POSTER SESSIONS

Poster Session 1 (uneven poster numbers)

Thursday, Sept. 15th, 2011 (16:00-18:00)

Poster Session 2 (even poster numbers)

Friday, Sept. 16th, 2011 (16:00-18:00)

Actions of Hepatoma-Derived Growth Factor related protein 2 (HDGF-2) in DNA damage response and lysosomal membrane stability

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A challenge in cancer therapy is drug resistance, which may be circumvented by developing new and improved treatments or identifying therapeutic targets such as lysosomal membrane permeabilisation (LMP). Lens epithelium-derived growth factor (LEDGF) is an oncogenic protein and has been found to be required for proper repair of double strand breaks (DSBs) as well as stabilising lysosomes, thereby preventing LMP. It is part of a family of proteins, HDGF family, whose members are characterised by their C-terminal PWWP domain. Hepatoma-derived growth factor related protein 2 (HDGF-2) is a close homologue to LEDGF and a member of the same protein family. Considering the striking structural similarity between LEDGF and HDGF-2, to date only the functions and mechanisms of LEDGF have been reported. Our project aims at elucidating the mechanisms of HDGF-2 in relation to DNA damage response, cancer cell survival and lysosomal membrane stability. Our findings show that HDGF-2 plays a crucial role in the recruitment of CtBP-interacting protein (CtIP) to DNA DSBs. CtIP is necessary for the DNA end resection and the following repair by homologous recombination. Our study will enlighten the molecular mechanisms of HDGF-2 in relation to DNA-end resection at DSBs. Also, if HDGF-2 shares more of the same functions of LEDGF, it may become a new target in triggering LMP. In cancer, many patients suffer from ineffective response to chemotherapy, thereby leading to drug resistance and further aggravation of the disease. By inducing LMP, one circumvents the traditional apoptosis cell-death pathway, which is often defective in cancer cells, and instead seeks to hit the disease in a soft spot – namely the lysosomes. HDGF-2 is a potential target in cancer treatment due to its marked homology to LEDGF and it may become a new target for chemotherapeutic drugs and thus help ameliorate cancer treatment.

RIP-1 is required for Smac mimetics-enhanced sensitization of neuroblastoma cells to TRAIL-induced apoptosis.

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Smac mimetics are being developed as a new class of anticancer therapies. Since the single agent activity of Smac mimetics is limited in most cancers, rational combinations represent a viable strategy for their development. Here, we report that Smac mimetics at subtoxic concentrations cooperate with specific monoclonal antibodies against TRAIL-R1 or TRAIL-R2 to induce apoptosis in a synergistic manner in neuroblastoma cells. This cooperativity of specific monoclonal antibodies to TRAIL-R1 or TRAIL-R2 in combination with Smac mimetics is confirmed in a panel of neuroblastoma cell lines (SH-EP, LAN-5, SH-SY5Y, KELLY, SK-NAS, CHP-212, NLF), demonstrating the generality of this finding. Of note, Smac mimetics also act in concert with TRAIL-R1 or TRAIL-R2 antibodies to trigger apoptosis in primary cultured neuroblastoma cells, underscoring the clinical relevance. Importantly, we identify RIP-1 as a critical regulator of this synergism that is critical for the formation of a RIP-1/FADD/Caspase-8 complex that drives caspase-8 activation. Indeed, knockdown of RIP-1 abolishes formation of this complex and subsequent activation of caspases and apoptosis. Similarly, inhibition of RIP-1 kinase activity by Necrostatin-1 inhibits Smac mimetics and agonistic TRAIL receptor antibodies –triggered apoptosis. Thus, our findings provide evidence that Smac mimetics cooperate with TRAIL to induce apoptosis in neuroblastoma cells in a RIP-1 dependent manner.

Identification of cytoskeleton-associated proteins essential for cancer cell survival and lysosomal stability

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The inhibition of lysosomal trafficking by microtubule disturbing drugs induces lysosomal membrane permeabilization and cathepsin-dependent cell death. In order to identify more specific trafficking-related proteins that control cell survival and lysosomal stability, we screened a molecular motor siRNA library in MCF7 breast cancer cells. Depletion of six microtubule motors (Kif11, Kif19, Kif20A, Kif21A, Kif25 and Dnah8), two actin motors (Myo1G and Myh1) and one actin stabilizer (Tpm2) induced non-apoptotic cell death preceded by significant changes in the endo-lysosomal compartment, i.e. decreased lysosomal stability (Kif11, Kif19, Kif20A, Kif21A, Dnah8, Myh1 and Tpm2), increased lysosomal volume (Kif11, Kif20A, Kif25, Dnah8, Myo1G and Myh1), increased cysteine cathepsin activity (Kif20A, Dnah8 and Myo1G), changed lysosomal localization (Dnah8, Myh1 and Tpm2), increased dextran accumulation (Kif20A) and reduced autophagic flux (Myo1G and Myh1). Importantly, the depletion of any of the nine proteins reduced cell viability also in other cancer cells. Whereas Kif11 inhibitors are already under clinical development for the treatment of cancer as mitotic blockers, our data reveals a new function for Kif11 in the control of lysosomal stability and introduces eight new lysosome-regulating proteins as attractive cancer drug targets.

Role of airway epithelium in engulfing apoptotic eosinophils

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Background: Airway epithelial cells may play an important clinical role in the apoptosis of eosinophils. To study recognition pathways, two types of large bronchial airway epithelial cells were used (LAECs and A549).
Methods: Both resting, and dexamethasone-stimulated epithelial cells, were used in an inhibition assay. Confocal microscopy was used to demonstrate engulfment of apoptotic eosinophils. Apoptotic eosinophils were recognized and phagocytosed by macrophages, and by LAECs.

Results: The ability of LAECs to engulf apoptotic eosinophils was enhanced by dexamethasone and interlukin-1 (IL-1β). Inhibition by monoclonal antibodies (Mabs) prevented the uptake of apoptotic cells by LAECs.

