the production of ROS and its involvement in FOXO3-induced apoptosis of human NB cells.

Methods: For studying subcellular shuttling of FOXO3 by live cell fluorescence imaging an ECFP-FOXO3-allele was retrovirally expressed in NB cells. Generation of ROS was assessed by fluorescence staining using the ROS-sensitive dye reduced MitoTracker Red CM-H2XROS. NB cell lines for constitutive or conditional expression of Bim, BclXL, or shRNA directed against Bim and Sestrin3 were generated by retroviral gene transfer.

Results: Etoposide and doxorubicin treatment activates FOXO3, induces ROS and elevates the expression of the proapoptotic proteins Noxa and Bim in NB cells. Conditional activation of FOXO3 induced two sequential waves of ROS the first one being associated with elevation of Bim and Noxa. Knockdown of Bim or retroviral overexpression of the prosurvival BclXL both prevented ROS production and delayed apoptosis which implies that FOXO3-induced ROS is downstream of Bcl2 proteins. The decline after the first ROS wave correlated with increased expression of the peroxiredoxin Sestrin3. Knockdown of Sestrin3 prevented the ROS decline and accelerated cell death in NB cells.

Conclusions: The combined data suggest that programmed cell death by FOXO3 involves ROS production downstream of Bcl2 rheostat and that FOXO3 in parallel activates ROS-protection by Sestrin3. Prolonged FOXO3 activation however overcomes Sestrin3 protection, induces a secondary ROS burst and eventually leads to cell death in human NB cells.

Keywords: reactive oxygen species, FOXO3, chemotherapeutics, neuroblastoma, apoptosis

P-98 Attenuation of the lysosomal death pathway by lysosomal cholesterol accumulation

Hanna Appelqvist(1), Cathrine Nilsson(1,2), Brett Garner(3,4), Andrew Brown(5), Katarina Kågedal(1), Karin Öllinger(1)

(1) Experimental Pathology, Department of Clinical and Experimental Medicine, Linköping University, SE-581 85 Linköping, Sweden (2) Division of Otorhinolaryngology, University Hospital, SE-581 85 Linköping, Sweden
(3) Prince of Wales Medical Research Institute, Randwick, NSW 2031, Australia (4) School of Biological Sciences, University of Wollongong, Wollongong NSW 2522, Australia (5) School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia

Neurodegenerative diseases such as Niemann-Pick type C (NPC) and Alzheimer's disease (AD) are characterized by neurofibrillar tangles, endosomal abnormalities, accumulation of β-amyloid, and disturbances in cholesterol metabolism. NPC is caused by mutations in the cholesterol transporting proteins NPC1 and NPC2, resulting in cholesterol accumulation in the endo-lysosomal system and a concomitant neuronal death. Lysosomal membrane permeabilization (LMP) is a significant component of the cellular death machinery and release of lysosomal proteases, cathepsins, to the cytosol have proapoptotic functions. Although the mechanism regulating LMP is still unknown, the lipid composition of the lysosomal membrane is important for lysosomal stability and might influence the susceptibility to lysosomal leakage. The aim of this study was to investigate lysosomal stability and cellular sensitivity to apoptosis in cells allowed to accumulate cholesterol in the endo-lysosomal system.

Human fibroblasts carrying NCP mutation and age and gender matched wildtypes were used. The NPC phenotype was also mimicked in wildtype fibroblasts by treatment with 3-beta-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A), a drug known to interfere with intracellular cholesterol transport and cause cholesterol accumulation in late endosomes and lysosomes.

Lysosomal cholesterol accumulation did not affect viability per se and was associated with upregulation of the lysosomal system shown as rose levels of bis(monoglycero)phosphate, and increased expression cathepsin D and LAMP-2. Apoptosis was induced by exposure to staurosporine or the lyosomotropic detergent O-methyl-serine dodecylamine hydrochloride (MSDH), which are known inducers of apoptosis via the lysosomal death pathway in human fibroblast. Cholesterol accumulation rescued cells from apoptosis and the decrease in apoptosis frequency was associated with diminished lysosomal release of cathepsins. The cholesterol content of lysosomes correlated to their susceptibility to LMP, suggesting that cholesterol content determines lysosomal stability. We conclude that cholesterol accumulation in lysosomes attenuates the lysosomal death pathway by increasing lysosomal membrane stability.

Keywords: lysosome, cathepsin, cholesterol

P-99 Inhibition of cyclin A /CDK2 protein/protein interaction: Re-sensitizing tumor cell lines resistant to Tarceva (erlotinib).

Orzaez M.(1), Guevara T (1), Bachs O. (1,2) and Pérez-Payá E.(1,3)

(1)Centro de Investigación Príncipe Felipe, Chemical Biology Department Avda. Autopista del Saler, 16 E-46012 Valencia , Spain
(2)Departament de Biologia Cellular i Anatomia Patològica, Facultat de Medicina, University of Barcelona, E-08036, Barcelona, Spain
(3)Instituto de Biomedicina de Valencia, CSIC, E-46010 Valencia, Spain

Progression through the cell division cycle is controlled by a family of cyclin-dependent kinases (CDKs), whose activity depends on their binding to the regulatory partners (cyclins A–H)[1]. In our research group, we have developed an hexapeptide, NBI1, that binds to a minimal structural domain within cyclin A (amino acids 257–345), which is highly conserved and inhibits the kinase activity of CDK2/cyclin A affecting the stability of the complex[2]. NBI1 does not compete with ATP nor with the CRS (substrate recruitment site). NBI1 treatment induces cell cycle arrest in G2/M and apoptosis in a broad panel of cell lines.

As the ATP-binding domains present a high level of conservation among different families of protein kinases, it makes difficult to develop specific inhibitors and increases the occurrence of unwanted side effects. Therefore, development of ATP-non competitive inhibitors could represent a clear advantage for this type of treatments.

The epidermal growth factor receptor (EGRF) is overexpressed in a variety of tumours and its activation has been correlated with increases in cell proliferation, angiogenesis and metastasis[3]. Inhibitors of this receptor such as Erlotinib, an EGFR tyrosine kinase inhibitor, have been extensively studied and are now implicated in a broad number of clinical trials[4, 5]. Nevertheless, there are some tumours which show resistance to Erlotinib treatments. The sensitivity to Erlotinib has been correlated with the CDK2 activity and the resistance with cytoplasmic mislocalization of the CDK inhibitor p27[6, 7].

The objective of this work has been to evaluate the selective inhibition of cyclin A using NBI1 as a way to sensitize to Tarceva resistant breast cancer cell lines. The molecular mechanism responsible of cell death induction after co-treatment will be discussed.

Keywords: apoptosis, cancer, cell cycle

P-100 Multiple pathways of granzyme B-induced cell death: result of evolutionary pressure from pathogens (tumors) to evade cytotoxic cells.

Aguiló, Jl. (1), Martin, P. (2), Sánchez-Martínez, D. (1), Galvez, EM. (3), Regner, M. (4) ,Mullbacher, A. (4), Anel, A. (1), Simon, MM (2) and Pardo, J (1) (5)

(1) Dpt. Bioquímica y Biología Molecular y Celular, University of Zaragoza, Spain
(2) Metchnikof lab, MPI for Immunobiology, Freiburg, Germany
(3) Instituto de Carboquímica, CSIC, Zaragoza, Spain
(4) John Curtin Shcool of Medical Research, ANU, Canberra, Australia
(5) Fundación Aragón I+D (ARAID), Zaragoza, Spain

Granzyme B (gzmB) of cytotoxic T (Tc) cells is essential for recovery from intracellular pathogens, but the molecular basis of its action is still unresolved. Using ex vivo virus-immune Tc cells we showed that gzmB kills targets using both caspase- and mitochondria-dependent and -independent pathways By analysing the underlying process (es) we have found out that gzmB is able i) to induce extramitochondrial radical oxygen species by activating NADPHoxidase and ii) to directly cleave the actin binding protein gelsolin promoting cytoskeleton disassembling. The data provide evidence for pleiotropic pro-cytotoxic functions of gzmB suitable to counteract the multiple evasion strategies of pathogens and to differentially control tumors. The biological relevance of these findings, which are currently being tested in in vivo mouse models and in the human system, will be discussed.

Keywords: cytotoxic cells, granzyme, virus, tumor

P-101 The regulation of co- and post-translational myristoylation of proteins during apoptosis: roles of N-myristoyltransferases and caspases

Maneka A. Perinpanayagam, Erwan Beauchamp, Jacky Y.W. Sim, Dale D.O. Martin, Megan C. Yap and Luc G. Berthiaume

Department of Cell Biology, University of Alberta, Edmonton, Alberta T6G2H7, Canada

Myristoylation is the irreversible attachment of myristate (C14) on the amino-terminal glycine residue of a protein via an amide bond and is catalyzed by N-myristoyltransferases (NMT1 and 2). This modification is essential for directing proteins to various membranes and sub-cellular compartments. Typically, it occurs in a co-translational manner on the nascent polypeptide after the initiator methionine has been removed by methionyl aminopeptidase, but can occur post-translationally after cysteinyl-aspartyl protease (caspase) cleavage and exposure of a cryptic myristoylation consensus sequence bearing an N-terminal glycine part of a myristoylation consensus sequence. In order to investigate the role of NMTs during apoptosis, we monitored the expression of NMTs in Jurkat T cells at different time points after the induction of apoptosis. For the first time, we demonstrate that both NMT1 and 2 are cleaved during apoptosis, with NMT1 cleavage preceding NMT2 by nearly 2 hours. Caspase inhibition studies suggest that NMT1 and 2 are caspase 8 and 3 substrates, respectively, which correlates with the timing of NMT cleavage during apoptosis. We have further validated these results by using cell lines devoid of caspase 3 or 8. To assess the impact of caspase-cleavage on NMT activity in cell lysates, we have developed a highly sensitive filter-based assay adapted from King et al.1991, Anal Biochem. using [3H]-myristoyl-CoA and various myristoylatable and non-myristoylatable decapeptides. Following induction of apoptosis, there is an apparent increased activity of both NMTs followed by a progressive decay in activity. It is apparent that 2 hours after induction of apoptosis a sudden and time-dependent change in substrate specificity is observed, likely as the cell “switches” from a co- to post-translational myristoylation “status” and new cryptic myristoylation sites are unraveled by the action of caspases. Additionally, our work shows that the inhibition of NMTs by 2-hydroxymyristic acid (HMA), a specific inhibitor of myristoylation, but not palmitoylation, potentiates cell death induced by apoptotic inducers staurosporine and anti-Fas in Jurkat T cells. Therefore, inhibitors of NMT could perhaps potentiate the effect of known chemotherapeutic agents and offer a novel chemotherapeutic avenue in the treatment for cancer.

Keywords: apoptosis, myristoylation, caspases

P-102 Designed receptor DR5-specific rhTRAIL variants accelerate induction of apoptosis in cancer cells

Wim J. Quax (1), Almer M. van der Sloot (1,2), Eva Szegezdi (3), Eline Duiker (4), Carlos R. Reis (1), Robbert H. Cool (1), Luis Serrano (2), Steven de Jong (4) and Afshin Samali (3)

(1)Dept. of Pharmaceutical Biology, University of Groningen, the Netherlands (2) CRG-EMBL Systems Biology Unit, Barcelona, Spain. (3)Cell Stress & Apoptosis Research Group, Dept. of Biochemistry, National University of Ireland, Galway, Ireland.(4) Dept. of Oncology, UMCG, Groningen, the Netherlands.

Tumor necrosis factor related apoptosis inducing-ligand (TRAIL, Apo2L) is attracting great interest as it selectively kills various types of cancer cells, and unlike other apoptosis inducing TNF-ligand family members, appears to be inactive against normal cells.

TRAIL is a promiscuous ligand as it binds to five different cognate receptors of the TNF-receptor family: the death receptor 4 (DR4, TRAIL-R1), death receptor 5 (DR5, TRAIL-R2) both containing a cytoplasmic death domain that transmits an apoptotic signal and to the decoy receptor 1 (DcR1, TRAIL-R3, TRIDD), decoy receptor 2 (DcR2, TRAIL-R4, TRUNDD) and the soluble secreted receptor OPG, that lack an intact death domain and therefore may act as antagonist receptors. The occurrence of DR4 or DR5 responsive tumor cells indicates that a DR4 or a DR5-receptor specific TRAIL variant will permit new and selective tumor therapies. Using the automatic design algorithm FOLD-X, we successfully generated DR5-selective TRAIL variants(1). These variants do not induce apoptosis in DR4-responsive cell lines but show a large increase in biological activity in DR5-responsive cancer cell lines. Even rhTRAIL wt insensitive ovarian cancer cell line could be brought into apoptosis. In a xenograft experiment using bioluminescent ovarian cancer cells the efficacy of the DR5-selective TRAIL variant in combination with cisplatin was demonstrated (2). Recently we have shown that DR5 selective variants lead to a 17 fold faster kinetics of Caspase-8 processing resulting in accelerated apoptosis induction of cancer cells. A model for the underlying mechanism will be presented.

Keywords: protein design, ovarian cancer, receptor selective TRAIL

P-103 Proteomic approaches to identify novel binding partners of the survival factor Bcl-xL

Nina Raulf (1,2), Martin Biniossek (1) and Christoph Borner (1,2)

(1) Institute of Molecular Medicine and Cell Research, Albert Ludwigs University Freiburg, Stefan-Meier-Str. 17, D-79104 Freiburg, Germany
(2) Spemann Graduate School of Biology and Medicine, Albert Ludwigs University Freiburg, Albertstr. 19a, D-79104 Freiburg, Germany

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. Bcl-xL protein complexes of various cell lines were purified by gel filtration analysis and analyzed via SDS-polyacrylamide gel electrophoresis. Furthermore, Bcl-xL immunoprecipitation and co-immunoprecipitations were performed. Preliminary data indicate that Bcl-xL is present in protein complexes in the range between 60 – 150 kD, both in the cytosol as well as on mitochondria. This pattern is independent of the cell type used and is clearly different from the elution pattern of recombinant Bcl-xL which is only found in lower molecular weight fractions. Since known Bcl-xL binding partners such as Bax, Bak or BH3-only proteins exhibit molecular masses between 20 – 30 kD and the possibility of Bcl-xL multimers could be excluded, these data indicate that ca. 28 kD Bcl-xL is likely to be bound to other, yet unidentified proteins. To identify these interaction partners currently mass spectrometry analyses are performed employing immunoprecipitations of endogenous as well as exogenous Bcl-xL.

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

P-104 Rapid and efficient cancer cell killing mediated by high affinity death receptor selective TRAIL variants

Carlos R. Reis(1), Almer M. van der Sloot(2), Alessandro Natoni(3), Eva Szegezdi(3), Rita Setroikromo(1), Michel Meijer(4), Klaas Sjollema(5), Francois Stricher(2), Robbert H. Cool(1,6), Afshin Samali(3), Luis Serrano(2), Wim J. Quax(1)

(1) Department of Pharmaceutical Biology, A. Deusinglaan 1, 9713 AV, Groningen, The Netherlands.
(2) Centre for Genomic Regulation, CRG-EMBL Systems Biology Unit, Dr. Aiguader 88, 08003, Barcelona, Spain.
(3) Department of Biochemistry and National Centre for Biomedical Engineering Science, National University of Ireland, Galway, Ireland.
(4) Department of Medical Physiology, University Medical Center Groningen, University of Groningen, A. Deusinglaan 1, 9713AV Groningen, The Netherlands.
(5) University Medical Imaging Center, University Medical Center Groningen, University of Groningen, A. Deusinglaan 1, 9713AV Groningen, The Netherlands.
(6) Triskel Therapeutics BV, A. Deusinglaan 1, 9713 AV, Groningen, The Netherlands.