Conclusion: This study therefore suggests that LAECs are capable of recognizing and engulfing apoptotic eosinophils, and that this process is enhanced by IL-1β and dexamethasone

The effects of ginger active component (Zerumbone) on Caco-2 human colon cancer cells

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Accumulating evidence indicates that spices that are commonly used in food have antioxidant and antimutagenic properties. In particular, zerumbone extracted from ginger (Zingiber zerumbet Smith) is reported to have anti-proliferative activities and can induce toxicity in colon cancer cells. However its molecular mechanisms are still poorly understood. In this study, in vitro apoptotic, antiproliferative and antioxidant activities of zerumbone were investigated in human colon cancer Caco-2 cells. The specific objective was to identify whether zerumbone-induced Caco-2 cell death occurs through apoptosis, autophagy, necrosis or a new form of cell death by undertaking morphological and biochemical characterisation. Caco-2 cell viability and activity with time and in the presence of different concentrations of zerumbone were investigated using LDH. In addition, characterisation of cell death induced by different concentrations of zerumbone including changes in cell size, phosphatidylserine externalization, caspase activation and PARP-1 involvement were studied. The results showed that Caco-2 cell death occurred in the absence of caspase activation at 5μg and DNA fragmentation. Additionally, Caco-2 cell death was characterised by cell shrinkage and an absence of necrotic cell death pathway. Other molecules involved in the cell death pathway such as cytochrome c, kinase activity of receptor interacting protein RIP1, and Bcl-2 family are being investigated. Anti-proliferative activity of zerumbone (5 and 10 μg/ml) on Caco-2 cells was also investigated by changes in the DNA content using flow cytometry. Zerumbone showed significant antiproliferative activity against Caco-2 cells by arresting the cells at G2/M phase of the cell cycle (30% compared to 12% in untreated cells). The effect of zerumbone on cyclins and cyclin dependant kinases related to the cell cycle activity will be investigated to elucidate the mechanism of action. Similar studies are planned for normal colon cells. In conclusion, the studies indicate that zerumbone may have potential therapeutic properties.

The regulation of PON2 expression, a new key enzyme in hematopoesis

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Paraoxonase-2 (PON2) is a ubiquitously expressed enzyme localizing to mitochondria and the endoplasmic reticulum. We recently found that PON2 diminished mitochondrial superoxide generation causing reduced cytochrome C release, which prevented cell death and protected various human tumor cell lines against chemotherapeutics. We also revealed that PON2 is frequently upregulated in lymphomas and other tumor tissues. While PON2 overexpression reduced cell death, PON2 knock-down led to spontaneous apoptosis in different tumor cell lines, among them the human chronic myeloid leukemia blast crisis cell line K562. Furthermore, imatinib-induced K562 cell death was enhanced upon PON2 knock-down, demonstrating additive effects. Thus, PON2 down-regulation may be beneficial in anti-cancer therapies. In light of these findings we analyzed the hematopoetic system of PON2-/- mice. These studies revealed an imbalance in long-term and short-term hematopoetic stem cells (HSCs) and subpopulations, e.g. of granulocyte / monocyte progenitors. Redox signaling is known to modulate self-renewal, differentiation and apoptosis of HSCs, which implies a role for the anti-oxidative PON2 in leukemic stem cell development and maintenance. Given its overexpression in leukemias and the in vivo and in vitro findings, PON2 may serve a role in cancer stem cells, establishing a strong interest in PON2 regulation. To this end, three unbiased approaches were used: (1) K562 nuclear proteins specifically interacting with the PON2 promoter were identified by mass spectrometry; (2) Putatively PON2 regulating transcription factors were proposed on in silico predictions, promoter primary sequence and inter-species conserved binding sites; (3) PON2 levels were determined after several major transcription factors were knocked-down. These studies implied a role for factors like LEF1 / TCF, HIF-1, GATA or FoxO. More specifically, we determined that Wnt / beta-catenin stimulation and GSK-3-beta inhibition induced PON2 promoter activity, which was confirmed by dominant negative LEF / TCF mutations. These findings may help to develop new strategies targeting tumor cell and HSC redox balance via PON2.

Endosomal acidification is required for efficient activation of TRAIL-induced proximal apoptotic signaling in colorectal cancer cells

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Tumour necrosis factor-related apoptosis inducing ligand (TRAIL) belongs to mainly hematopoetically-expressed so-called "Death Ligands" from the TNF-α family. Upon binding to its signal-transducing receptors TRAIL-R1/DR4 or TRAIL-R2/DR5, TRAIL can trigger apoptosis of transformed, damaged or stressed cells, and plays an important role in the immune surveillance. The core TRAIL receptor signalling complex consists, in addition to the ligand-receptor(s) couple, also from the adaptor protein FADD, pro-caspases-8 or -10 and can contain some other regulatory or associated proteins such as FLIPL/S, PEA-15/PED and others. This Death-Inducing Signalling Complex (DISC) then undergoes rapid endocytosis during which the initiator pro-caspases are activated by proximity-driven self-processing. Fully-processed caspase-8 then cleaves and activates both the effector caspase-3 and the BH3-only protein Bid - crucial mediator of the mitochondrial apoptotic signalling. The first and essential step in TRAIL-induced pro-apoptotic signalling is productive and efficient assembly of the DISC complex - a process that has been for TNF-α or FasL-triggered signalling dependent on effective endocytosis of the receptor signalling complexes. However, several reports claimed that endocytosis of TRAIL receptors was unnecessary and even inhibitory for TRAIL-induced apoptotic signalling. In contrast to the published results, here we show the data that point out to the enhancing role of endocytosis or endocytosis-accompanied acidification in the efficient DISC formation and caspase-8 activation in several colorectal cancer cell lines. Blocking endocytosis with specific dynamine-1 inhibitor Dynasore, siRNA-mediated downregulation of CHC significantly decelerates the early activation of the caspase-8 – caspase-3 axis. Endosomal acidification apparently plays an important role in this process, as inhibition of the vacuolar ATPase (vATPase) by bafilomycin A1 or concanamycin has similar decelerating effect on DISC formation and caspase processing. However, this inhibitory effect is just transient, observable mainly within the first hour of TRAIL treatment, and could be related to less effective clustering of TRAIL receptor complexes in endosomes of cells with inhibited vATPase. We will also present data analyzing a role of endosomal maturation addressed via downregulation of Rab 5, 7 and acid sphingomyelinase/ceramides in this process.

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HEMA-induced DNA damage, cell cycle arrest, and apoptosis in BEAS 2B cells

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Background and aim: The methacrylate monomer 2-hydroxyethyl methacrylate (HEMA)is a constituent in commonly used resin based dental restorative materials. These materials are cured in situ and HEMA and other monomers have been identified in ambient air in dental surgeries. In vitro studies have demonstrated a toxic potential of methacrylates, and concerns regarding possible health effects due to inhalation have been raised. In this study we have investigated the mechanisms of HEMA-induced toxicity in the human epithelial lung cell line (BEAS 2B).

Results and discussion: Depletion of cellular glutathione (GSH) and an increased level of reactive oxygen species (ROS) were seen after two hours of HEMA exposure, but the levels were restored to control levels after 12 h. After 24 h, reduced cell proliferation and increased apoptotic cell death were observed. Results of Comet assay and western analysis showed phosphorylation of DNA-damage associated signalling proteins including Chk2, H2AX, and p53. This suggests that the toxicity is mediated by DNA-damage. Co-treatment with antioxidant and Buthionine sulphoximine (BSO; an inhibitor of glutathione synthease) did not reduce the HEMA-induced cell cycle arrest. Taken together, the results indicate that HEMA induces DNA-damage of non-oxidative origin.

Conclusion: Exposure to HEMA markedly reduced cell density in cultured BEAS 2B cells. The effect was explained by a HEMA-induced inhibition of proliferation in combination with an enhanced death rate. The induced depletion of GSH and enhanced ROS formation appeared to be a parallel event rather than directly involved in these processes.

Identification and characterization of Ambra1 interactome: New insights in the crosstalk between autophagy and ubiquitin/proteasome system

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Two major pathways are responsible for protein degradation within the cell: the ubiquitin/proteasome System (UPS) and autophagy. Both processes are essential for cell homeostasis and survival and their impairment is directly correlated to several pathologies, such as neurodegenerative diseases and cancer. Recently, many evidences of a crosstalk between the UPS and autophagy have been reported, but the molecular mechanism behind this crosstalk has not been fully elucidated yet. Ambra1 has been recently identified as an important regulator of autophagy by interacting and regulating the Beclin1-Vps34 complex activity. In an attempt to understand how Ambra1 function is regulated during autophagy, we performed a systematic analysis of Ambra1 interacting proteins using a Tandem Affinity Purification (TAP) associated to mass spectrometry. Notably, we found that Ambra1 interacts with several proteins involved in the UPS, including different subunits of Cullin-RING E3 ubiquitin-ligases. These interactions prompted us to investigate the role of Cullin complexes in autophagy. We found that downregulation of distinct proteins of Cullin complexes that interact with Ambra1, by RNA interference approaches, modulates either the initial or the late steps of autophagy, suggesting that these E3 ligases could be involved in the regulation of this process. On the basis of these results, we propose Ambra1 as a novel regulative factor involved in the crosstalk between autophagy and UPS.

UVA irradiation of human keratinocytes cause exocytosis of lysosomes and caspase-8 mediated apoptosis

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Irradiation of human keratinocytes with UVA causes plasma membrane damage, which is rapidly repaired by calcium dependent exocytosis of lysosomes forming a patch to seal the wound. This was shown by detection of the lysosomal associated membrane protein-1 (LAMP-1) on the surface of the plasma membrane using immunocytochemistry. Moreover, cathepsin D was found extracellularly. Simultaneously, acidsphingomyelinase (aSMAse) appeared at the plasma membrane and lipid rafts were formed as detected by staining with fluorescent cholera toxin subunit B. Subsequently, caspase-8 activity was detected and apoptotic nuclear morphology evident. Caspase-8 activation and apoptosis was prevented by pretreatment with the aspartic cathepsin inhibitor pepstatin A, or when ammonium chloride was used to increase lysosomal pH. In addition, caspase-8, immunoreactivity, was detected in intracellular vesicles expressing LAMP-1. Using the endocytosis inhibitor phenylarsine oxide revealed that caspase-8 activation was dependent of endocytosis. Moreover, rise of lysosomal pH with ammonium chloride and inhibition of cathepsin D reduced caspase-8 location in endosomes. We conclude that UVA induced apoptosis is mediated by a lysosomal dependent mechanism that involves participation of active cathepsins and endosomal location of caspase-8.

Involvement of lipid rafts in macrophage apoptosis induced by cationic liposomes

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The success of gene therapy depends on the development of vectors that can selectively deliver therapeutic genes to target cells with efficiency and safety. Vectors proposed for gene delivery are classified into two categories, viral and non-viral vectors. Numerous studies have been reported on the use of cationic liposomes as non-viral vectors. However, many researchers have pointed out the cytotoxicity of cationic liposomes, and care must be taken when using as non-viral vectors. We have demonstrated that the cytotoxicity of cationic liposomes is a result of apoptosis, and the apoptosis exhibited the following; cationic liposome-induced apoptosis involves the ROS-mediated activation of p38 MAP kinase and subsequent activation of caspase-8, and cleavage of Bid, a member of bcl-2 family. Then, caspase-8-mediated cleavage of Bid and its translocation to the mitochondria was associated with the release of cytochrome c from mitochondria. We have recently reported that the activation of PKC-delta is involved in macrophage apoptosis induced by cationic liposomes. However, it is not fully understood how cationic liposomes interact with the cells and induce subsequent activation of PKC-delta at the membrane surface. In this paper, we clarified whether lipid rafts are involved in the PKC-delta activation induced by cationic liposomes composed of stearylamine (SA-liposomes). Co-localization of SA-liposomes and Cholera toxin B subunit, which specifically binds to GM1 on lipid rafts, was observed by microscopic observation, and the incorporation of liposomes into lipid rafts was clearly inhibited by the pretreatment of cells with an agent that disrupts lipid rafts, 2,6-di-O-methyl-α-cyclodextrin (DM-α-CD). Activation of PKC-delta and externalization of phosphatidylserine induced by SA-liposomes were also suppressed by DM-α-CD, which extracts sphingolipids and proteins from lipid rafts. Reactive oxygen species (ROS) generation, which could be involved in the macrophage apoptosis, was also inhibited by DM-α-CD. Furthermore, apoptosis induced by SA-liposomes was clearly inhibited when the cells were pretreated with DM-α-CD, but not nystatin, a cholesterol-sequestering agent that disrupt lipid rafts. These findings suggest that sphingolipids in lipid rafts are involved in the activation of PKC-delta which leads to apoptosis induced by cationic liposomes.