The TNF family member TRAIL selectively induces apoptosis in a variety of cancer cells through activation of death receptors 4 (DR4) and 5 (DR5) and is considered a promising anti-cancer therapeutic agent. Since apoptosis seems to occur primarily via only one of the two death receptors in many cancer cells, the introduction of death receptor selectivity is thought to create more potent TRAIL agonists with superior therapeutic properties. Here we report the design of highly active, DR4-specific TRAIL variants by using an improved homology model of DR4, computational protein design and a rational combination strategy. This resulted in DR4-selective variants that not only have an enhanced selectivity to DR4, but also show a significant increase in apoptosis-inducing activity in many cancer cells in vitro. The increased receptor selectivity and increased affinity for the target receptor both appear to contribute to the more efficient apoptosis induction by these TRAIL variants. These developed DR4-selective variants in addition to previously described DR5-selective variants allowed us to establish the individual contribution of death receptors in apoptosis-signalling of different cancer cells. Cell lines signalling via both death receptors showed a large increase in efficacy upon combined treatment with selective TRAIL variants when compared to wild-type rhTRAIL. Our results demonstrate that the use of these novel DR4-specific variants alone or in combination with a DR5-specific variant as a powerful strategy in targeting DR4 and DR5 responsive cancer cells.

Keywords: TRAIL, cancer, selectivity, death receptors

P-105 Neutrophil extracellular trap cell death requires both autophagy and superoxide generation

Quinten Remijsen(1,2,3), Tom Vanden Berghe(1,2), Ellen Wirawan(1,2), Bob Asselbergh(4,5), Eef Parthoens(4,5), Riet De Rycke(4,5), Sam Noppen(4,5), Michel Delforge(6), Jean Willems(3), and Peter Vandenabeele(1,2)

(1)Laboratory of Biochemistry, Department of Medicine, K.U. Leuven campus Kortrijk, Kortrijk, Belgium.
(2)Molecular Signaling and Cell Death Unit, Department for Molecular Biomedical Research, VIB, Ghent, Belgium.
(3)Molecular Signaling and Cell Death Unit, Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium.
(4)Microscopy Core Facility, Department for Molecular Biomedical Research, VIB, Ghent, Belgium.
(5)Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium.
(6)University Hospital Gasthuisberg, Department of Hematology, Leuven, Belgium.

Neutrophil Extracellular Traps (NETs) are extracellular chromatin structures that can trap and degrade microbes. They arise from neutrophils that have activated a cell death program called neutrophil extracellular trap cell death, or NETosis. Activation of NETosis has been shown to involve NADPH oxidase activity, disintegration of the nuclear envelope and most granule membranes, decondensation of nuclear chromatin, and formation of NETs. We report that in PMA-stimulated neutrophils, intracellular chromatin decondensation and NET formation follow autophagy and superoxide production, both of which are required to mediate PMA-induced NETosis and occur independently of each other. Neutrophils from patients with chronic granulomatous disease, who lack Nox2 activity, still exhibit PMA-induced autophagy. Conversely, PMA-induced NADPH oxidase activity is not affected by pharmacological inhibition of autophagy. Interestingly, inhibition of either autophagy or NADPH oxidase prevents intracellular chromatin decondensation, an essential event for NETosis and NET formation, and results in cell death characterized by hallmarks of apoptosis. These results indicate that apoptosis might function as a backup program for NETosis when autophagy or NADPH oxidase activity is prevented.

Keywords: neutrophil extracellular trap, granulocyte, chronic granulomatous disease, superoxide, autophagy, live cell imaging

P-106 LeuLeuOMe-induced apoptosis is associated with early autophagosome accumulation and critically depends on cytosolic cathepsin activity

Urska Repnik (1), Marusa Hafner (1), Dejan Caglic (1), Catherine Miniejew (2), Tina Zavasnik (1), Gregor Kosec (1), Marko Mihelic (1), Gabriela Droga-Mazovec (1), Saska Ivanova (1), Vito Turk (1), Ulrich Wendt (2), Boris Turk (1)

(1) Department of Biochemistry, and Molecular and Structural Biology, Jozef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia
(2) R&D Chemical and Analytical Sciences, Sanofi-Aventis Deutschland GmbH, Industriepark Park Hoechst, 65926 Frankfurt, Germany

LeuLeuOMe is a lysosomotropic dipeptide methyl ester that is polymerized in lysosomes to give hydrophobic polyleucine chains. These cause lysosomal membrane permeabilization and can thus initiate lysosomal pathway of apoptosis that is mediated by cysteine cathepsins. In the present study, we have investigated the early events upon LeuLeuOMe treatment and looked more closely at the fate of cysteine cathepsins in order to explain the window of concentrations that fail to initiate apoptosis. Apoptosis was determined by annexin V and PI staining or DEVD-ase activity. Lysosomal membrane permeabilization was monitored by acridine orange uptake and NAG-activity. Total and cytosolic cysteine cathepsin activities were measured using fluorogenic synthetic substrate Z-FR-AMC. In addition, activity based probe was used to discriminate between the activities of different cysteine cathepsins. Immunoblotting of endogenous LC3 and transfection with LC3-EGFP-plasmid were used to evaluate the effect on autophagy. We have found that already 15 minutes after LeuLeuOMe treatment lysosomes are destabilized over a wide range of LeuLeuOMe concentrations. The total activity of cysteine cathepsins decreases, however their cytosolic activity increases and represents the bulk of total cysteine cathepsin activity. The reduction in activity is due to cysteine cathepsin degradation caused by progressive unfolding due to lysosomal pH rise and hydrophobic interaction with polyleucine chains. The remaining total and cytosolic cysteine cathepsin activities are increasing along with the increasing LeuLeuOMe concentration. We therefore suggest that LeuLeuOMe concentration determines the speed and the extent of lysosomal membrane destabilization. These two factors in turn determine the cytosolic cysteine cathepsin activity. At the threshold apoptotic concentration of LeuLeuOMe cysteine cathepsins are rapidly released to the cystosol and their cytosolic activity becomes sufficient to initiate the signaling pathway leading to apoptosis. Below the threshold apoptotic concentration cysteine cathepsins are inactivated and degraded before they can be released to the cytosol. Cells therefore survive but are largely devoid of cysteine cathepsin activity and depend on novel synthesis of lysosomal proteases to resume normal lysosomal degradation.

Keywords: lysosomes, cysteine cathepsins, LeuLeuOMe, apoptosis

P-107 XPC silencing in normal human keratinocytes increases reactive oxygen species and triggers metabolic alterations that drive the formation of squamous cell carcinomas

Hamid Reza Rezvani (1,2,3), Frédéric Mazurier (1), Arianna L. Kim (2), Ali Nsrein (1), Meaghan Daly (2), Hubert de Verneuil (1), David R. Bickers (2), Alain Taïeb (1,3)

(1) INSERM U876, Univ Victor Segalen Bordeaux 2, Bordeaux France.
(2) Department of Dermatology, College of Physicians and Surgeons, Columbia University, NY.
(3) National Reference Centre for Rare Skin Disorders, Bordeaux, France.

Genomic mutations, the Warburg effect, and alterations in the levels of reactive oxygen species (ROS) are consistently observed in a variety of cancers. However, the inter-relationships among these factors and their impact on the neoplastic process remain poorly understood. We took advantage of the intrinsic genomic instability arising in the nucleotide excision repair disease xeroderma pigmentosum C (XPC) to look at underlying molecular mechanisms. Here we show that shRNA-mediated knockdown of XPC in normal keratinocytes (XPCKDNHK) leads to 1) increased reactive oxygen species (ROS) and 2) metabolic alteration through reduction of mitochondrial function but increased glycolysis, as defined by decreases in the NADH dehydrogenase subunit 1 and cytochrome c oxidase subunit III; and increases in proteins involved in glucose uptake and lactate generation. We also detected high levels of basal cell layer markers keratin 14, α;6 and β1 integrin in epidermis reconstructed with XPC-deficient keratinocytes, which manifested epithelial hyperplasia compared to control epidermis. Furthermore, XPC-deficient keratinocytes formed SCCs in immunodeficient mice. The critical step in tumoral transformation of XPCKDNHK was bypassing the XPC silencing–induced stalled cell cycle progression. In fact, the majority of XPCKDNHK cells harboring ATR-mediated stalled cell cycle progression underwent apoptosis and only subsets of cells with metabolism modification were selected. Our results show that overactivation of DNA-dependent protein kinase (DNA-PK) and consequently increased activity of nonhomologous endjoining (NHEJ) repair pathway is indispensable for escaping from XPC silencing-induced apoptosis. Impairment of DNA-PK activation in XPCKDNHK blocked their neoplastic transformation, indicating that NHEJ repair activation in cells lacking XPC contributes to high susceptibility to carcinogenesis.

Keywords: Carcinogenesis, Metabolism, Xeroderma pigmentosum, DNA-PK, NHEJ, Warburg effect

P-108 Finding the prerequisites for synergy between ABT-737 and conventional therapy in T-cell malignancies

Rogier Rooswinkel(1), Marcel Verheij(2), Jannie Borst(1)

(1) Division of Immunology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
(2) Department of Radiotherapy, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Tumor cells are often depending on high expression of anti-apoptotic Bcl-2 proteins to survive their harsh environment. Neutralization of these proteins is often sufficient to drive tumors cell towards apoptosis. In addition, inhibition of the anti-apopototic proteins represents a promising strategy to counteract resistance to conventional anticancer therapies. We have used overexpression of anti-apoptotic proteins in Jurkat cells to explore under what conditions the Bcl-2 inhibitor ABT-737 and conventional therapy are synergistic. We show that Bcl-2 and Bcl-xl overexpression cell-lines are completely resistant to etoposide while remaining sensitive to ABT-737. In these cell-lines ABT-737 is able to restore sensitivity to etoposide induced apoptosis. Mcl-1 and Bfl-1 overexpressing cell-lines were resistant against ABT-373 and could not be sensitized by etoposide treatment. This is surprising since overexpression of Mcl-1 and Bfl-1 had no impact on sensitivity to etoposide alone. We are currently exploring the need for activation or induction of specific BH3-only proteins to sensitize cells to ABT-737 using inducible proteins. Furthermore, we are testing the possible involvement of p53 by comparing p53 wild-type and p53 KD cell lines.

Keywords: Therapy, ABT-737, Lymphoma

P-109 Targeting of XIAP combined with systemic Mesenchymal Stem Cell-mediated delivery of sTRAIL inhibits metastatic growth of pancreatic carcinoma cells

Andrea Mohr (1), Stella Albarenque (1), Laura Deedigan (1), Rui Yu (1), Mairead Reidy (1), Sina Rupprecht (1), Simone Fulda (2), Ralf M. Zwacka (1)

(1) National University of Ireland, Galway, National Centre for Biomedical Engineering Science, Molecular Therapeutics Group, Galway, Ireland
(2) Children's Hospital Ulm, Ulm University, Ulm, Germany

Disseminating tumors are one of the gravest medical problems. We combined the tumor-specific apoptosis-inducing activity of TRAIL with the ability of mesenchymal stem cells (MSCs) to infiltrate both tumor and lymphatic tissues to target primary tumors as well as disseminated cancer cells in a human pancreatic cancer mouse model. Furthermore, we targeted XIAP by RNAi inside the cancer cells to make use of the apoptosis sensitisation as well the anti-metastatic effect that is afforded by XIAP silencing.

We generated MSCs that express and secrete a trimeric form of soluble TRAIL using adenoviral vectors. When mixed with the two human pancreatic carcinoma cells Panc-1 and PancTu1, these MSCs, termed MSC.sTRAIL showed that these normally TRAIL resistant cells were slightly sensitive to MSC.sTRAIL, which is most likely due to the enhanced effect of the direct, cell mediated delivery of trimeric TRAIL. When we additionally knocked-down XIAP by RNAi in the cancer cells, cell death was markedly increased. These findings were confirmed in xenograft models, in which tumors from the parental pancreatic carcinoma cells showed only partial responses, whereas tumors with silenced XIAP that were treated with MSC.sTRAIL went into remission. Moreover, animals with XIAP-negative xenografts treated with MSC.sTRAIL were almost free of lung metastasis, whereas animals treated with control MSCs showed substantial malignant growth in the lungs. In summary, a combined approach using systemic MSC-mediated delivery of sTRAIL together with inhibition of XIAP inside the cancer cells tackles metastatic growth of pancreatic carcinoma.

Keywords: TRAIL, MSCs, XIAP, pancreatic cancer, metastasis

P-110 MicroRNA profiling in small and non-small cell lung carcinomas with different response to radio- and chemotherapy

Hogir Salim[1], Nadeem S.Akbar[2], Kristina Viktorsson[1], Rolf Lewensohn[1], Ali Moshfegh[1] and Boris Zhivotovsky[2].

[1] Department of Oncology and Pathology, Karolinska Biomics Center
[2] Institute of Environmental Medicine, Division of Toxicology, Karolinska Institutet, Stockholm, Sweden

Lung cancer (LC) is one of the most common tumor diseases and is the number one of cancer-related deaths worldwide. Based on histopathology it is roughly divided into small (SCLC) and non-small cell lung cancer (NSCLC). Despite the advances in diagnosis and treatment modalities, the outcome of LC patients remains poor. Several approaches have been tried to improve the survival of LC, such as inhibitors of growth factor receptor signalling pathways like (EGFR) and (IGF-1R), but the outcome of LC still remains poor. Nowadays, high attention is paying to the microRNAs. These RNAs are small single stranded endogenous RNA molecules of 18-24 nucleotides in length that act as negative regulators of gene expression at the posttranscriptional level. They are involved in the regulation of many cellular processes, such as development, cell growth, differentiation, apoptosis and carcinogenesis. However, their role in regulation of tumor response to chemo- and radiotherapy is unknown. To address this, we performed miRNA expression profiling of a panel of SCLC and NSCLC cell lines with different chemo- and radiosensitivity using Affymetrix miRNA arrays. In addition to some specific expression we found that around 450 miRNAs are expressed in one or more of the cell lines. Both SCLC and NSCLC cell lines were divided into two groups according to their SF2 (surviving fraction after 2-Gy irradiation) values. Those having SF2 values of more than (0.3) regarded as radio-resistant (RR) and those with SF2 values of less than (0.3) considered as radiosensitive (RS). Certain miRNAs being differentially expressed in RR cell lines were validated by performing qRT-PCR. To check their significance in radiotherapy response, we are performing the down-regulation of the highly expressed miRNAs in the RR cell lines using antagomirs and up–regulation of those, which have low expression level by transfecting them with mimics of the miRNAs. Transfected cells are irradiated and their apoptotic response (caspase-3 activation, cell cycle distribution and nuclear morphology) is analyzed. The precise role of these miRNAs in response to radiotherapy will be discussed.

Keywords: microRNA, lung cancer, small cell and non-small cell lung cancer, radio-therapy and chemo-therapy.