BIRC5/Survivin enhances aerobic glycolysis by altered regulation of fusion/ fission machinery

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The gain of chromosome 17q leads to increased expression of the anti-apoptotic protein BIRC5/Survivin, which correlates with an adverse clinical outcome and high stage in neuroblastoma (NB). We have shown before that cellular Survivin defines a threshold for the sensitivity of NB cells to certain DNA-damaging agents that require FOXO3 activation for cell death induction. To investigate the molecular basis for apoptosis inhibition by Survivin we analyzed its effects on mitochondria and uncovered that Survivin induces mitochondrial fragmentation, reduces oxidative phosphorylation and lowers BCL1L11/Bim levels. These striking effects on mitochondrial architecture and activity were accompanied by the increased recruitment of the fission protein DNM1L/Drp1 to mitochondria. Thereby, Survivin participates in the prevention of mitochondrial ROS via inhibition of the redoxenzyme p66/SHC, which in turn impairs FOXO3-induced apoptosis in NB. The Survivin-triggered reduction of mitochondrial respiration is compensated by increased glycolysis contributing to the so called Warburg effect. Treatment with glycolysis-inhibitors neutralizes the apoptosis-protective effect of Survivin and sensitizes high-stage NB to DNA-damaging agents. These data suggest that glycolysis-inhibitors target an “archilles heel” of Survivin-overexpressing NB and may be highly useful as chemosensitizers in the treatment of high-stage NB.

Far upstream element-binding protein-1, a novel caspase substrate, acts as a cross-talker between apoptosis and the c-myc oncogene

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Far upstream element-binding protein-1 (FBP-1) binds to an upstream element of the c-myc promoter and regulates the c-myc mRNA level. Earlier, FBP-1 was identified as a candidate substrate of caspase-7. Here, we report that FBP-1 is cleaved by executor caspases, both in vitro and during apoptosis. Cleavage occurs at the caspase consensus site (DQPD74) located within the classical bipartite nuclear localization signal sequence. In cells subjected to apoptotic stimuli, the caspase-mediated cleavage of FBP-1 leads to its decreased presence in the nucleus, concomitant with the marked downregulation of c-Myc and its various target proteins. By contrast, cells transfected with a non-cleavable mutant of FBP-1 (D74A) maintain higher levels of c-Myc and are protected from apoptosis. On the basis of these results, we suggest that the oncogenic potential of c-Myc is 'switched off' after apoptosis induction as a consequence of the caspase-mediated cleavage of FBP-1.

Endoplasmic reticulum stress and inflammatory caspases are involved in the pathogenesis of non-alcoholic steatohepatitis

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Nonalcoholic fatty liver disease (NAFLD) is a component of the metabolic syndrome. Non-alcoholic fatty liver disease is fast becoming one of the top concerns for clinicians due to the obesity epidemic. NAFLD is characterized by a broad spectrum of manifestations, ranging from simple steatosis to inflammatory non-alcoholic steatohepatitis (NASH) in which patients are at greater risk for progression to cirrhosis and even hepatocarcinoma. The mechanistic basis of NAFLD and NASH is incompletely understood. There is no specific treatment for NAFLD. In obesity, several mechanisms as defective autophagy induce endoplasmic reticulum (ER) stress. ER stress leads to apoptosis, inflammation and insulin-resistance, mechanisms that have been shown to be crucial in the pathophysiology of NASH. The aim of our study is to study ER stress induced apoptosis and inflammation in an animal model of NASH and to analyze molecular mechanisms after treatment with the ER stress inhibitor tauro-ursodeoxycholate (TUDCA). We used as experimental model the genetically obese (ob/ob) mice which develop severe steatosis. Hepatic inflammation was induced by LPS challenge. We observed (i) increased histological injury; (ii) increased serum transaminases, indicative of more hepatocyte death; (iii) increased percentages of TUNEL-positive hepatocytes; (iv) elevation of inflammatory caspases 11 and 1 correlated with inflammatory markers (TNF-α, TGF-beta); and (v) activation of the ER stress protein inositol requiring enzyme 1 (as shown by increased levels of spliced XBP-1 protein) and upregulation of the C-EBP homologous protein (CHOP). In contrast, treatment of ob/ob mice with TUCDA before LPS injection leads to reduced serum transaminases levels (75%), decreased percentages of TUNEL-positive hepatocytes (50%), decreased number of inflammatory foci (50%) and reduced inflammatory caspases activation (50%) compared with LPS injected ob/ob mice. Consistent with those changes, there was an increase in the expression of protective chaperone Grp78 associated with a strong decrease in expression of spliced XBP-1 and CHOP. We conclude that treatment with TUCDA protects the liver of ob/ob mice from ER stress induced apoptosis and inflammation typically found in NASH. To our knowledge, this is the first report showing more specifically that activation of inflammatory caspases 11 and 1 play a role in NASH and that their activation is reversed by treatment with TUCDA.

The effects of wedelolactone on cancer cells depend on its redox state

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Wedelolactone is one of the active polyphenolic compounds in extracts of Wedelia calandulaceae and Eclipta prostrata. Anti-tumor effects of this drug have been demonstrated in vitro and in vivo. The growth inhibitory and pro-apoptotic effects of wedelolactone on cancer cells have been largely attributed to inhibition of either IKK kinase, the key enzyme regulating activity of NFκB, or the androgen receptor activity. Our group has recently described that wedelolactone acts as catalytic inhibitor of DNA topoisomerase IIα. The aim of this study was to further characterize the mechanism of wedelolactone action on topoisomerase IIα and cancer cells. Using electrophoretic mobility shift assay we found that wedelolactone inhibited binding of topoisomerase IIα to supercoiled plasmid DNA. Compounds inhibiting formation of the topoisomerase IIα-DNA complexes can antagonize DNA damage by topoisomerase II poisons. In agreement with this hypothesis, wedelolactone antagonized the etoposide-induced DNA breaks in topoisomerase II-mediated DNA cleavage assay. The inhibitory effect of wedelolactone on the topoisomerase IIα activity was reversed by excess of enzyme but not DNA suggesting that wedelolactone exerted its inhibitory effect by interaction with the topoisomerase II protein. The in vitro inhibitory effect of wedelolactone on the topoisomerase IIα activity was redox-dependent as it diminished in the presence of reducing agents, such as DTT, glutathione, N-acetylcysteine and L-ascorbic acid. Similarly, cytotoxicity of wedelolactone in breast cancer MDA-MB-231 cells was inhibited by N-acetylcysteine but enhanced by buthionine sulfoximine, an inhibitor of glutathione synthesis. Finally, we found that wedelolactone can be oxidized in the presence of copper ions to semiquinone/quinone radicals resulting in induction of DNA strand breaks, activation of DNA damage signaling and induction of apoptosis in MDA-MB-231 cells. We conclude that the cytotoxic-, topoisomerase IIα; inhibitory- and DNA damaging activities of wedelolactone depend on its redox status and could be at least partially reversed by antioxidants.

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Sensitization of melanoma cells for TRAIL-induced apoptosis by the kinase inhibitor indirubin is mediated through upregulation of p53 and death receptors

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Background: No effective therapy is available for metastatic melanoma so far. An anti-tumour activity of indirubin is known from traditional Chinese medicine, and its derivative 8-Rha-beta has been described as a cyclin-dependent kinase inhibitor. However, the molecular basis underlying 8-Rha-beta-induced apoptosis remained elusive. TNF-related apoptosis-inducing ligand (TRAIL) is known to trigger apoptosis in a variety of human cancer cells, while normal cells are largely spared. However, prevalent or inducible resistance prevented its efficient use in cancer therapy so far. TRAIL resistance in melanoma cell lines is frequently associated with downregulation of its agonistic receptors DR4 and DR5.

Methods: TRAIL-sensitive melanoma cell lines A-375 and Mel-HO were compared to permanently resistant MeWo and Mel-2a as well as to cell lines selected for death ligand resistance A-375-TS, Mel-HO-TS (TRAIL-selected) and A-375-CS, Mel-HO-CS (selected with an agonistic CD95 antibody, CH-11, for resistance to the death ligand CD95L).

Results: Both death ligand-sensitive cell lines (A-375 and Mel-HO) responded with enhanced apoptosis to combinations of death ligands (TRAIL, CH-11) with 8-Rha-beta. The indirubin was further able to sensitize resistant Mel-2a and A-375-TS (DR4+, DR5+) for death ligand-induced apoptosis. In contrast, MeWo and Mel-HO-TS (DR4-, DR5+) remained without effect. The unraveling of proapoptotic signaling pathways in A-375-TS revealed strong enhancement of the effector caspase-3 in the combination. Significant loss of the mitochondrial membrane potential, release of cytochrome c and apoptosis-inducing factor (AIF) as well as processing of caspase-9 was evident for activation of intrinsic apoptosis pathways. On the other hand, enhanced surface expression of DR4 and DR5 as well as processing of initiator caspase-8 was indicative for activation of extrinsic apoptosis pathways. Remarkably, this combination was able to overcome an apoptosis block due to ectopic Bcl-2 overexpression. The effects may be explained by downregulation of antiapoptotic proteins Mcl-1 and XIAP as well as by activation of the master regulator p53 seen in course of 8-Rha-beta treatment.

Conclusions: Apoptosis resistance to TRAIL may be overcome by kinase inhibitors, and the indirubin 8-Rha-beta appears as a promising therapeutic strategy for melanoma cells, dependent on their expression of TRAIL receptors.

The Drosophila Retinoblastoma protein in apoptosis regulation: specific properties induced by punctual mutation at a conserved caspase cleavage site

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The tumor suppressor retinoblastoma protein (pRb) is inactivated in a wide variety of cancers. While its role during cell cycle is well characterized, its role in apoptosis is much less clear. Indeed, pRb has been found to be either pro- or anti-apoptotic. To clarify how the proliferative status of the cells impacts on the role of pRb in apoptosis, we used Drosophila to induce RBF (the pRb fly homologue) expression in different cellular and developmental contexts. We showed that RBF expression induces apoptosis in different proliferative tissues in a caspase-dependent manner, whereas RBF has an anti-apoptotic effect on Dmp53-induced cell death in post-mitotic cells. These data showed that RBF can exert a dual role in apoptosis and that its properties depend on the proliferative status of the cells [1].Conversely, apoptosis could regulate pRb. Indeed, pRb is cleaved during apoptosis at caspase cleavage sites in several cellular contexts. However, it is unclear whether these cleavages participate in the degradation of pRb, or if they could be part of a modulation of pRb properties. Only the LExD site is conserved between mammalian pRb and its Drosophila homologue, as a TELD site in RBF. This conservation may indicate that the cleavage of RBF could have a physiological role in Drosophila as in mammals. We generated a punctual mutant form of RBF at the TELD site in order to study the properties of a cleavage-resistant form of RBF named RBFD253A. This mutant form conserved RBF pro-apoptotic properties but displayed a specific ability in inducing overgrowth phenotypes resulting from a non-autonomous cell proliferation. Results from these studies will be presented.

1. C. Milet, A. Rincheval-Arnold, B. Mignotte and I. Guénal (2010) "The Drosophila retinoblastoma protein induces apoptosis in proliferating but not in post-mitotic cells". Cell Cycle 9, 97-103.

cIAP1/2 are Direct E3 Ligases Conjugating Diverse Types of Ubiquitin Chains to Receptor Interacting Proteins Kinases 1 to 4 (RIP1-4)

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The RIP kinases have emerged as essential mediators of cellular stress that integrate 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. 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 show that cIAP1 and cIAP2 are direct E3 ubiquitin ligases for all four RIP proteins and that cIAP1 is capable of conjugating the RIPs with diverse types of ubiquitin chains, including linear chains. Consistently, we show that repressing cIAP1/2 levels affects the activation of NF-κB that is dependent on RIP1, -2, -3 and -4. Finally, we identified Lys51 and Lys145 of RIP4 as two critical residues for cIAP1-mediated ubiquitination and NF-κB activation.