P-111 In vitro-expanded and ex vivo-derived human NK cells as a tool to study cell death induced by cytotoxic cells on hematologic neoplasias

Diego Sánchez1, Juan Ignacio Aguiló1, Javier Naval1, Alberto Anel1 and Julián Pardo1,2

1 Department of Biochemistry, Molecular and Cell Biology, Faculty of Sciences, University of Zaragoza, 50009 Zaragoza (Spain).
2, ARAID, Fundación Aragón I+D, Zaragoza, Spain

Natural Killer cells are critically involved in the elimination of tumor cells that express reduced levels of MHC-I. Recently it has been reported that diverse hematologic malignant cells are sensitive to allogeneic NK cells even if MHC-I expression was highly expressed in those tumor cells. This effect is due to non-recognition of allogeneic MHC-I by the inhibitory receptors of the NK cells (KIR). Despite these advances nothing is known about the mechanism involved in the elimination of hematologic neoplasias by allogeneic human NK cells. In a first approach to elucidate such a mechanisms, we have compared the cytotoxic potential of ex vivo-isolated human NK cells from healthy donors with in vitro-expanded NK cells from the same donor, against human multiple myeloma (MM) and T leukaemia cell lines. For it, NK cells were enriched from fresh blood and apoptosis induction on the target cells was analysed. In parallel, peripheral blood lymphocytes were activated in vitro for 5 days by using 3 different activation protocols: i) IL-2, ii) mitomycin C-treated human B lymphoblastoid cells (R69) or iii) mitomycin C-treated K562 cells together with IL-2. Ex vivo-NK cells were already able to induce apoptosis on target cells. However, in vitro activation increased very much their ability to kill target cells. Expansion in the presence of R69 cells was the best treatment to increase cytotoxic potential of NK cells even in the absence of exogenous cytokines. All NK cell populations induced cell death by a perforin/granzyme-mediated mechanism, although expression of those molecules did not change significantly during activation. We are currently analysing cell death induced by those NK cell populations on diverse leukaemia and MM tumor cell lines in which specific molecules involved in apoptosis are targeted. On this way, we will be able to elucidate the pro-apoptotic requirements of hematologic tumor cell lines to be susceptible to NK cells and predict the ability of this new therapy to kill specific tumor cells.

Keywords: NK cell, cancer, cytotoxic

P-112 Use of 10-N-nonyl acridine orange for flow cytometric analysis of apoptosis

Gennady V. Lutsenko, Elena I. Kovalenko, Alexander M. Sapozhnikov

Lab. Cell Interactions, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia

Cytometric method for detection of phosphatidylserine on the external surface of cells using fluorochrome labeled annexin V is one of the main assays used at present for the analysis of apoptosis. Recently it has been shown that redox catalytic interactions of cytochrome c with cardiolipin (CL) and subsequent oxidation of CL occurs during apoptosis. Oxidation of CL is accompanied by a release of cytochrome c from the mitochondria into the cytosol, a key event in development of apoptosis. 10-N-Nonyl acridine orange (NAO), a fluorophor that forms a strong complex with reduced form of CL but not with oxidized CL can be used for flow cytometric analysis of this effect. It can be combined with detection of annexin V-positive cells as an additional tool in the study of process of apoptosis.

CTLL-2, Jurkat and Raji cell lines were used for the experiments. Apoptosis was induced by deprivation of growth factor (w/o interleukin-2, 48 h), incubation of cells with ethanol (7%, 90 min) or with actinomycin D (100 ng/ml, 24 h). NAO (100 ng/ml), annexin V - FITC, annexin V-Alexa Fluor 647 (AF647), and propidium iodide (PI, 2 µg/ml) were used for vital cell staining. FACScan and FACSVantage DiVa flow cytometers (Becton Dickinson Biosciences, USA) were used for cell analysis.

Flow cytometric analysis of the cells stained with NAO and PI showed that the induction of apoptosis resulted in a population with low levels of fluorescence of NAO and PI (NAO-negative/PI-negative). Comparison of these data with the results obtained by using of the conventional assay for apoptosis (annexin V-FITC/PI) demonstrated that the percentage of NAO-negative/PI-negative cells are almost identical with that for annexin V-FITC-positive/PI-negative cell pool (apoptotic cells). Similar correlations were obtained for other cell populations: NAO-positive/PI-negative – annexinV-FITC-negative/PI-negative (alive cells) and NAO-negative/PI-positive – annexinV-FITC-positive/PI-positive (necrotic cells). The data obtained by the both tested methods had a very high level of correlation (0.953, $p<0.0001$). The three-color cytometric analysis (NAO/annexinV-AF647/PI) also showed that the population of NAO-negative cells coincided with population of apoptotic, annexin V-AF647-positive cells.

The results of flow cytometric detection of apoptosis using NAO is in a good accordance with the conventional assay (annexin V-FITC/PI), and can be used as a new additional methodical tool for analysis of process of programmed cell death.

Keywords: apoptosis, cardiolipin, flow cytometry

P-113 Specific targeting of human Caspases with DARPin binders

Thilo Schröder1, Andreas Flütsch1, Jens Sobek2 *, Markus Grütter1

1 University of Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland
2 Functional Genomics Center Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland

Despite the well-recognized importance of Caspases in apoptosis and inflammation there are no reliable techniques available to monitor caspase activity in apoptotic or inflammatory cells. Current studies based on commercially available short peptide-based substrates lack sufficient specificity [1]. We have developed a protein array using a combined approach with Designed Ankyrin Repeat Proteins (DARPins) [2] as capture reagents and peptide substrates to specifically detect and monitor caspase activity in cells. This approach will ultimately allow us to dissect the various Caspase pathways in human apoptotic cells.

DARPins represent ideal binding proteins for in vitro applications due to their superior biophysical properties. We have established expression and purification protocols for caspase-1 through to caspase-9 [3]. Using ribosome display [4], DARPins have been selected against these caspases showing tight binding with single digit nanomolar affinities. In order to make a comprehensive screen of over 300 potential binders to human Caspases, we developed a high throughput DARPin microarray. The specificity and affinity of DARPins towards their target Caspase was confirmed using Surface Plasmon Resonance.

Based on crystal structures we can show how DARPins inhibit [5] and bind Caspases in an allosteric manner with high specificity. Atomic resolution at 1.7A of a Caspase-8/DARPin complex structure revealed atomic interactions on the Caspase surface. Based on this structure we can imply possibilities to activate proCaspase-8 with a DARPin linker DARPin construct in vitro.

We anticipate that the selected DARPins can be widely applied to address key questions concerning Caspase activation, activity and intracellular localization. In addition, this approach represents a valuable tool to inhibit or activate Caspases in human cells.

Keywords: Caspase, DARPin

P-114 Annexin A5 based strategy to improve phagocytosis of apoptotic cells

Kristof Schutters (1), Heidi Kenis (1) Leon Schurgers(1) Jagat Narula (2) Chris Reutelingsperger (1)

(1)Department of Biochemistry, Cardiovascular Research Institute Maastricht, 6200 MD Maastricht, The Netherlands
(2)Department of Cardiology, University of California Irvine, United States of America

Apoptosis is characterized by the externalization of phosphatidylserine (PS), which is an aminophospholipid that resides predominantly in the inner leaflet of the plasma membrane of healthy cells. Cell surface expressed PS acts as an 'eat me'-signal towards phagocytes. Impaired clearance of apoptotic cells has been shown to be associated with, and even to contribute to, progression of chronic inflammatory diseases such as atherosclerosis. Enhancing phagocytic clearance of apoptotic cells has been proposed as strategy to treat chronic inflammation. Annexin A5 (anxA5), a 35 kD human protein that binds PS with high affinity, is being exploited as a Molecular Imaging probe to visualize apoptosis non-invasively in animal models and in patients. AnxA5 inhibits clearance of apoptotic cells by shielding cell surface expressed PS.

Here we report the construction of an anxA5-variant that binds PS and that enhances phagocytosis of apoptotic cells. Inspired by the findings that the RGD-motif of MFG-E8 triggers engulfment by engaging αVbeta3/5 integrins of phagocytes if bound to apoptotic cells we incorporated an RGD-motif in the N-terminal tail of anxA5. In addition we extended the anxA5-variant at the N-terminus with a Histidine-tag and introduced a Cysteine-residue in the protruding loop at the concave side connecting domains II and III. The Histidine-tag can be easily labeled with 99mTechnetium via Isolink-technology for in vivo nuclear imaging of apoptosis with SPECT. The Cysteine at position 166 functions as a chemical anchor for making conjugates of 1:1 stoichiometry with maleimide-functionalized fluorochromes for optical imaging of apoptosis. Here we report the design, generation and testing of the RGD-anxA5-variant in vitro and in vivo. The RGD-anxA5 variant binds PS and can be used to image apoptosis in vivo using nuclear and optical imaging. In addition, the RGD-anxA5 variant binds αVbeta3/5–integrins in vitro and in vivo and reverses inhibition of phagocytosis into stimulation. In conclusion, the RGD-anxA5-variant is a multifunctional protein that combines diagnostic and therapeutic functionalities and that has potential in diagnosis and treatment of chronic inflammatory diseases such as atherosclerosis and lupus erythematosous.

Keywords: apoptosis, annexin A5, RGD-motif, Molecular Imaging, therapeutic agent, phagocytosis

P-115 Real Time Analysis of Mitochondrial Events and Caspase Activation by Procaspase Activator : An Essential Role of Mitochondria for Effective Cell Death

Mahendra Seervi and Santhosh Kumar T.R.

Apoptosis & Cell Signaling Group, Department of Molecular Medicine & Cancer Biology, Rajiv Gandhi Centre for Biotechnology , Thiruvananthapuram , Kerala , India.

Most anticancer drugs induce cytotoxicity through signaling events that funnel through the mitochondria, either in a Bax/Bak dependent or Bcl2/ Bcl-xL regulated manner. Thus, the activation of downstream caspases necessary for effective tumor cell death is often impaired in drug resistant tumors upon Bcl2 /Bcl-xL overexperssion or Bax /Bak deficiency, leading to drug resistance. An approach to activate executioner caspases directly within the cells is expected to ensure direct demolition of drug resistant cells and so can be considered as a better strategy to kill cancer cells. PAC1, PAC2 and1541 are some of the recently discovered small molecules that have been reported as direct activators of the caspase 3. The complex signaling potential of Bcl2 family proteins in the regulation of cell death downstream of mitochondria and the ability of certain cancer cells to survive despite caspase activation warrants systematic analysis of the potential of direct caspase activation to bypass drug resistance. Moreover most of the direct caspase activators were identified using cell free recombinant protein based models, because of which the ability of these compounds to trigger direct enzymatic activation in the absence of mitochondrial signaling needs to be substantiated using well defined cellular platforms.

We have developed live cell based system to evaluate the central events of apoptosis like cytochrome C release , Bax activation , caspases activation in real time and hierarchical manner . Such tools can clearly solve the ambiguity and effectiveness of direct caspases activating compounds in killing cancer cells. We have studied PAC1 which has recently been discovered as direct activator of caspase 3 employing these cell based models that substantiate the importance of mitochondrial events for effective death execution .We have observed that PAC1 effectively induces cytochrome c release and Bax activation even in the absence of caspases 3 and the absence of Apaf 1 blocks caspase 3 activation and cell death. It clearly suggests that PAC1 requires mitochondrial events for caspase activation and effective cell death. Interestingly, we found that PAC1 induce cytochrome c release and caspase activation in Bax and Bak knock out cell which suggests that PAC1 has the potential to induce Bax and Bak independent cell death . We observed that overexperssion of Bcl2 and BclxL could not block cytochrome c release and caspase activation completely, rather delayed it slightly. Our study suggest that PAC1, like a classical apoptosis inducing drug, requires cytochrome c release and Apaf1 for caspase activation and effective cell death. However it has the potential to bypass the role of antiapoptotic Bcl2 and Bclxl proteins and can effectively induce Bax and Bak independent cell death . The study highlighted the importance of novel cellular systems to identify direct activation of caspases in tumor cells and the potential use of PAC1 in eliciting Bax/ Bak independent cell death that is partially regulated by Bcl2 and Bclxl.

Keywords: Mitochondria , caspase , cytochrome c , Bcl2

P-116 Transglutaminase type 2 and autophagy: its involvement in pexophagy

Sara Sepe (1,2), Roberta Nardacci (2), Manuela D'Eletto (3), Maria Grazia Farrace (3), Flavie Strappazzon (3), Andrea Baiocchini (2), Franca Del Nonno (2), Sandra Moreno (1) and Mauro Piacentini (2,3).

(1) Department of Biology, University of Rome 'Roma Tre', Viale G Marconi 446, 00146, Rome, Italy
(2) National Institute for Infectious Diseases 'Lazzaro Spallanzani', Via Portuense 292, Rome, Italy
(3) Dept of Biology, University of Rome 'Tor Vergata', Via della Ricerca Scientifica 1, Rome, Italy

"Tissue" or type 2 transglutaminase (TG2) is a peculiar multifunctional enzyme catalysing Ca(2+)-dependent post-translational modification of proteins, by establishing covalent bonds between peptide-bound glutamine residues and either lysine residues or mono- and poly-amines. In addition, TG2 may also act as a G protein in transmembrane signalling, as a kinase, as a protein disulphide isomerase, and as a cell surface adhesion mediator. Our recent findings indicate a role for TG2-mediated post-translational modifications of proteins in the maturation of autophagosomes (D'Eletto M. et al. Autophagy. 2009 Nov;5(8):1145-54).

Autophagy is a highly conserved cellular process responsible for the degradation of long-lived proteins and organelles. This process occurs at low levels under normal conditions, and is enhanced in response to stress, nutrient deprivation, hypoxia, mitochondrial dysfunction and infection. The ablation of TG2 protein both in vivo and in vitro, results in accumulation of microtubule-associated protein 1 light chain 3 cleaved isoform II (LC3 II) on pre-autophagic vesicles, suggesting induction of autophagy. By contrast, the formation of the acidic vesicular organelles in the same cells is reduced, indicating impaired maturation of autophagolysosomes.

In this work we crossed TG2 -/- mice with GFP-LC3 mice. We analysed the participation of TG2 in a selective autophagic pathway, referred to as pexophagy, leading to vacuolar degradation of damaged and/or excess peroxisomes.

Peroxisomal proliferation was induced by dietary administration of ciprofibrate for 8 days. It is well known that this hypolipidemic drug produces an increase in the number and size of peroxisomes in rodent liver, leading to oxidative stress, and altered cell proliferation and apoptosis. Excess liver peroxisomes are normally degraded within a week, but this rapid removal is impaired in autophagy-deficient liver. Peroxisomal proliferation was similarly induced in WT and TG2 -/- mice. The number of organelles was monitored at 0, 3 and 7 days after the end of treatment, (T0, T3, T7 respectively). WT mice gradually restored peroxisomal hepatic content during the recovery period, while TG2 -/- mice showed an impaired clearance process. In fact, the peak of peroxisome removal in WT liver is found at T3, when a 4-fold-increase of the number of autophagic vacuoles (AVs), compared to T0, is observed. Strikingly, almost invariant numbers of AVs are detected in TG2 -/- mouse liver, throughout the recovery period. Ultrastructural analysis shows numerous AVs containing indigested peroxisomes are still present at T7. WB data confirm the morphological observations, indicating a progressive accumulation of LC3 II protein in TG2 -/- mouse liver.

Histological examination suggests higher susceptibility to liver damage of TG2 -/- mice, compared to WT. Periportal hepatocytes show a a mild cytoplasmic swelling, accompanied by condensation and displacement of organelles. The hypothesized autophagic impairment, could thus result in insufficient ability to restore proper liver cytoarchitecture.

Although the molecular mechanism(s) whereby TG2 possibly regulates autophagy are yet to be characterized, an effect on the regulation and arrangement of the cytoskeleton could be hypothesized, based on the postulated action of TG2 on posttranslational modification of its major components tubulin, actin and vimentin.

Keywords: TG2, autophagy, pexophagy

P-117 Two mechanisms of antioxidant activity of SkQs

Fedor Severin, Dmitry Knorre, Olga Markova, Ekaterina Smirnova, Dmitry Zorov, Vladimir P. Skulachev

Belozersky Institute of Physico–Chemical Biology and Institute of Mitoengineering, Moscow State University, Vorobyevy Gory 1, Moscow 119991, Russia

Reactive oxygen species (ROS) are one of the major inducers and mediators of apoptosis. SkQ type molecules were recently shown to act as mitochondrial antioxidants. SkQs display two basic activities: they deliver the rechargeable antioxidant(plastoquinone) to mitochondria and they also act as mitochondrially-targeted protonophores. The latter activity depends on the non-quinone part and is self-inhibitory (“mild”) because the molecules are positively-charged and so their mitochondrial accumulation depends on the membrane potential.

It is known that both hyper-polarization and deep depolarization of mitochondria may cause ROS accumulation. Thus, mild protonophoric activity of SkQs may contribute to their antioxidant properties. To explore this, we studied the antioxidant effects of the hydrophobic tail of SkQ-Rh19, C12-Rh19, in vitro and in vivo. We found that both a conventional protonophore (FCCP) and C12-Rh19 improve the survival in the model of yeast cell death driven by mitochondrial hyperpolarization. As expected, the concentration window for C12-Rh19 was much (approximately twenty times) wider than for FCCP.

There are established experimental models of death of rats which are mediated by mitochondrial ROS accumulation, e. g. the ones caused by infarctions of kidney or brain. It was found that SkQ-Rh19 significantly improves the survival in these models. Here we show that the non-quinone part of this molecule (C12-Rh19) also active as the death protector: its effects are approximately two-fold lower than that of SkQ-Rh19. Importantly, the quinone moiety of SkQs can act as pro-oxidant when overdosed. Therefore, hydrophobic tails of SkQs are medically promising antioxidant compounds. It is tempting to speculate that they also can be used as mimetics of caloric restriction.

Keywords: mitochondria, antioxidant, ROS, uncoupling

P-118 USP18 modulates the responsiveness to Interferon-α (IFN-a) induced apoptosis in glioblastoma cells: identification of the critical players.

Andrea Sgorbissa(1), Harish Potu(1), Claudio Brancolin (1).

(1): Dipartimento di Scienze e Tecnologie Biomediche, Universita' di Udine, Italy P.le Kolbe 4, 33100 Udine

Downregulation of USP18, a type I IFN-induced protein that deconjugates the ubiquitin-like modifier ISG15 from target proteins, augments drugs-induced apoptosis. This increased apoptotic susceptibility depends on the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and the interferon pathway (Harish et al Cancer res. 2010, 70:655).

USP18 is an important negative regulator of the IFN pathway that protects cells by IFN induced apoptosis by preventing TRAIL up regulation.

Glioblastoma multiforme is among the most lethal tumors with a median survival of about 1 year from the time of diagnosis. Glioblastoma cells are highly resistant to apoptosis as induced by chemotherapy. We have decided to investigate the role of USP18 in regulating resistance to IFN-induced apoptosis in different glioblastoma cell lines. We have observed that in T98G but not in U87MG cells the downregulation of USP18 potently promotes IFN-induced apoptosis. This apoptotic response is elicited by a dramatic up-regulation of TRAIL.

In U87MG cells silencing of USP18 is not enough to boost TRAIL expression although several IRFs are up-regulated. When the TRAIL promoter was transfected in U87MG and T98G cells transcription of the reporter gene in response to IFN was similarly observed. To evaluate differences in the TRAIL promoters of T98G and U87MG cells we cloned both promoters and evaluated their transcriptional activity when transfected in glioblastoma cell lines. Both promoters equally elicited transcription of a reporter gene in response to IFN. Studies are in progress to unveil the methylation status of the TRAIL promoter in the two cell lines. The responses to IFN in terms of apoptosis, TRAIL up-regulation and the contribution of USP18 are also evaluated in primary glioblastoma cells obtained from different patients.

In addition to defect in the TRAIL up-regulation in response to IFN, U87MG cells are resistant to recombinant TRAIL-induced apoptosis. Gene array data, confirmed by western blot analysis, revealed that U87MG, compared to T98G cells, have several apoptosis related gene differentially expressed, in particular FLIP (high), BID (low) and IAPs (high). Finally, to efficiently kill U87MG cells in addition to USP18 silencing and IFN treatment, cells were challenged with several drugs in order to find a compound capable of synergizing with IFN to induce apoptosis. Preliminary data show that ER stress inducing drugs are the most promising candidates in synergizing with IFN.

Keywords: apoptosis, glioblastoma, TRAIL, DUBs

P-119 Divergences in the TRAIL signaling pathway – differences between mice and men.

Sarah Shirley (1), Pascal Schneider (2), Olivier Micheau (1),

(1) INSERM U866, Faculty of Medicine and Pharmacy, University of Bourgogne, Dijon, F-21079, France
(2) Dept. of Biochemistry, University of Lausanne, Boveresses 155, CH-1066 EPALINGES, Switzerland

TNF-related apoptosis inducing ligand (TRAIL) has great potential as a cancer therapeutic due to its ability to induce apoptosis in tumour cells, but not in healthy tissue. Though there is a large body of work on TRAIL signaling in human cell lines, the action of TRAIL in murine cell lines remains largely uncharacterized. In order to establish mouse models for further study of TRAIL as a therapeutic agent, it is important to understand the molecular mechanisms of TRAIL in murine cell lines.

Contrary to human tumor cell lines, most murine tumour cell lines were found to be resistant to TRAIL ligand-induced apoptosis. However, the MD5 antibody directed at the agonistic DR5 receptor, when cross-linked on protein-A coated plates, was able to induce some killing in L929 cells, derived from an immortalized mouse fibroblast cell line, and EMT6H cells, a mouse mammary carcinoma, but not B16 cells, a mouse melanoma cell line. Combining MD5 antibody and soluble TRAIL ligand resulted in killing of the L929 and EMT6H cells, but not the B16, which were found to express little mDR5 at the cell surface. Retroviral-mediated overexpression of the mouse DR5 receptor restored B16 cell sensitivity to the MD5 and TRAIL ligand combined treatment. Interestingly, overexpression of the human DR5 receptor failed to restore apoptosis induced by the combined treatment. In line with the actions of the human TRAIL receptor set, overexpression of the mouse “decoy” receptor DcR2L reduced sensitivity to the combined treatment, providing the first evidence that the inhibitory function of this receptor is conserved in these two species.

These initial results hint at a multifaceted system of control for TRAIL signaling in the murine system, echoing the complex signaling pathway in the human cell. However, it remains unclear why soluble TRAIL ligand fails to engage the apoptotic machinery in mouse cell lines, while this signaling pathway is easily engaged in human cell lines. Further work is still needed to understand the molecular mechanisms involved in the regulation of the TRAIL system in the mouse.

Keywords: TNF-signalling, TRAIL, murine, death receptor, decoy receptor

P-120 Serine protease inhibitor-2 blocks inflammatory and cell cytotoxicity responses in mousepox infection

Carolina R. Melo-Silva (1), David C. Tscharke (2), Mark R. Buller (3), Arno Müllbacher (1), Mario Lobigs (1), Matthias Regner (1)

(1) Department of Emerging Pathogens and Vaccines, John Curtin School of Medical Research, Australian National University, Canberra ACT, Australia.
(2) School of Biology, Australian National University, Canberra ACT, Australia.
(3) Department of Molecular Microbiology and Immunology, St. Louis University Health Sciences Center, St Louis, Missouri, USA

Ectromelia virus (ECTV) is a natural pathogen of mice, causing mousepox. Many of its genes have been implicated in viral evasion of the host immune response. The serine protease inhibitor-2 (SPI-2) is one of these putative ECTV host response modifier proteins. We are studying the role of SPI-2 in ECTV infection by deleting the serine protease inhibitor active domain from the SPI-2 gene. In vitro, the resultant mutant virus replicates as efficient as wild-type virus in a number of cell lines, suggesting that SPI-2 protein does not affect viral replication per se. However, in the absence of SPI-2 protein, ECTV is attenuated in susceptible mice, resulting in lower viral loads in the liver and substantially increased host survival. Moreover, mice infected with SPI-2-deficient ECTV present with altered immune responses compared with wild-type or revertant virus-infected mice: they mount an earlier serum IFNgamma response by four days post-infection. By six days post-infection, there are similar levels of CD8+ and NK cells circulating in the blood of mice infected with SPI-2 expressing virus and SPI-2 deficient virus. However, a greater percentage of NK cells express granzyme B in the blood and spleen of mutant virus-infected mice. The data indicate that the SPI-2 protein delays the antiviral inflammatory response and thereby dampens adaptative antiviral responses, leading to death of the host by 7 to 10 days post-infection.

Keywords: ectromelia virus, poxvirus, SPI-2, caspases

P-121 Apoptosis during luteolysis and its associations with the expressions of Bax and NF-kB in pseudopregnant rats

Helle Evi Simovart1, Raivo Masso(1), Gunnar Selstam(2), Kersti Kokk(1), Marina Aunapuu(1), Andres Arend(1)

(1)Department of Anatomy, University of Tartu, Biomedicum, 19 Ravila St. Tartu 50411, Estonia
(2)Department of Molecular Biology, University of Umea, Sweden

The corpus luteum (CL) develops from residual follicular granulosal and thecal cells after ovulation. Apoptosis contributes to luteal regression in many species and is one of the physiological processes which takes place during the luteolysis of CL in the ovary. However, which molecules are involved in the series of events leading to the CL apoptosis is not clearly known yet.

The aim of our study was to investigate the apoptosis of luteal cells of the corpus luteum on different days of pseudopregnancy in rats and examine its associations with the expressions of Bax and the nuclear transcription factor-kB (NF-kB). The pro-apoptotic protein Bax is involved in the regulation of intrinsic pathway of apoptosis, while NF-kB is implicated in multiple physiological and pathological processes as well as cell survival and apoptosis.

Adult Wistar rats were mated with sterile males for inducing pseudopregnancy. The ovaries were removed and blood samples were taken from three rats per group on Days 4, 8, 13, 18 and 21. The ovaries were fixed in 10% neutral-buffered formalin, embedded in paraffin and cut into sections of 3-4 micrometer thickness. Blood sera were separated and analysed for progesterone concentration. Luteal cell apoptosis in the CL was determined in situ, using the TUNEL method, the expressions of Bax and NF-kB were investigated immunohistochemically.

In our study observations of apoptosis by the TUNEL method in the CL of rats during 4-21 days of pseudopregnancy showed that the apoptotic luteal cell count was at a low level till Day 8, after that it increased from Day 13 to Day 18, and there were statistically significant differences between the days in early pseudopregnancy, namely Days 4-8 and Days 13-18.

Expression of Bax was found in the cytoplasm of luteal cells throughout the period of pseudopregnancy. It rose from Day 4 to Day 18 and decreased on Day 21. Expression of NF-kB rose till Day 13, and on Day 18 there was noticed a decrease, while on Day 21 it rose again. The progesterone levels in blood sera were high until Day 8 and declined thereafter.

Our investigations with pseudopregnant rats indicate that luteal cell apoptosis was more prevalent on Days 13 to 18 and that the expression of Bax correlated with the occurrence of apoptosis in luteal cells during corpus luteum regression. Expression of NF-kB followed the expression of Bax and luteal cell apoptosis in early days of pseudopregnancy, but in later days it showed the opposite tendency.

Keywords: apoptosis, Bax, NF-kB, corpus luteum

P-122 Determinants of the inhibitory effects of COX-2 inhibitors on cell proliferation in hematopoietic malignancies

Cyril Sobolewski (1), Claudia Cerella (1), Sebastien Chateauvieux (1), Cindy Grandjanette (1), Mario Dicato (2), and Marc Diederich (1)

(1) Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg, Luxembourg, Luxembourg.
(2) Department of Hematology-Oncology, Centre Hospitalier, Luxembourg, Luxembourg.

The cyclooxygenases (COX) are a family of enzymes, which catalyze the biosynthesis of prostaglandin from arachidonic acid. COX-2, the inducible isoform, is upregulated during inflammation and over-expressed in various cancers. There is evidence about a role of COX-2 in proliferation of colorectal, breast and prostate cancer, whereas this role remains to be elucidated in hematopoietic malignancies.

In this study, we investigated the role of COX-2 in cell proliferation of a panel of leukemic and lymphoblastic cell lines, including cells highly expressing COX-2 (U937, Jurkat, Hel and Raji) and one cell line (K562), which expresses COX-2 at very low level. For our approach, we used three different COX-2 inhibitors: nimesulide, NS-398 and celecoxib.

We found that these inhibitors were able to reduce cell proliferation in all COX-2-positive cell lines whereas they were completely ineffective in K562 cells. The inhibitory effects on cell proliferation led to an accumulation in G0/G1 phase of the cell cycle as assessed in U937 cells, chosen as a COX-2-positive model. Results were paralleled by altered expression levels of proteins involved in cell cycle progression. First we observed a strong downregulation of c-Myc protein, followed by the up-regulation of p27 and the downregulation of PCNA and cyclin D1, which are normally implicated in blocking G1/S transition. None of these regulators was modulated in K562 cells.

Reduced cell proliferation as well as alterations of c-myc/p27 protein levels are events described as occurring during senescence. We then investigated the potential of COX-2 inhibitors to promote cellular senescence. We found that these compounds triggered alterations in cellular morphology, induced accumulation of vacuoles and increased cell granularity. Furthermore, we observed appearance of Senescence-Associated Heterochromatin Foci and detected beta-galactosidase activity.

Altogether, our results suggest that targeting COX-2 functions may play a role in activating a senescence program in cancer cells, thus offering an alternative to cytocidal anti-cancer therapy.

Keywords: COX-2, cell proliferation, c-myc, senescence

P-123 Selective cytotoxicity of epidermoid cancer cells through apoptosis induction by RW002 essential oil

Jérémie Soeur (1,2), Laurent Marrot (2), Philippe Perez (2), Ismail Iraqui (1), Guy Kienda (1), Michèle Dardalhon (1), Jean-Roch Meunier (2), Dietrich Averbeck (1), Meng-Er Huang (1)

(1)UMR3348 Centre National de la Recherche Scientifique, Institut Curie, Université Paris Sud-XI, 91405 Orsay, France
(2) L'OREAL Advanced Research Phototoxicology Unit, Safety Research Department, 1, avenue Eugène Schueller, BP22, 93601 Aulnay-sous-Bois Cedex, France

Essential oils are complex mixtures of odorous and volatile compounds from secondary plant metabolism. They can be isolated from many plants by mechanical pressing or hydro- and steam-distillation and are known to possess a wide range of biological activities including antibacterial, antiviral, antifungal, insecticidal, cytotoxic, antioxidant and antimutagenic activities. In order to explore their beneficial properties on human skin cells, we investigated the effects of a essential oil, designated RW002, on the human epidermoid carcinoma cell line A431, immortal HaCaT cells thought to represent an early stage of skin carcinogenesis, immortalized normal keratinocytes HEK001 and primary normal keratinocytes NHEK. We showed that in a defined range of concentrations, RW002 essential oil selectively killed A431 cells and, to a lesser degree, HaCaT cells. Same treatment exerted only a minor cytotoxic effect on HEK001 and NHEK cells. The selective cytotoxicity of epidermoid cancer cells by RW002 essential oil is revealed to be through apoptosis induction. The detailed results of this work will be presented.