Induction of autophagy-dependent necroptosis to overcome drug resistance in acute lymphoblastic leukemia

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Inclusion of new agents into current treatment regimens is an attractive strategy to resensitize drug resistant cancer cells to standard chemotherapy. We have shown that low dose of the small molecule BCL2 family antagonist obatoclax resensitized cells from steroid resistant acute lymphoblastic leukemia (ALL) patients to glucocorticoids and other cytotoxic agents such as daunorubicine, vincristine and cytarabine. Surprisingly, this resensitization to glucocorticoids occurred through induction of autophagy-dependent necroptosis, while combination of obatoclax with other cytotoxic agents induced classical apoptosis. Activation of the necroptotic response was critically dependent on RIP1 kinase activity. We here report that not only cells from de novo resistant, but also from heavily pretreated, relapsed or refractory ALL patients respond to obatoclax and dexamethasone. This is of particular importance, since these patients represent the population eligible for a clinical phase I trial. Obatoclax disrupted a complex of Beclin-1 and MCL-1, and combination with dexamethasone triggered dephosphorylation of the mTOR target S6 protein. In accordance with a role for RIP1 kinase activity in the execution of necroptosis upon treatment with obatoclax and dexamethasone, presence of its putative deubiquitinase CYLD was also required. While all analyzed samples from precursor B-ALL patients responded to obatoclax and dexamethasone, several samples from T-ALL patients were refractory to this combination. These samples did neither show dephosphorylation of S6 protein. In vivo, in immunodeficient mice transplanted with primary ALL cells from steroid resistant patients, dephosphorylation of S6 protein was evident in human leukemia cells already 5 h after treatment, remained detectable over several days and was paralleled by a decrease of disease burden. Based on these data, we are currently preparing a phase I trial to evaluate combination therapy with obatoclax and dexamethasone. We will also assess if analysis of the phosphorylation status of S6 protein could be used as marker of biological response in patients treated with the combination of obatoclax and dexamethasone.

miR-143 overexpression impairs growth of human colon carcinoma xenografts in mice with induction of apoptosis and inhibition of proliferation

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We have previously shown that miR-143 is down-regulated in colorectal cancer and that miR-143 overexpression increases sensitivity to 5-fluorouracil, reduces cell viability and increases apoptosis in HCT116 cells. In the present study, we evaluated the role of miR-143 overexpression on HCT116 xenograft tumor growth in nude mice. HCT116 cells with stable miR-143 overexpression (over-143) and control (empty) cells were subcutaneously injected into the backs of nude mice, and tumor growth was evaluated. Tumors arose approximately 14 days later, and the experiment was ended 40 days after injection. miR-143 was confirmed to be significantly overexpressed in over-143 versus empty xenografts, by Taqman real-time PCR ($p < 0.05$). Over-143 xenografts displayed slower tumor growth compared to empty xenografts from 23 until 40 days in vivo ($p < 0.05$), with final volumes of 928±338 and 2312±387 mm3, respectively. Evaluation of apoptotic proteins showed that over-143 versus empty xenografts displayed reduced Bcl-2 expression, and increased caspase-3 activation and PARP cleavage ($p < 0.05$). In addition, the incidence of apoptotic tumor cells, assessed by TUNEL, was increased in over-143 versus empty xenografts ($p < 0.01$). Finally, over-143 versus empty xenografts displayed significantly reduced NF-κB activation and ERK5 expression ($p < 0.05$), as well as reduced proliferative index, evaluated by Ki-67 immunohistochemistry ($p < 0.01$). Collectively, our results suggest that the reduced tumor volume in over-143 versus empty xenografts may result from increased apoptosis and decreased proliferation induced by miR-143. Our data reinforces the relevance of miR-143 in colorectal cancer, suggesting an important role in the control of in vivo tumor progression. This further expands its anti-proliferative, pro-apoptotic and chemosensitizer role that we have previously demonstrated in vitro.

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A novel copper (I) complex induces ER stress-mediated apoptosis in leukemia cell lines

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Recently, a novel phosphine copper(I) complex [Cu(thp)4][PF6] (CP), was identified as an efficient, in vitro antitumoral agents. It has also been demonstrated that it induces in colon cancer cells a programmed non-apototic cell death called paraptosis or type III cell death. In this study we evaluated CP antiproliferative activity on a panel of leukemia cell lines and it significantly inhibited cancer cells growth at micro and submicromolar concentration, especially against SEM and RS4;11 cell lines. Flow cytometric analysis demonstrated that CP did not affected cell cycle in RS4;11 and SEM cell line but we observed a concentration-dependent increase of the cell population with a hypodiploid DNA content peak (subG1), suggesting that CP may induce apoptosis. To better characterize the mode of cell death induced by CP, a biparametric cytofluorimetric analysis was performed using propidium iodide (PI), which stains DNA and is permeable only to dead cells, and fluorescent immunolabeling of the protein annexin-V, which binds to PS in a highly selective manner. We found a concentration-dependent increase in annexin-V positive cells in well agreement with the appearance of hypodipolid peak. Western blot analysis demonstrated that the activation of the apical caspase-9 and the two effector caspase-3 and 7 occur after treatment with CP, while caspase-8 was not affected by the treatment. Interestingly we did not observed mitochondrial depolarization or cytochrome c release into the cytoplasm, suggesting that mitochondria was not involved in the process of cell death. Previous observations indicated that CP may induces functional suppression of the ubiquitin–proteasome pathway thus triggering endoplasmic reticulum stress in solid tumor cells. We also evaluated if CP exerts ER stress in leukemia cell lines. Western blot analysis showed a remarkable increase of GRP78 in RS4;11 and SEM, a well known marker of ER stress. Moreover the protein synthesis inhibitor cicloheximide significantly protected the cells from CP-induced cell death suggesting that protein synthesis machinery is involved in the mechanism of action. To evaluate if CP directly inhibits the proteasome, semipurified proteasome from cell extracts were incubated with increasing concentrations of CP and the chymotrypsin-like activity was measured. The results showed a decreased activity in a concentration-dependent way, with an IC50 of approximately 12 μM. It is well known that copper represent an excellent catalyst of redox cycle and therefore it can stimulate ROS production. Interestingly the CP-induced cell death was significantly reduced in presence of ROS scavenger such as tocopherol, n-acetyl cysteine (NAC) and butylated hydroxyanisole (BHA) suggesting that also ROS may contribute to the process of cell death. Further experiments are in progress to elucidate the mechanism of action of this copper complex and the results will be discussed.