Keywords: Apoptosis, essential oil, cancer,

P-124 Role of mitochondria in cell senescence-induced death of yeast Saccharomyces cerevisiae

Sviatoslav Sokolov (1), Natalya Bocharova (1), Sergey Kochmak (1), Dmitry Knorre (1), Fedor Severin (1)

(1) A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University

Eukaryotic cells are capable to arrest cell cycle in G0/G1 state (quiescence) for very long periods of time. However, a prolong arrest in other stages of cell cycle can result in cell senescence and subsequent apoptosis. Recent data indicate that S. cerevisiae cells arrested with non-replicated telomeres increase the mitochondrial DNA content per cell and undergo senescence-like cell death which can be rescued by either antioxidant or rapamycin treatment (Qi et al. 2008). We asked first whether this type of yeast cell death is specific to telomere replication defect or can be induced by other factors arresting cells the in the division phase. It appeared that long-term mitotic arrest induced by cdc15-1 or cdc26delta mutations in Anaphase-Promoting Complex (APC) displays the same features: causes rapamycin- and antioxidant-sensitive cell death.

Why the prolonged arrest causes death? Unlike higher cells, yeast lack specialized proteins (p53, etc.) which trigger cell senescence-dependent apoptosis. One possible reason for the arrest-induced death can be multiplication of mitochondria in the absence of nuclear DNA replication, resulting in the imbalance between mitochondrial- and nuclear-encoded proteins in mitochondria. Supporting this, we found that mitochondrial translational inhibitor chloramphenicol or rho0 mutation rescue the senescence-induced death while mutations in mitochondrial retrograde signaling reduce the survival. We also found that S. cerevisiae daughter cells posses more mitochondria per cell volume than the mother cells and are more sensitive to yeast apoptosis-inducing conditions: acetic acid treatment and heat shock. Together, it suggests a novel conservative pathway for senescence-induced cell death.

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Keywords: cell cycle, mitochondria, senescence

P-125 Lysosomes and lysosomal proteases are only amplifiers of apoptosis in death receptor pathway

Aleš Špes1, Ana Petelin1, Martina Klarić1, Peter Vandenabeele4, Thomas Reinheckel2, Henning Walczak3, Vito Turk1, Boris Turk1

(1)Department of Biochemistry and Molecular and Structural Biology, Jožef Stefan Institute, Jamova 39, Ljubljana, Slovenia
(2)Institut für Molekulare Medizin und Zellforschung, Albert-Ludwigs-Universität Freiburg, Stefan-Meier-Str. 17, Freiburg, Germany
(3)Tumour Immunology Unit, Division of Medicine, Imperial College London, London W12 ONN, United Kingdom
(4) VIB,Department for Molecular Biomedical Research, University of Ghent, Technologiepark 927, 9052 Gent, Belgium

Tumor necrosis factor α (TNFα), TNF-related apoptosis-inducing ligand (TRAIL) and FasL are members of the TNF superfamily. They are expressed by the cells of the immune system and are known to induce apoptosis (death-receptor or intrinsic pathway) in a wide variety of cancer, transformed or sensitized cells. Moreover, activation of this pathway by agonistic antibodies or recombinant cytokines is currently in clinical trials for cancer treatment. However, the molecular mechanism of the intrinsic apoptosis pathway is still controversial. Several reports suggested an important role of lysosomes in this model. Lysosomal proteases were thus reported to be released from the lysosomes into the cytosol, where they were suggested to contribute to the apoptotic cascade upstream of mitochondria. Among them cathepsin B was suggested to be of major importance, based on RNAi studies or the use of cathepsin B-deficient cells.

In our work we focused on the role of lysosomes and lysosomal cysteine cathepsins in TNFα, Fas and TRAIL-induced apoptosis in mouse embryonic fibroblast (MEFs). In order to address this issue, we measured progression of apoptosis by Annexin V-PE staining, lysosomal stability by acridine orange staining and mitochondrial stability by Mitotracker Red CMXRos as a function of time. Our results suggest that destabilization of mitochondria occurs simultaneously with the initiation of apoptosis and Bid cleavage, whereas destabilization of lysosomes was observed at a later time point. In order to further investigate the role of lysosomes and lysosomal cathepsins in this apoptotic model, we have been using different inhibitors (CA-074Me, E-64d, Z-FA-fmk) and cathepsin B knock-out cells. Although apoptosis was attenuated in cathepsin B-deficient cells, it was not prevented. Moreover, this was only observed at later time points, consistent with the idea that cathepsins contribute to apoptosis only at the late stage after disruption of mitochondria. Therefore, it can be concluded that lysosomal proteases are released into the cytosol only after mitochondrial damage and that they contribute to apoptosis progression only as amplifiers of the apoptotic signal and not as major players. However, involvement of some other factors in this pathway cannot be excluded, as the link between mitochondria and lysosome remains elusive.

Keywords: apoptosis, death receptor, lysosomes

P-126 Interaction of Hepatitis B virus (HBV) with apoptosis signaling pathways is involved in viral clearance

Steinebrunner N. (1), Joschko N. (1), Stremmel W. (1), Müller M. (1)

(1) Department of Internal Medicine IV, Hepatology and Gastroenterology, University Hospital Heidelberg, Germany

Introduction and aim: One major risk factor for development of hepatocellular carcinoma (HCC) is chronic infection with Hepatitis B Virus (HBV). We have previously shown in vivo that a functional p53 pathway and an intact CD95-apoptosis signaling pathway are essential for clearance of HBV infection. Furthermore, upregulation of the antiapoptotic, dominant negative isoform of p73, deltaNp73, in patients with HCC correlated with reduced survival. The aim of this study was to analyze if p53 family-mediated apoptosis is involved in clearance of HBV-infected hepatocytes.

Material and methods: TAp73, deltaNp73 and expression plasmids of the HBV proteins (knock out constructs of L protein (AdHBV-L-), X protein (AdHBV-X-) as well as HBV wild type) were transferred into Hep3B and HepG2 cells using adenoviral vectors. Apoptosis signaling pathways were investigated by flow cytometry (staining for death receptors, mitochondrial membrane potential) and luciferase reporter assays.

Results: HBV cooperated with TAp73 in the induction of both, the extrinsic as well as the intrinsic apoptosis pathway. Combined adenoviral transfer of TAp73 and HBV led to a synergistic transactivation of the CD95-receptor and an enhancement of mitochondrial membrane depolarization as well as an increase in the activity of the pro-apoptotic Bcl-2 protein family member Bax and of the BH3-only proteins Puma and Noxa. HBV-infection led to a transactivation as well as a consecutive increase of mRNA and protein expression of Bax shown via gene activation by luciferase reporter assays and on mRNA- and protein-level by qPCR, immunoblot and flow cytometry. The activation of pro-apoptotic proteins and the depolarization of the mitochondrial membrane resulted in an increased cytochrome c induction and a subsequent activation of Apaf-1. DeltaNp73 inhibited both, the synergistic induction of the extrinsic as well as the intrinsic apoptotic signaling pathway.

Conclusion: These data support the fact that the p53 family-related interaction with HBV and the extrinsic and intrinsic apoptosis signaling pathways is an underlying mechanism of the diverse outcomes of an HBV infection (HBV clearance vs. chronicity), suggesting a new mechanism by which oncogenic deltaNp73 could support viral persistence.

Keywords: apoptosis, hbv, p53

P-127 Why does HAMLET preferentially kill tumour cells? p38- and Ras-dependent death in carcinoma but upregulation of innate immunity in healthy, differentiated cells

Petter Storm1, Soyoung Min2, Chimène Moreilhon1, Maria Trulsson1, Yinxia Chao3, Tomas Westergren1, Lotta Gustafsson1, Sonja Aits1, Ann-Kristin Mossberg1, James Ho3, Priscilla Lay Keng Lim3, Henry Yang3, K. Hun Mok1,2 and Catharina Svanborg1,3*

(1)Department of Microbiology, Immunology and Glycobiology (MIG), Institute of Laboratory Medicine, Lund University, Sölvegatan 23, S-223 62 Lund, Sweden
(2)Trinity College Dublin, School of Biochemistry and Immunology, Dublin 2, Ireland
*(3)Singapore Immunology Network (SIgN), A*STAR, 8A Biomedical Grove, 04-06, Singapore, 138648*

New approaches are needed to develop improved, more tumor-specific cancer therapies. HAMLET (human α-lactalbumin made lethal to tumor cells) is a tumoricidal, partially-unfolded protein-fatty acid complex that has shown a remarkable selectivity for carcinoma cells in vitro and tumor-specificity in clinical studies and animal models without adversely affecting healthy differentiated cells. Here, we show that perturbations of oncogenic Ras and activation of p38 by HAMLET underlie the divergent effects of lethality in carcinoma and immunity in healthy, differentiated cells. HAMLET directly bound to nine Ras family members, inhibited Ras signaling, and triggered a p38 MAP kinase-dependent response, involving increased phosphorylation and the expression of several p38 pathway members. Specific inhibition of p38 activity attenuated the lethal effects of HAMLET in carcinoma and lymphoma cells, linking the p38 MAPK response to cell death. In parallel, HAMLET triggered an ER stress response in carcinoma cells, characterized by rapid XBP1 mRNA splicing, eIF2α; phosphorylation, and ATF6 cleavage. Healthy, differentiated cells, in contrast, survived HAMLET challenge with a multifaceted innate immune response that was absent of p38 activation. We propose that this divergent response by HAMLET between tumor cells and healthy, differentiated cells potentially supports an immune-mediated removal of dying cells and debris from the carcinoma site.

Keywords: HAMLET / Ras / p38 / ER stress / innate immunity / cell death

P-128 Retinoids enhance glucocorticoid-induced apoptosis of T cells by facilitating glucocorticoid receptor-mediated transcription

Katalin Tóth, Zsolt Sarang, Beáta Scholtz, László Fésüs and Zsuzsa Szondy

Department of Biochemistry and Molecular Biology, Apoptosis and Genomics Research Group, Hungarian Academy of Sciences, University of Debrecen, Debrecen, Hungary

Glucocorticoid-induced apoptosis of thymocytes is one of the first recognized forms of programmed cell death. It was shown to require gene activation induced by the glucocorticoid receptor (GR) translocated into the nucleus following ligand binding. In addition, the necessity of the glucocorticoid-induced, but transcription-independent phosphorylation of phosphatidylinositol-specific phospholipase C (PI-PLC) and the mitochondrial translocation of GR have also been demonstrated. Here we report that retinoic acids, physiological ligands for the nuclear retinoid receptors, enhance glucocorticoid-induced death of mouse thymocytes both in vitro and in vivo. The effect is mediated via retinoic acid receptor (RAR) α/retinoid X receptor (RXR) heterodimers, and occurs when both RAR α and RXR are ligated by retinoic acids. We demonstrate that the ligated RARα /RXR interacts with the ligated GR resulting in an enhanced transcriptional activity of the GR. Interestingly, ligated GR and RAR α colocalised also in the mitochondria and transcription of various mitochondrial genes was also enhanced. Phosphorylation of PI-PLC was not affected. Besides thymocytes, retinoids also promoted glucocorticoid-induced apoptosis of various T cell lines suggesting that they could be used in the therapy of glucocorticoid sensitive T cell malignancies.

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Keywords: thymocyte, T lymphocytes, retinoid, glucocorticoid, transcription, mitochondria

P-129 Novel Insights into Cell Death of Malaria Parasites: Opportunities for Therapeutic Exploit

Kevin SW Tan (1), Chng Jun Hong (1) and Martin J Lear (2)

(1) Laboratory of Molecular and Cellular Parastiology, Dept of Microbiology, National University of Singapore, Singapore.
(2) Dept of Chemistry, National University of Singapore, Singapore.

Programmed cell death (PCD) is a crucial process in the growth and development of multicellular organisms. The discovery of unique PCD features in the unicellular Protozoa provides novel opportunities for anti-parasitic chemotherapy. In the current study, we report that the anti-malarial drug chloroquine (CQ) induces a cathepsin-dependent apoptotic-like PCD pathway in both CQ-sensitive and -resistant strains of the malaria parasite Plasmodium falciparum. The use of a new fluorescent-labeled chloroquine molecule revealed, for the first time, CQ concentration-dependent relocalization of the drug, from the digestive vacuole (DV) to the parasite cytosol at low versus high concentrations respectively. The observation that the chemoreversal compound verapamil restores PCD features in drug resistant parasites suggests a role for the lysosome-like DV in mediating parasite PCD, possibly through the release of cathepsins via a breached DV membrane. Our study sheds new light on PCD mechanisms in the Protozoa and implicates parasite DV and associated cathepsins as good targets for therapeutic exploit.

Keywords: Malaria, Apoptosis, Clan CA, Lysosome, Fluorescent Drug

P-130 Rescuing melanoma susceptibility to apoptosis by inhibiting IAPs expression

Tassi E.(1), Vegetti C.(1), Molla A.(1), Zanon M.(1), Pennati M. (2), Zaffaroni N.(2), Milella M(3)., Ferrone S.(4), and Anichini A(1).

(1) Human Tumor Immunobiology Unit and (2) Molecular Pharmacology Unit, Department of Experimental Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, 20133 Milan, Italy.
(3) Division of Medical Oncology A, Regina Elena National Cancer Institute, 00144 Rome, Italy.
(4) University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA 15232, USA.

Metastatic melanoma is characterized by a severe chemoresistance, which contributes to its poor prognosis. One of the mechanisms that account for this phenomenon is the inactivation of the apoptotic machinery; the inhibitors of apoptosis (IAPs) proteins negatively regulate the apoptotic process, and some of them have been shown to be expressed by malignant melanoma.

With the aim of clarifying the role of IAPs in melanoma chemoresistance and of finding new strategies to increase drug-induced apoptosis, we first evaluated the expression of IAPs in melanoma cell lines at steady-state and after cytotoxic treatments. Our results show that IAPs proteins are generally highly expressed by melanoma cells, and suggest that they might have a role in the defense mechanisms of melanoma against pro-apoptotic drugs. Then, with a small interfering RNA (siRNA) specific for Apollon/BRUCE we demonstrated that the inhibition of expression of this protein can sensitize melanoma cells to pro-apoptotic treatments. Finally, our data show that both the association of an anti-human leukocyte antigen (HLA) class II monoclonal antibody (mAb) with cytotoxic drugs and the combination of MAPK and ERK kinase (MEK) and mammalian target of rapamycin (mTOR) inhibitors increase apoptotic cell death compared to the single treatments, which is associated with a reduced IAPs expression.

Collectively, our results suggest that IAPs proteins, and especially Apollon/BRUCE, might be involved in melanoma chemoresistance, and that both the association of anti-HLA class II mAbs with pro-apoptotic treatments and the combined inhibition of MEK and mTOR pathways could be efficient strategies to inhibit IAPs expression and to increase melanoma chemosensitivity and apoptosis.

Keywords: melanoma, IAPs, chemoresistance

P-131 Differentiation-Dependent Apoptosis (DDA) accompanies induced erythroid differentiation of Murine Erythroleukemia (MEL) cells

Triviai I.(1,2,3), Badbaran A(3), Nowak R(2), Stübig T(3), Kröger N(3), Fehse B(2) and Tsiftsoglou A(1)

(1)Laboratory of Molecular Pharmacology, Dept. of Pharmaceutical Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece
(2)Laboratory for Cell and Gene Therapy, Clinic for Stem Cell Transplantation, University Medical Center (UMC) Hamburg-Eppendorf, Hamburg, Germany
(3)Clinic for Stem Cell Transplantation, UMC Hamburg-Eppendorf, Hamburg, Germany

Self-renewal, apoptosis and differentiation can maintain the appropriate balance between the number of hematopoietic stem cells or early progenitors entering the differentiation pathways and mature blood cells produced during life-long hematopoiesis. Using murine erythroleukemia (MEL) cells (virally transformed murine hematopoietic progenitors) as a suitable model for culture erythropoiesis, we investigated whether erythroid differentiation is accompanied by differentiation-dependent apoptosis (DDA). Cells were treated separately with and without DMSO, HMBA and/or Etoposide (an pro-apoptotic agent) and then assessed for differentiation, cell viability, cell cycle profiling, DNA damage, and apoptosis by flow cytometry, expression of cell cycle related proteins as well as cytochrome C exit from mitochondria by Western blot analysis and visible changes in morphology during differentiation. Viable, early and late apoptotic populations were sorted by FACS and commitment to erythroid differentiation was assessed based on mRNA expression levels of various erythroid differentiation genes as determined by RT-PCR. Our results indicate that: (a) Etoposide caused extensive apoptotic damage in more than 90% of cells without promoting erythroid cell differentiation. (b) DMSO and HMBA induced apoptosis in 20 – 30% of terminally differentiated MEL cells which was characterized by activation of caspase-3, reduction in Bcl-2, Bad and Bcl-x protein synthesis and expression of Annexin V; propidium iodide-positive cells exhibited nuclear fragmentation and characteristic apoptotic ladder. (c) Both differentiation inducers caused reduction in PCNA, p36, cyclin D3, CdK2 and promoted cell cycle arrest. (d) Viable MEL cells sorted after 96h-treatment with both inducers exhibited up-regulated mRNA levels of B-major globin gene and Glycophorin A, indicators of committed late erythroid progenitors. Annexin V-positive sorted populations, after 96h of DMSO/HMBA incubation, exhibited relatively lower expression of aforementioned genes in comparison to live MEL subsets, while stably expressing CD36 gene, characteristic of early erythroid progenitors. In summary, the apoptotic mechanism is activated early during erythroid in vitro differentiation of MEL cells at the proerythroblast compartment while 70-80% of induced CFU-Es are fully committed to the erythroid lineage expressing erythroid clusters of differentiation and producing hemoglobin. These findings indicate that induction of erythroid differentiation is accompanied by differentiation-dependent apoptosis (DDA) presumably as an integral part of hematopoiesis. Elucidation of the molecular basis of DDA induced by chemical inducers during erythroid differentiation, may uncover exploitable potential molecular targets for leukemia therapies.

Keywords: erythroleukemia, apoptosis, differentiation

P-132 HAMLET binding to α-actinin disrupts focal adhesions and facilitates tumor cell detachment

Maria Trulsson (1), Hao Yu (1), Lennart Gisselsson (2), Yinxia Chao (3), Alexander Urbano (3), Sonja Aits (1), Ann-Kristin Mossberg (1), K. Hun Mok (4) and Catharina Svanborg (1)

(1) Institute of Laboratory Medicine, Department of Microbiology, Immunology and Glycobiology, Lund University, Lund, Sweden.
(2) Phase Holographic Imaging AB, Lund, Sweden.
*(3) Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), Singapore.*
(4) Trinity College Dublin, School of Biochemistry and Immunology, University of Dublin, Dublin, Ireland.

HAMLET (human α-lactalbumin made lethal to tumor cells) is a protein-lipid complex, composed of α-lactalbumin and oleic acid, which triggers tumor cell death in a wide range of tumor cell lines. In addition, HAMLET cause a change from a distended to a rounded morphology and from an adherent to a detached state. The aim of this study was to further study the detachment process. HAMLET was shown to interact with α-actinin-4, a major F-actin cross-linking protein and focal adhesion constituent, in a proteomics screen with membrane-associated proteins. The interaction with α-actinin-4 as well as with α-actinin-1 was confirmed by co-immunoprecipitation. Synthetic peptides covering the entire α-actinin-4 protein were used to further characterize the interaction between HAMLET and α-actinin. The mapping revealed that HAMLET recognizes the N-terminal actin-binding domain of α-actinin as well as part of the beta1 intergrin binding site, suggesting that HAMLET might disrupt the α-actinin-dependent scaffold between the actin cytoskeleton and the beta integrins in focal adhesions. Confocal microscopy was used to study focal adhesions constituents and focal adhesions containing paxillin, vinculin and α-actinin-4 were readily observed in adherent tumor cells before HAMLET was added but were rapidly lost after HAMLET treatment. Confocal microscopy also showed that HAMLET colocalize with α-actinin-4 at the cell periphery of carcinoma cells. Focal adhesion assembly leads to downstream signaling through focal adhesion kinase (FAK). This signaling cascade was studied by Western blot and showed a loss in FAK and ERK1/2 phosphorylation upon HAMLET treatment, consistent with a disruption of focal adhesions. In addition, beta1 integrin staining was lost in detached cells. Cell detachment in response to HAMLET was previously observed in bladder cancer patients following intra-vesical HAMLET instillation. In this study, cell detachment in response to HAMLET was confirmed in vitro, using adherent lung carcinoma cells. The detachment in response to HAMLET was shown to be both dose- and time-dependent. siRNA targeting both α-actinin-1 and -4 were shown to increase the detachment of tumor cells. In contrast, α-actinin-4 over-expression significantly delayed morphological changes in response to HAMLET. Preliminary results show that α-actinin siRNA makes adherent cells but not cells in suspension more sensitive to HAMLET, indicating that the increase in detachment rather contributes to cell death than the opposite. The results suggest that the interaction between HAMLET and α-actinins may disrupt focal adhesion complexes, leading to tumor cell detachment, in parallel with their cell death response.

Keywords: Cancer, HAMLET, actinin, detachment, cell death

P-133 Unconventional ubiquitination regulates Bid apoptotic activity

Bert van de Kooij1, Evert de Vries1, Stephen Tait1,*, Peter van Veelen2 and Jannie Borst1

*1Division of Immunology, The Netherlands Cancer Institute, 2Department of Immunohematology and Blood Bank, Leiden University Medical Center, Leiden, The Netherlands, *Current address: Department of Immunology, St Jude Children's Research Hospital, Memphis, TN,*

Upon death receptor stimulation, the BH3-only protein Bid is activated through cleavage by Caspase-8. It is the C-terminal fragment (tBidC) that harbours the pro-apoptotic activity, mediated via its BH3 domain. NMR data demonstrate that after cleavage the Bid N-terminal (tBidN) and C-terminal (tBidC) fragment remain associated via intramolecular interactions. This is surprising, as biochemical data indicate that the pro-apoptotic activity of tBidC is inhibited by the association with tBidN. Our lab has shown, that this inhibition is relieved by ubiquitination and subsequent proteasomal degradation of tBidN. Ubiquitination was unconventional, as ubiquitin conjugation to either a lysine or the N-terminal residue was ruled out. Instead, biochemical and mutagenesis data implicated cysteine, serine and possibly threonine residues as the ubiquitin acceptor sites. We are currently exploring the conjugation to these unconventional residues using biochemical techniques and mass spectrometry. Also, we are testing candidates and setting up a siRNA based screen to identify the machinery that regulates tBidN ubiquitination, which includes a specific deubiquitination (DUB) enzyme, according to our findings. Lastly, we assess the role of mono- and polyubiquitination in the process of Bid activation and function at mitochondrial membrane.

Keywords: Death receptor induced apoptosis, Bid, ubiquitination

P-134 Heterogenous nuclear ribonucleoprotein K is a pan-granzyme substrate and is essential for cell survival

Robert van Domselaar, Robert L. Tatsis, Astrid M. van der Made, Razi Quadir, Roel Broekhuizen, J. Alain Kummer, Niels Bovenschen

Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands

Granule exocytosis by cytotoxic lymphocytes is the key mechanism to eliminate virus-infected cells and tumor cells. These lytic granules contain the pore-forming protein perforin and a set of five serine proteases called granzymes (GrA, GrB, GrH, GrK, GrM). All human granzymes display distinct substrate specificities and induce cell death by cleaving critical intracellular death substrates. Previously, we and others have used proteomic screens to identify heterogenous nuclear ribonucleoprotein K (hnRNP K) as a potential substrate of GrA, GrB, and GrK in tumor cell lysates. In the present study, we show that all human granzymes cleaved hnRNP K, which makes it the first known pan-granzyme substrate. All five human granzymes directly cleaved hnRNP K, and GrH and GrK were most efficient. All granzymes, except GrK, cleaved hnRNP K in an apparent proteolysis-sensitive amino acid region. Furthermore, hnRNP K was cleaved under physiological conditions when intact human tumor cells were challenged with living cytotoxic effector cells. Interestingly, RNA interference-mediated knockdown of hnRNP K in human tumor cell lines triggered spontaneous apoptosis that coincided with increased reactive oxygen species production. Our data indicate that hnRNP K is indispensable for cell viability, suggesting that targeting of hnRNP K by granzymes contributes to the mechanism by which cytotoxic lymphocytes eliminate tumor cells.

Keywords: granzyme, hnRNP K, apoptosis

P-135 Susceptibility for E. coli LPS-induced lethality is differentially regulated by caspase-1 and -3

Tom Vanden Berghe1,2, Dieter Demon1,2, Pieter Bogaert1,2, Alain Goethals1,2, Jill Vandenbroecke1,2, Nozomi Takahashi1,2 , Anje Cauwels1,2 and Peter Vandenabeele1,2

1Department for Molecular Biomedical Research, VIB, Technologiepark 927, 9000 Ghent, Belgium. 2Department of Molecular Biology, Ghent University, Technologiepark 927, 9000 Ghent, Belgium.

Caspases are involved in inflammation and apoptosis, two major mechanisms in the pathology of septic shock. To study their involvement in a comparative way, we analyzed the susceptibility for E.coli LPS-induced lethal shock in caspase-1-, caspase-3-, caspase-7- and IL1b/IL18-deficient mice. We found that caspase-1-deficient mice are protected, as previously reported, and that from its well-established substrates IL1b/18-deficiency, but not caspase-7-deficiency, also protected. Caspase-1- and IL1b/IL18-deficiency protected against both hypothermia and mortality induced by LPS. Interestingly, caspase-3-deficient mice are also protected against LPS-induced mortality, but not against LPS-induced hypothermia. In addition, differential levels of IL-18, IL-1b, IL-1a, IL-12(p40) and IL-6 at early (2h or 6h) time points indicate that caspase-1 and caspase-3 act through different mechanisms. Bone marrow transplantation, macrophage- and T cell-depletion studies, as well as apoptotic ex vivo analyses, will be set up in caspase-1 and -3 deficient mice to further analyze the specific and differential roles of these caspases in LPS-induced lethal shock.

Keywords: Caspases, cell death, inflammation, endotoxemia

P-136 Alteration in mitochondrial superoxide production by shRNA gene silencing of AIF in various cell lines

Miroslav Varecha (1), Daniela Paclova (1), Jirina Prochazkova (2), Pavel Matula (1) and Michal Kozubek (1)

(1) Centre for Biomedical Image Analysis, Faculty of Informatics, Masaryk University, Botanicka 68a, Brno 602 00, Czech Republic.
(2) Department of Cytokinetics, Institute of Biophysics, Kralovopolska 135, Brno 61265, Czech Republic.

During caspase-independent apoptosis AIF is released from mitochondria to the cytoplasm, and subsequently it is translocated into the nucleus, where it mediates caspase-independent chromatin condensation and degradation. Apart from this function in cell death, AIF also plays largely unknown role in cell life. We used AIF gene silencing using shRNA vectors followed by flow cytometry and confocal fluorescent microscopy to detect the superoxide production during oxidative phosphorylation in mitochondria. We managed to decrease the expression of AIF by transient or stable transfection in various human cell lines. Interestingly, mitochondrial superoxide productions in various cell lines were affected by gene silencing differently. Cell line U-2 OS transiently or stably transfected with shRNA was found to produce less superoxide in comparison to control U-2 OS cells. Interestingly, HeLa cell line shows opposite results with significantly increased mitochondrial superoxide production in AIF-silenced HeLa cells. We are also in process of examination of other healthy and cancer cell lines. Conclusions about possible role of AIF in mitochondria based on our results with various AIF-silenced cell lines will be presented.

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Keywords: AIF, shRNA, gene silencing, image analysis, superoxide, mitochondria, oxidative phosphorylation

P-137 Signaling of p75 neurotrophin receptor in breast cancer cells

Stephanie Verbeke (1), Samuel Meignan (1), Emmanuelle Germain (1), Hubert Hondermarck (1), Eric Adriaenssens (2) and Xuefen Le Bourhis (1).

(1) INSERM U908, University of Sciences and Technologies of Lille, F-59650 Villeneuve d'Ascq, France
(2) CNRS UMR 8161, Institut Pasteur de Lille, F-59800 Lille, France

The common neurotrophin receptor p75NTR has been shown to initiate intracellular signaling that leads to cell survival, differentiation or apoptosis depending on the cell types examined. In breast cancer cells, we and others have shown that all the neurotrophins are able to induce an anti-apoptotic effect via p75NTR. Nevertheless, the commonly used breast cancer cell lines express relatively low levels of p75NTR compared to tumor biopsies. To elucidate further the role of p75NTR in these cells and its mechanism of action, we established and characterized breast cancer cells which stably overexpress p75NTR. We showed that p75NTR overexpression per se promoted cell survival to apoptogens with a concomitant slowdown of cell growth. The pro-survival effect is associated with an increased expression of the inhibitor of apoptosis protein-1 (c-IAP1), a decrease of TRAIL-induced cleavage of PARP, procaspase 9 and 3, and a decrease of cytochrome C release from the mitochondria suggesting a regulation of the intrinsic pathway. The anti-proliferative effect is due to a cell accumulation in G0/G1, associated with a decrease of Rb phosphorylation and an increase of p21waf1. Interestingly, inhibition of p21waf1 with siRNA not only restores proliferation but also abolishes the pro-survival effect of p75NTR, indicating the key role of p21waf1 in the biological functions of p75NTR. Finally, using a SCID mice xenograft model, we showed that p75NTR overexpression favors tumor growth and strongly increases tumor resistance to anti-tumoral treatment.

Together, our findings suggest that p75NTR overexpression in breast tumor cells could favor tumor survival and contribute to tumor resistance to drugs. This provides a rationale to consider p75NTR as a potential target for the future design of innovative therapeutic strategies.

Keywords: p75NTR, p21waf1, survival, cell cycle, breast cancer

P-138 PERK deficiency distorts the ER – mitochondrial interface and protects against ROS-induced intrinsic apoptosis

T. Verfaillie(1), N. Rubio(1), M. Dewaele(1), G. Bultynck (2), J.P. Decuypere(2), W. Martinet(3), S. Gupta(4), A. Samali(4) and P. Agostinis(1)

(1)Laboratory of Cell Death Research and Therapy, Dept. of Molecular Cell Biology, K.U.Leuven, Belgium
(2)Laboratory of molecular and cellular signaling, Dept. of Molecular Cell Biology, K.U.Leuven, Belgium
(3)Division of Pharmacology, University of Antwerp, Belgium
(4)AS Lab, Department of Biochemistry, NUI, Galway, Ireland

Any insult that disturbs ER homeostasis, like alteration of the cellular redox status or release of ER Ca2+, ultimately results in ER stress due to the accumulation of misfolded proteins. ER stress subsequently triggers the Unfolded Protein Response (UPR) which sustains both pro-survival and pro-apoptotic pathways. Interestingly, the hostile environment encountered by tumor cells also activates the UPR and has been shown to alter the chemosensitiviy of cancer cells. Therefore, a better understanding of the molecular mechanisms underlying the proapoptotic and prosurvival mechanisms governed by the UPR is of great importance for current and future cancer therapies.