Emergence of mTRAIL+IFN-α+ pDCs as a consequence of NK-pDC crosstalk in the context of HIV-1 infection

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Background. Plasmacytoid dendritic cells (pDCs) mainly contribute to antiviral immunity through recognition of viral components resulting in the production of type-I interferon (IFN), a powerful innate antiviral cytokine. IFN-α production by pDCs is promoted by a cross-talk with NK cells, that triggers in return the cytotoxicity of NK cells. Given the essential role of pDCs and NK cells in viral control, we addressed the question of the impact of HIV on NK-pDC cross-talk, and the consequences on viral innate immunity.
Methods. pDCs and NK cells were negatively sorted from PBMC of healthy donors. NK cells were kept either unstimulated (rNK) or activated with PMA/ionomycine for 2 hrs (aNK). pDC were either uninfected or infected with R5-HIV-1 BAL (pDCHIV) at various concentrations and cocultured with NK cells at different ratios for 24 h. The fate of both cell types was studied by multiparametric flow cytometry combined to Multianalyte Profiling technology and Gene array approach.
Results. HIV-1-infection of primary pDCs induced their maturation, characterized by the expression of maturation markers (HLA-DR, CD80, CD83, CD86) and the homing receptor CCR7. In addition, HIV-1 induced the emergence of TRAIL expressing IFN-α-producing pDCs. The crosstalk of pDCHIV with aNK cells strongly increased the differentiation of pDCs into killer pDCs, and also triggered the production of the beta-chemokines RANTES, MIP-1α and MIP-1beta (inhibitor of HIV-1 entry). At high concentrations of HIV-1, pDCs were able to activate rNK cells, and to induce IFN-gamma and TNF-α expression as well as perforin degranulation by aNK cells.
Conclusion. We report for the first time that NK-pDCHIV crosstalk potentiates the emergence of TRAIL-expressing IFN-α-producing pDCs, and also triggers beta-chemokines synthesis and NK cell killing activity. Overall these data suggest that the dialogue of HIV-infected pDCs with NK cells favors the emergence of both killer pDC and cytotoxic NK cells and promote host innate immunity through the activation of potent anti-viral effectors.

Thiazolides, GSTP1 and colon cancer cell apoptosis

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Thiazolides are antibiotics with potent anti-microbial activities used for the treatment of intestinal infections. Although so far no (side) effects on mammalian cells have been described, our lab has recently shown that thiazolides promote apoptosis in colon cancer cells. They potently synergized with other apoptosis inducers, such as chemotherapeutic drugs and TRAIL. As the main mammalian target of thiazolides we identified the glutathione S-transferase GSTP1. Interestingly, GSTP1 enzymatic activity was required for the apoptosis inducing activity of these compounds. Furthermore, we have seen that cell cycle progression was a prerequisite for thiazolide-induced apoptosis in colon cancer cells. We are currently investigating the molecular requirements of the thiazolide structure and derivatives to induce a GSTP1-depedent apoptotic cell death in colon cancer cells. Interestingly, even less complex derivatives were still capable of promoting cell death in CaCo2 cells. Thiazolides appear to induce apoptosis via a MAP kinase- and the BH3-only protein Bim-dependent manner. These substrates induced activation of Jun kinase and p38, and their inhibition strongly inhibited thiazolide-induced cell death. They also induced the expression of the Jun kinase target Bim, and downregulation of Bim attenuated thiazolide-induced apoptosis. As GSTP1 sequesters and inhibits Jun kinase and other signaling molecules we are currently investigating whether thiazolides induce Jun kinase and p38 activation and subsequent apoptosis induction via the release of these MAP kinases from GSTP1. Interestingly, GSTP1 is barely expressed in normal colonic mucosa, but abundantly expressed in colorectal tumor cells. As we have previously shown that inhibition of cell cycle progression blocks thiazolide-induced cell death in CaCo2 cells, we are investigating whether cell cycle arrest causes reduced GSTP1 expression in colon cancer cells or affects GSTP1 enzymatic activity. Current data indicate that GSTP1 expression is not affected by cell cycle arrest. Our study proposes thiazolides as a novel therapeutic for the treatment of colorectal tumors and GSTP1 as an Achilles' heel of thiazolide-induced cell death.

CNS neurons dying back pattern involves mitochondrial fission and axonal caspase activation: implication for Alzheimer Disease.

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If apoptosis is mainly at stake, the precise molecular mechanisms involved in axonal destruction during neurodegenerative processes are poorly understood. Peripheral axotomy has shown that axon severing triggers a caspase-independent axonal degeneration process. However, in chronic diseases involving aggregated proteins such as Alzheimer's disease, the consequences of CNS neurons exposure to limited and focal insults on axonal fragmentation are not known. Hypothesizing differential consequences of local modifications of transduction pathways in terms of degenerative signals propagation, we used primary CNS neurons cultured in microfluidic devices allowing the compartmentalization of axons from their somas. Application of pro-apoptotic inducers or beta-amyloid peptides on either compartment showed that somato-dendritic initiation of apoptosis triggers the diffusion of a degenerative signal in intact axonal shafts, leading to progressive cytoskeleton alteration and ultimately to axonal destruction. However, axonal pro-apoptotic insults result in similar axonal destruction only when supplemented by sub-toxic somato-dendritic stress. Altogether, our data show that CNS progressive dying back patterns, in contrast to Wallerian degeneration, involve rapid axonal activation of JNK which controls mitochondrial fission and axonal caspases activation.