Real time PCR studies revealed an upregulation of several targets of the PERK branch of the UPR after perturbation of ER homeostasis through the generation of reactive oxygen species (ROS) by photodynamic therapy (PDT) with the ER localizing photosensitizer hypericin, in both MEFs and T24 bladder cancer cells. Cell death analysis, involving detection of PARP and caspases 3 and 9 cleavage, loss of mitochondrial transmembrane potential as well as clonogenic assays, revealed that PERK-KO MEFs display an increased resistance to ROS-mediated mitochondrial apoptosis, while being sensitized against thaspigargin. Moreover, our data indicate that the contact site between ER and mitochondria as well as Ca2+ signaling is deranged in PERK deficient cells. Interestingly, Ca2+ signaling in IRE1 deficient cells is not perturbed, thus indicating a PERK specific effect. Experiments assessing direct mitochondrial Ca2+ uptake with a mitochondrially targeted cameleon and ROS-mediated mitochondrial damage in WT and PERK deficient cells are ongoing to clarify the crucial role in the ER-mitochondria interplay in ROS-mediated intrinsic apoptosis.

Keywords: ROS, ER stress, apoptosis, mitochondria

P-139 Induction of autophagy and caspase dependent cell death by the flavonoid Luteolin specifically in malignant skin cells.

Lien Verschooten(1), Kathleen Barrette(1), Sofie Van Kelst(1), Rita De Vos(2), Patrizia Agostinis(3) and Marjan Garmyn(1)

(1)Laboratory of Dermatology, Catholic University of Leuven, Belgium
(2)Laboratory of Morphology and Molecular Pathology, Catholic University of Leuven, Belgium
(3)Laboratory for Cell death Research and therapy, Department of Molecular Cell Biology, Catholic University of Leuven, Belgium

Flavonoids interfere with multiple cellular pathways and processes resulting in antioxidative, anti-inflammatory and anticarcinogenic effects in mammalian cells. The cellular response on flavonoid compounds differs often depending on the cell type (tissue origin, malignancy) and on the doses. In general however, flavonoids inducing cell death in cancer cells show a relative lack of toxicity towards normal cells, which makes them promising chemotherapeutic molecules. In this study, we investigated the possible differential effect of 3',4',5,7-tetrahydroxyflavone or luteolin (LUT) on normal human keratinocytes (NHK) and on cells derived from squamous cell carcinoma (SCC) (MET1, MET4 ,A253).

LUT induced cell death through apoptotic signaling in all tested SCC cell lines starting from a concentration of 50µM, whereas no caspase activation was detected after treatment of NHK with LUT up to at least 200µM. In SCC-cells, both initiator caspases, caspase 8 and 9, were activated upon LUT treatment. Addition of zVAD-fmk, a pan-caspase inhibitor, significantly reduced cell death induced by LUT, which suggests a caspase-dependent cell death modality.

Interestingly, the induction of cell death was preceded by a highly vacuolisated cytoplasm of the SCC cells. Using acridine orange, we identified the vacuoles as part of an enlarged lysosomal compartment, which might indicate the involvement of autophagy. Indeed, we detected an increased number of autophagosomes using transmission electron microscopy (TEM) analysis and a punctuated pattern in GFP-LC3 transiently transfected cells. An increase in the number of autophagosomes can be the result of a block of autophagosome-lysosome fusion on the one hand or of a stimulation of autophagic flux on the other hand. Degradation of p62, a multidomain protein adaptor which acts as a receptor protein during early autophagy, and the appearance of mRFP-LC3 puncta in mRFP-GFP-LC3 transiently transfected SCC cells upon LUT-treatment, confirmed that LUT induced autophagic flux in these malignant cells.

Previously, we could show that all used SCC cell lines exhibit enhanced activation of PI3K/AKT-signaling, which is prominent in the regulation of both apoptosis and macroautophagy. Here we show that LUT treatment resulted in a strong and immediate inhibition of the enhanced activation status of AKT in MET1 and MET4 cells, as demonstrated by a decrease in AKT phosphorylation and in an inhibition of AKT-activity.

These results suggest that LUT induces autophagy-associated cell death in SCC cells, possibly by interference with the PI3K/AKT signaling pathway. Further experiments are needed to further clarify the molecular interplay between LUT-induced autophagy and apoptosis.

Keywords: autophagy, apoptosis, flavonoid, SCC

P-140 MG-2477, a new tubulin inhibitor with potent antitumor activity in vitro and in vivo induce autophagy and delayed apoptosis in A549 cells

Giampietro Viola (1), Roberta Bortolozzi (1), Maria Grazia Ferlin (2) Paola Brun (3), Ignazio Castagliuolo(3), Ernest Hamel (4), Giuseppe Basso (1)

(1)Department of Pediatrics, Laboratory of Oncohematology University of Padova, Italy
(2) Department of Pharmaceutical Sciences, University of Padova, Italy
(3) Department of Histology, Microbiology and Medical Biotechnologies University of Padova, Italy
(4)Toxicology and Pharmacology Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute at Frederick, National Institutes of Health, Frederick, Maryland, USA

We have previously demonstrated that MG-2477 (3-Cyclopropylmethyl-7-phenyl-3H-pyrrolo[3,2-f]quinolin-9(6H)-one) remarkably inhibited the growth of several cancer cells in vitro. Here we identified MG-2477 as an antimitotic agent since it inhibited tubulin polymerisation bound to colchicines site, induce arrest of cells in metaphase and disrupted the tubulin network as assessed by immunofluorescence staining of A549 cells. The mechanism of action of MG-2477 has been investigated in vitro. Treatment of A549 cells with MG-2477 indicate that the drug induce an arrest of the cell cycle in G2/M phase, confirmed also by accumulation of cyclin B. Western blot analysis also reveal increased levels of p53 and p21 protein in response to treatment with MG-2477. As other microtubule interacting agents the compounds induces phosphorylation of Bcl-2 and Bcl-xL proteins.

Interestingly the drug induces autophagy followed at later times of incubation by apoptotic cell death. Autophagy was early detected (12 h) by the conversion of the microtubule associated protein 1 light chain 3 (LC3-I) into its cleaved and lipidated form (LC3-II) which is exclusively localized to autophagosomes during autophagy. This effect was also confirmed by appearance of large acidic vacuoles that were detected by flow cytometry after acridine orange staining. At longer times of exposure (48 h) phosphatidylserine externalization on the outer membrane along with caspase-3 and PARP activation take place revealing that apoptotic cell death occur. Notably pharmacological inhibition of autophagy with 3-Methyladenine, Bafilomycin A1 increase apoptotic cell death suggesting that the induced autophagy by MG-2477 plays a protective role that delay apoptotic cell death. A key regulator of cell fate decision, including regulation of autophagy is the kinase mTOR. Treatment of A549 cells with MG-2477 resulted, just after 12 h of incubation, in a marked decreased of the phosphorylation of the mTOR targets S6 protein and 4EB-P1. Interestingly MG-2477 also induce dephosphorylation of AKT at Ser473 but at Thr308 suggesting that autophagy is controlled by the Akt-mTOR pathway. The activated pathway(s) involve late activation of caspase-3 and 7 but not caspase-9. Neither significative changes in mitochondrial potential nor cytochrome c release were observed, suggesting that the apoptosis induced by MG-2477 do not follow a mitochondrial pathway. On the other hand a sharp decrease of the antiapoptotic proteins XIAP and Mcl-1 was observed at 48h of treatment.

Altogether our results show that death signals activated by the novel antimitotic agent MG-2477 are dependent on mTOR and this effect could be attributed to damaged microtubules. In addition these findings may have implications for cancer therapy and provide new clues for anticancer drug design and development.

Keywords: Microtubule, autophagy, apoptosis, mTOR, Akt

P-141 The Regulation of HIF-1 by Vitamin C and its Impact on Cell Survival in Inflammation and Cancer

Caroline Kuiper (1), Usha Rani Pujary(1), Gabi U Dachs (2), Margaret J Currie (2), Peter H Sykes (3) and Margreet C M Vissers (1),

(1)Free Radical Research Group,Pathology Department,University of Otago, Christchurch.Christchurch, New Zealand.
(2) Angiogenesis Research Group,Pathology Department,University of Otago, Christchurch.Christchurch, New Zealand.
(3) Dept of Obstetrics and Gynaecology,University of Otago, Christchurch.Christchurch, New Zealand.

The response to metabolic stress is determined by hypoxia-inducible factor(HIF)-1, a ubiquitous transcription factor that controls the expression of genes involved in cell metabolism, glycolysis, angiogenesis, erythropoeisis, iron metabolism, and cell life and death pathways. HIF-1 is an α-beta heterodimer and its activity is under dual control via the hydroxylation of proline and asparagine residues on the α subunit, regulating both stabilisation of the protein and its transcriptional ability. The HIF hydroxylases responsible for these reactions belong to the family of 2-oxoglutarate(OG)-dependent dioxygenases and are non-haem iron proteins that require O2, 2-OG, Fe and ascorbate for activity. When any one of these factors is limiting, hydroxylation of HIF-1α is compromised and transcription of HIF-1 target genes is likely. As a co-factor for the HIF hydroxylases, ascorbate is thought to keep the active site Fe in the ferrous state required for optimal activity. This action is specific for ascorbate, and cannot be adequately substituted by other reducing agents. In our studies we have investigated the importance of maintaining adequate intracellular ascorbate for the optimal control of HIF-1 in immune cells and in cancer.

HIF-1 is an important regulator of immune cell function and changes in the phagocytic ability of macrophages and in neutrophil apoptosis have been described. We have shown that when neutrophils are deficient in ascorbate, HIF-1 is up-regulated under normoxic conditions and apoptosis is impaired. This has a major impact on the clearance of neutrophils and the resolution of inflammation.

HIF-1 is a major pro-survival factor in solid tumours through the promotion of angiogenesis and glycolysis: these effects combine to contribute to an aggressive phenotype and to evasion of chemotherapy. We have monitored ascorbate levels and HIF-1 activation in human endometrial tumours and have found (i) that delivery of ascorbate to cancer cells is compromised in high-grade tumours and (ii) that low ascorbate content is associated with up-regulation of HIF-1. This study demonstrates for the first time a likely in vivo relationship between ascorbate and HIF-1 with important implications for tumour growth and survival.

Keywords: cancer, inflammation, hypoxia, angiogenesis,

P-142 Apoptosis induced by lipid peroxidation end products: cellular and molecular basis

Beyza Vurusaner (1), Ozgur Kutuk (2) and Huveyda Basaga (1)

(1) Biological Sciences and Bioengineering Program, Sabanci University, 34956, Orhanli-Tuzla, Istanbul, Turkey
(2)Medical Oncology- Dana-Farber Cancer Institute, Harvard Medical School, Boston,MA 02115, USA

Peroxidation of lipids such as cholesterol, triglycerides, lipoproteins available in blood, are considered as the initiating factor of chronic inflammatory diseases such as atherosclerosis. Low density lipoproteins are oxidized enzymatically or non-enzymatically in the vessel wall and modified to oxidized low density lipoproteins which is a very toxic mixture that plays an important role in initiation and rupture of atherosclerotic plaques. Reactive aldehydes are often formed during peroxidation of lipids and 4-hydroxynonenal (HNE) is a highly reactive and stable end product of this process. HNE has been demonstrated to induce apoptosis and oxidative stress in a dose dependent manner in various cell types. Several signaling pathways have been shown to be modulated by HNE, including MAP kinases, PKC isoforms, cell cycle regulators, receptor tyrosine kinases, some Bcl-2 proteins and caspases. In order to get insight into the mechanism that HNE induces apoptosis, we studied MAP kinase and caspase activation pathways in 3T3 fibroblasts.

HNE activated JNK and p38 MAP kinases, but downregulated the basal activity of ERK 1/2 in 3T3 fibroblasts. We have showed that HNE triggered caspase-9 and 3 activation, release of cytochorome C from mitochondria and PARP cleavage. Utilization of specific kinase inhibitors and overexpression studies in 3T3 fibroblasts showed that activation of JNK and c-Jun/AP1 transcriptional acitivy are involved in HNE-induced apoptosis. We have also demonstrated that pre-treatment with resveratrol, a widely distributed polyphenol, protected against HNE induced apoptosis through inhibiting HNE-induced JNK and p38 activation, c-Jun expression and phosphorylation.

Recent data on the cellular mechanism of apoptotic response to HNE with a specific focus on the involvement of pro and anti-apoptotic members of Bcl-2 protein family will be presented and discussed.

Keywords: apoptosis, oxidative stress, lipid peroxidation

P-143 Serum biomarkers of cell death for monitoring therapy response of gastrointestinal carcinomas

Kristin Wahl (1), Doreen Brandt (1), Matthias Anstätt (1), Klaus Schulze-Osthoff (2), Heike Bantel (1)

(1)Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany
(2)Interfaculty Institute of Biochemistry, University of Tübingen, Tübingen, Germany

Purpose: Antitumour treatments are thought to exert their therapeutic efficacy mainly by induction of apoptosis in tumour cells. In epithelial cells, caspases, the key enzymes of apoptosis, cleave the intermediate filament protein cytokeratin (CK)-18 into specific fragments that are released into circulating blood and can be detected by a specific ELISA.

Experimental design: To investigate the use of CK-18 fragments as a potential biomarker for the treatment response, we examined the association of serum CK-18 levels and clinical response in 35 patients with gastrointestinal cancers.

Results: While both cleaved and total CK-18 levels were intrinsically elevated in tumour patients, they were further increased during 5-fluorouracil (5-FU)-based therapy. Importantly, the increased levels of CK-18 could discriminate between patients with different clinical response. Cancer patients with a partial response or stable disease revealed a significantly higher increase of cleaved CK-18 during chemotherapy as compared to patients with progressive disease.

Conclusions: Our results suggest that detection of circulating caspase-cleaved CK-18 might be a useful serum biomarker for monitoring treatment response and should merit further

evaluation in larger patient groups.

Keywords: gastrointestinal cancer, CK-18

P-144 Proteome-wide analysis reveals that caspase-2 shares the cleavage and substrate specificity with caspase-3

Magdalena Wejda1,2, Francis Impens3,4, Petra Van Damme3,4, Tom Vanden Berghe1,2, Nozomi Takahashi1,2, Kris Gevaert3,4, Peter Vandenabeele1,2

(1) Department for Molecular Biomedical Research; VIB; Ghent, 9052; Belgium
(2) Department of Molecular Biology; Ghent University; Ghent, 9052; Belgium
(3) Department for Medical Protein Research; VIB; Ghent, 9000; Belgium
(4) Department of Biochemistry; Ghent University; Ghent, 9000; Belgium

Caspase-2 is most often considered a member of initiator caspases group, as it contains long prodomain (CARD) and appears to be activated by proximity induced autocatalytic processing. On the other hand, the cleavage specificity analysis, based on a combinatorial library of synthetic peptides, placed caspase-2 closer to executioner caspases, namely caspase-3 and -7. Since the specificity for synthetic peptidic substrates does not always correspond directly to the specificity that a protease exerts on native proteins, we decided to screen systematically for both unique and common caspase-2 and -3 substrates in cell lysates by means of COFRADIC method.

The analysis aiming at complete proteome coverage was performed on caspase-2 or -3-treated cell lysates from mouse macrophage cell line (Mf4/4) or from human non-small cell lung carcinoma cell line (A549). Of total 108 cleavage events in mouse and 92 cleavage events in human, no unique caspase-2 cleavage sites were identified, contrary to caspase-3-unique substrates (34 in mouse and 19 identified in human). The IceLogo analysis of P and P' site amino acid sequence showed that both caspases show similar preference, P4-P1 DEVD being the most favored by both caspase-2 and -3. The results obtained by COFRADIC and in silico analyses are being validated experimentally.

Based on these results it appears that caspase-2 shares the cleavage and substrate specificity with caspase-3, all proteins being cleaved by caspase-2 are also cleaved by caspase-3, suggesting overlapping degradome, although caspase-2 cleaves less efficiently. Taken into account the fact that caspase-2 of all mouse or human caspases has the largest sequence homology to CED3 in C. elegans (55%) and that it seems to be a rather inefficient protease compared to other family members, we hypothesize that caspase-2 may be an evolutionary more executioner caspase, while caspase-3 has developed as a more efficient dismantling system and executioner amplifier downstream of a broad variety of apoptotic stimuli. Therefore, caspase-2 in some cases may operate as an apoptotic executioner caspase in conditions where caspase-3 and -7 are absent. We are currently exploring this possibility.

Keywords: caspase-2, COFRADIC

P-145 Improving the survival of pancreatic beta cells

Christian Widmann, Natasa Bulat, Evrim Jaccard, Jannick Pétremand

Department of Physiology, Faculty of Biology and Medicine, University of Lausanne, 1005 Lausanne, Switzerland

Apoptosis of pancreatic beta cells leads to type 1 diabetes and may contribute to the development of type 2 diabetes. Apoptosis also mediates beta cell loss in islet transplantation both during isolation of the islets and during engraftment. Finding ways of increasing the resistance of beta cells towards apoptotic stimuli would therefore be beneficial in the context of diabetes therapy.

We have characterized in the last few years an amino-terminal, caspase-generated, RasGAP fragment, called fragment N, that protects various cell types against a series of apoptotic stimuli. Pancreatic beta cells, in particular, are rendered more resistant towards apoptotic stresses when they express fragment N. We have recently derived a transgenic mouse line, called RIP-N, which expresses fragment N under the control of the rat insulin promoter (RIP). The presence of fragment N in the beta cells of these mice confer resistance to experimentally induced diabetes and islets isolated from RIP-N mice are more resistant to cell death induced by inflammatory cytokines, hyperglycemia, palmitate or hypoxia. Fragment N also delays the onset of diabetes in NOD mice, a well-known type 1 diabetes mouse model. Importantly, the presence of fragment N in beta cells has no impact on their ability to secrete insulin in response to increased glucose levels.

Another strategy to protect beta cells that we employ relies on high density lipoproteins (HDLs). These lipoproteins are indeed known to turn on anti-apoptotic signals in various cells types, including beta cells. We have found in particular that HDLs are efficient blocker of ER stress-induced apoptosis in beta cells and we are currently characterizing the mechanisms of this protection. HDLs may therefore be particularly useful to protect beta cells in situations where the beta cells have to produce and secrete more insulin via the ER such as in insulin-resistant pre-diabetic patients

Keywords: Diabetes, RasGAP, Caspase substrate, Anti-apoptotic signals

P-146 The role of PON3 in reduction of oxidative stress and cell death

Ines Witte, Eva-Maria Schweikert, Petra Wilgenbus, Ulrich Förstermann, Sven Horke

Department of Pharmacology, University Medicine Mainz, Obere Zahlbacher Str. 67, 55131 Mainz, Germany

Paraoxonase-3 (PON3), member of the paraoxonase family of enzymes, is a barely characterized protein. In recent studies it has been shown that overexpression of transgenic PON3 reduced atherosclerosis and adiposity in mice fed an atherogenic diet. Importantly, in human cells, PON3 localized to the endoplasmic reticulum (ER) and mitochondria. Since we recently found that its paralogue PON2, which is ~70% identical to PON3, protects against oxidative stress and apoptosis in a calcium-sensitive fashion, we performed a similar study with human PON3. We analyzed PON3 expression levels in a multitude of human cell lines and we show that its expression seems relatively restricted to certain tissues. When addressing its anti-oxidative function, PON3, like PON2, was able to diminish superoxide formation particularly at the mitochondria, or e.g. in response to the Pseudomonas aeruginosa bacterial signaling molecule pyocyanin. Unlike PON2, however PON3 mRNA and protein were not degraded in response to major Ca2+-disturbances. With respect to apoptosis, we recently found that PON2 stabilized tumor cells and was overexpressed in several cancers. Therefore, we also addressed this for PON3 and tested its anti-apoptotic potential, with the use of different chemotherapeutics. Interestingly, PON3 was also found overexpressed in several human cancer samples. Thus, our study characterizes PON3 functions and addresses a putative role in human tumors.

Keywords: mitochondria, oxidative stress, apoptosis, cancer

P-147 Stage-specific expression of TNFα to regulate sequential apoptotic and necrotic cell death in birnavirus-infected fish cells

Wei-Lun Wang (1,2), Jiann-Ruey Hong (3), Gen-Hwa Lin (2), Wangta Liu (2), Hong-Yi Gong (4), Ming-Wei Lu (4), and Jen-Leih Wu (1,2)

(1) Institute of Fisheries Science, National Taiwan University, Taipei, Taiwan
(2) Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan
(3) Institute of Biotechnology, National Cheng Kung University, Tainan, Taiwan
(4) Department of Aquaculture, National Taiwan Ocean University, Keelung, Taiwan

Infectious pancreatic necrosis virus (IPNV) can induce Bad-mediated apoptotic cell death and secondary necrosis; whether there is a connection is still unknown. We demonstrated that the pro-inflammatory cytokine TNFα was up-regulated following IPNV infection. Importantly, TNFα is required for regulating both the Bad/Bid-mediated apoptotic death pathway and RIP1/ROS mediated necrotic death pathway in the ZF4 cell line. Using DNA microarray and quantitative RT-PCR analyses, we identified two major subsets of differentially expressed genes following IPNV infection, including those involved in the innate immune response and in the induction of apoptosis. During the early stage of IPNV replication, the pro-inflammatory cytokine TNFα was quickly induced up to sixfold. Then, the pro-apoptotic bcl-2 family members Bad and Bid were up-regulated during the early-middle IPNV replication stages. Therefore, we examined whether TNFα may modulate the apoptotic and secondary necrotic death pathways in this system. The treatment of the IPNV-infected cells with siRNA of TNFα and AG-126, a specific inhibitor of TNFα expression, significantly inhibited the expression of the pro-apoptotic genes Bad and Bid as well as the activation of the downstream molecules caspase-8, -9, and -3 during viral replication. Furthermore, siRNA of TNFα and AG-126 treatment seemed to impact the necrotic death pathway, diminishing the expression of the necrosis-related gene RIP-1 and the downstream production of ROS during the middle-late stages of replication. Our results suggest that IPNV infection up-regulates the TNFα-mediated cell death signals that may regulate the Bad/Bid-mediated apoptotic death pathway during the early-middle stages of replication and the RIP1/ROS-mediated necrotic death pathway during the middle-late stages of replication.

Keywords: IPNV, microarray, apoptosis, necrosis, TNFα;, ROS, zebrafish

P-148 Intracellular GTP level determines cell fate by differentiation and apoptosis

Azadeh Meshkini, RaziehYazdanparast

University of Tehran

Since the provision of adequate supply of guanine nucleotides is vital for many aspects of cellular metabolism and signaling pathways, limitation of their synthesis might result in growth inhibition, differentiation or apoptosis. A point for speculation is whether a sensing mechanism exists in cells for monitoring guanine nucleotide levels. The aim of this study was to disclose the effect of different level of GTP depletion on signaling pathways and consequently cell fate. In that regard, we measured intracellular GTP level among K562 cells exposed to mycophenolic acid (MPA) or 3-hydrogenkwadaphnin (3-HK) as potent inosine monophosphate dehydrogenase inhibitors. Our results revealed that maturation developed when intracellular GTP level reduced 30-40% of the control level. Under this experimental condition, both 3-HK and MPA caused marked up-regulation of PKCα; and PI3K/AKT pathways; however these responses along with differentiation were abrogated by exogenous addition of guanosine and restoration of intracellular GTP. Further, co-treatment of cells with hypoxanthine plus 3-HK or MPA, caused the reduction of intracellular GTP level to less than 60% of the control value which led to occurrence of apoptosis. Under this circumstance, mitochondrial pathway was activated along with inverse regulation of Bcl-2/Bax expression and finally activation of caspase-3. Moreover, reduction of GTP level to less than 60% caused the augmentation of intracellular ROS formation and nuclear localization of p21 and subsequent cell death. Collectively, these results suggest that a threshold level of GTP is needed to initiate apoptosis and oxidative stress rather than differentiation.

Keywords: GTP, mycophenolic acid, 3-hydrogenkowadaphnine, K562, differentiation, apoptosis

P-149 TGF-β1 regulates apoptosis and autophagy in bovine mammary epithelial cells during involution

1Joanna Zarzynska, 2Malgorzata Gajewska, 2Agnieszka Sobolewska, 3Barbara Gajkowska, 2Tomasz Motyl

(1)Department of Food Hygiene and Public Health, Faculty of Veterinary Medicine,Warsaw University of Life Sciences, Poland
(2) Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, Poland
(3)Department of Cell Ultrastructure, Medical Research Center, Polish Academy of Science, Warsaw, Poland

Mammary gland undergoes intensive remodeling during each lactation cycle. The gland's involution, which occurs after weaning, is characterized by intensive apoptosis of secretory tissue. In contrast to other species bovine mammary gland undergoes a regenerative involution, because of the characteristic overlap in time between mammogenesis controlled by pregnancy hormones, and regression of the senescent tissue, during which the death of a part of secretory cells is observed. The state of temporary malnutrition of mammary epithelial cells, forces the cells to induce autophagy, as a mechanism stabilizing intracellular supplies of energy. The present study shows the existence of a balance between apoptosis and autophagy in bovine mammary gland throughout the lactation cycle. The range of both processes was assessed on the basis of expression of apoptotic markers: caspase3, 89kDa PARP, and autophagic markers: beclin1, and LC3 in the ex vivo studies on tissue sections obtained from cows in different stages of lactation cycle, as well as in the in vitro studies on bovine mammary epithelial cell line BME-UV1. Additionally the effect of TGF-β1 – an antiproliferative and proapoptotic growth highly expressed during involution - was studied in the context of autophagy and apoptosis regulation.

The highest intensity of apoptosis and autophagy in bovine mammary gland was observed during dry period, which was manifested by the highest IHC staining of the mammary gland tissue section with the antibodies against active caspase-3, and the 89kDa PARP degradation product, as well as high beclin1 expression. Additionally increased levels of TGF-β1 and its receptor – TβRII - in the parenchymal tissue was noted. The in vitro studies have shown that TGF-β1 caused an increase in the expression of proapototic protein Bax, and in the number of apoptotic cells. At the same time the level of beclin1 was also elevated in cells treated with TGF-β1. The ex vivo studies showed further, that the increase in the expression of TβRII during the dry period was accompanied by the decrease in IGF receptor (IGF-IRα;) expression, and an increase in the level of IGFBP-4, and -5. The obtained results suggested that TGF-β1 is a factor stimulating the processes of apoptosis and autophagy during bovine mammary gland involution. The cytokine may act via the regulation of IGF signaling, since the intensification of both processes correlates with downregultion of IGF-IRα;, and upregulation of IGF binding proteins.

Keywords: bovine mammary gland, TGF-β1, apoptosis, autophagy

P-150 Beyond cell death: role of p66shc and redox stress on autophagy, adipogenic differentiation and metabolism.

Zecchini E(1)., Granatiero V.(1) Mammucari C. (1), Murgia M. (1), Pallafacchina G. (1), Raffaello A.(1) and Rizzuto R. (1)

(1)Dept. Of Biomedical Science, University of Padova, via G.Colombo 3, 35131 Padova

Reactive oxygen species (ROS) are short-living and highly reactive molecules formed by incomplete one-electron reduction of oxygen. Mitochondria produce low levels of ROS as an inevitable consequence of oxidative metabolism. Low levels of ROS are normally reduced by non-enzymatic and enzymatic oxidizing agents, such as glutathione, thioredoxin, superoxide dismutase (SOD), catalase and peroxidases. Oxidative stress results from exposure to high levels of ROS, which are not detoxified by cellular antioxidizing agents, and produces cellular damage due to the oxidation of cellular constituents. ROS, however play not only a damaging role: recent studies have demonstrated that they also actively participate in a diverse array of biological processes, including normal cell growth, induction and maintenance of the transformed state, and cellular senescence. We focused our attention on the role of p66shc, a Shc protein involved in stress responses, in particular it has been demonstrated that it is activated by redox stress and that it produces ROS itself. Cells lacking p66shc, indeed, are protected by the apoptotic effect of redox stress; moreover mice KO for p66shc show a significantly extended lifetime. Our purpose is to investigate a possible involvement of p66shc in different phenomena, such as autophagy, adipogenic transdifferentiation and metabolism of skeletal muscle cells, by comparing wt mice with p66shc KO mice. Autophagy is a general term referring to pathways for the degradation of cellular constituents (cytosol and organelles) by the autophagolysosome; it is activated mainly by nutrient starvation and it plays a dual role: it is primarly a surviving mechanism, but it also leads to cell death thus possibly acting as an alternative to apoptosis. Starting from this notion, and from the fact that p66shc KO cells are protected from apoptosis, we investigated a possible role of p66shc as a key element in the switch from apoptosis to autophagy. We observed that, while in wt cells redox stress induces apoptosis, cells lacking p66shc in the same condition activate the autophagic pathway. We are now trying to investigate the biological effect of these observation. The second aspect of our work is the investigation of a putative role of p66Shc in the adipogenic transdifferentiation of skeletal muscle precursor cells. To this purpose we used an in vivo model and we observed that mice lacking p66shc exposed to muscle damage (e.g. freeze injury or redox stress) show lower adipocyte accumulation than wt mice, thus suggesting a role of p66 in the activation of adipogenic differentiation pathway. Finally, we compared wt and p66shc KO mice analyzing the activation of the Akt pathway and we observed that p66shc KO show a lower activation of this pathway, suggesting a ROS-dependent regulation of the Akt-S6 axis.

Keywords: p66shc, adipogenesis, autophagy, metabolism, ROS

P-151 Decitabine synergistically with valproic acid restores caspase-8 expression and sensitizes small cell lung carcinoma cells to TRAIL

Vitaliy Kaminskyy, Olga Surova, Alena Vaculova and Boris Zhivotovsky

Institute of Environmental Medicine, Division of Toxicology, Karolinska Institutet, Box 210, SE-171 77 Stockholm, Sweden

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a promising drug for treatment of tumors because of its specificity to kill cancer cells while sparing most normal cells. However, certain types of tumors were shown to be resistant to this cytokine. One of the mechanisms of the resistance of small cell lung carcinomas (SCLCs) to TRAIL is lacking of caspase-8 expression in most of these cell lines and clinical samples. Although methylation of caspase-8 promotor has been suggested as a potential mechanism of silencing of caspase-8, we showed that the reduction of the main enzymes involved in DNA methylation, DNA methyltransferases 1, 3a and 3b, by pharmacologically-relevant doses of decitabine was not sufficient to significantly restore expression of caspase-8 in a panel of SCLC cells lines. Recently, the combination of decitabine with an anticonvulsant and mood-stabilizing drug valproic acid (also known as an inhibitor of HDAC) has been suggested for clinical trials of advanced cancer. We found that this combination significantly increased expression of caspase-8 in SCLCs at the level of mRNA. Importantly, the combination of these drugs sensitized SCLC cells to apoptosis induced by TRAIL. The cell death induced by TRAIL treatment involved mitochondrial apoptotic pathway and accompanied by cleavage of Bid, activation of Bax and release of cytochrome c. Both initiator caspase-8 and -9 were required for sensitization of SCLC cells to TRAIL. Thus, here for the first time we showed that for efficient restoration of caspase-8 expression in SCLC cells inhibitors of DNA-methyltransferases might be used in combination with HDAC inhibitor valproic acid. Our results suggest combination of decitabine and valproic acid as a potential sensitizing factor to TRAIL for treatment of SCLC cells lacking caspase-8.

Keywords: apoptosis, lung cancer, caspase-8