Platinum(IV) complex LA-12 sensitizes human colon cancer but not normal epithelial cells to TRAIL-induced apoptosis independently on p53 status

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Platinum-based chemotherapeutic drugs are clinically used in therapy of many solid tumors, but their effective application is hampered by intrinsic or acquired resistance of cancer cells to these compunds and by their serious side effects to normal tissues. Novel platinum(IV) adamantylamine ligand-containing complex (LA-12) seems to overcome these obstacles. In addition to its cancer cell death-inducing ability, LA-12 can also cooperate with other apoptosis-inducing agents, which may finally lead to more efficient elimination of treatment-resistant cancer cells. In our previous work we showed that LA-12 enhanced tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis in human colon cancer cells, and triggered a significant increase of the expression of its pro-apoptotic TRAIL-R2/DR5 receptor. The significance of the role of TRAIL-R2/DR5 in the effects observed was addressed by its siRNA-mediated downregulation (1). Here we demonstrate that sensitizing effect of LA-12 on TRAIL-induced apoptosis of colon cancer cells is independent on p53 status, as similar apoptotic response to the combined treatment with LA-12 and TRAIL was detected in colon cancer cells expressing wt or mutant p53 as well as in those lacking p53. Moreover, LA-12-induced upregulation of DR5 was triggered regardless of the p53 status in the examined cancer cells. Next, we evaluated the response of normal colon epithelial cells to combined treatment with LA-12 and TRAIL. In contrast to the cancer cells, no significant cytotoxic effects were observed in normal colon cell line pretreated with LA-12, and subsequently treated with TRAIL. At the same time, LA-12 was not effective in modulation of DR5 levels in the normal cells. Our results support a promising application of the combination of LA-12 and TRAIL also in tumors with non-functional p53, which represent a high percentage among all cancer types. Importantly, selected combination of these agents in the concentrations that are effective to cancer cells do not seem to be harmful to normal colon epithelial cells, which may be beneficial with regard to substantially lower side effects compared to the conventionally used anticancer therapy.

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DNA damage score as predictive factor for response and toxicity for NSCLC treatment

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Non Small Cell Lung Cancer (NSCLC) represents the 80% of pulmonary tumors and it causes the majority of cancer-related deaths. At a primary stage, surgery together with adjuvant chemotherapy is the best healing treatment option, but at advanced stages chemoradiotherapy (QRT) becomes the main tool for NSCLC treatment, with very low response rates (10-15% of 5 years survival). This situation makes necessary the early detection of both treatment response and toxicity rates. Most of the employed QT schemes interfere with DNA synthesis and cell division, inducing a DNA damage and subsequent cellular apoptosis. The detection of such apoptotic cells in vivo is more difficult than in vitro due to phagocytosis of surrounding cells. So, the identification of early apoptotic events such as DNA fragmentation is crucial in order to quantify apoptosis upon QT/QRT.

Aim: Determination of DNA damage as a predictive factor for response and toxicity rates induced by QT or QRT from peripheral blood samples in patients with non-operable NSCLC.

Methods: Comet assay was used to determine the DNA damage of 60 patients where 38 of them had an IIIA-IIIB stage and 22 had an IV-IIIB (wet) stage. Based on the treatment scheme, 4 peripheral blood samples were obtained from each patient with IIIA-IIIB stage, including a sample taken before the treatment to determine the "basal DNA damage"; On the other hand, 5 peripheral blood samples were obtained from patients with IV-IIIB (wet) stage, also including the "basal DNA damage" sample. Mononuclear cells were isolated from blood samples using BD Vacutainer® CPT™ tubes. Obtained cells were stored at -80ºC until analysis. Comet assay was performed under alkaline conditions and micrographs at 200x were obtained using a fluorescence microscopy Nikkon 730037. 150 cells from each sample were analyzed using Comet Assay IV™ image analysis software (Perceptive Instruments, UK). Among the different parameters measured with this software, the Tail Moment was took as the most representative one. DNA damage was divided into four levels of damage, where O represented the intact cell and the 4th level the most severe damage. In order to minimized errors analyzing the huge among of images, the analysis process has been automated as much as possible. First of all, the range defining each level of damage based on "visual score" was established. Validation of these ranges was carried out using a different set of images. Obtained Tail Moment values were loaded into a template where a score was assigned to each cell depending on its DNA damage level: 0 points to level O; 1 point to level one… Finally, a total score was obtained for each sample, with the resulting DNA damage pattern for each patient.

Results: Obtained results let us to identify different behaviour patterns among analyzed patients allowing the association of samples depending on the DNA damage levels.

Conclusion: This behaviour association could be of interest for the evaluation of chemotherapy response and/or toxicity.

Apoptosis-inducing factor mediates dopaminergic cell death in response to LPS-induced inflammatory stimulus: evidence in Parkinson's disease patients.

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Neuronal inflammation is a very important phenomenon that affects the survival of different types of neurons. Its implication in neurodegenerative diseases such as Parkinson's disease or Alzheimer's disease has been well established. Microglia plays a critical role in this process. This role is considered as detrimental for neuronal survival during chronic inflammation. In this study we try to uncover the possible cell death mechanisms in dopaminergic (DA) neurons implicated in Parkinson's disease. For this purpose we use both in vitro and in vivo approaches (a co-culture system with the microglial BV2 and the DA neuronal MN9D murine cell lines and an intranigral lipopolysaccharide –LPS- injection, which provokes specific degeneration of DA neurons). In vivo we observe a strong induction of cleaved caspase 3 only in the glial cells (microglia and astroglia) but not in the degenerating dopaminergic cells. In contrast, there is a translocation into the nucleus of Apoptosis-inducing factor (AIF) in DA-degenerating neurons and also a significant decrease of the ratio Bcl-2/Bax protein after LPS injection. These observations were confirmed by comparing the survival of the dopaminergic neurons using different inhibitors (a caspase 3 inhibitor, an inhibitor of poly(ADP-ribose)polymerase-1- an upstream regulator of AIF release, and also a calpain inhibitor). These data support the role of AIF-dependent pathway in LPS-induced nigral DA cell death. We next confirmed these results in vitro, where the silencing of caspase-3 or AIF by small interfering RNAs exclusively in the MN9D cells demonstrated the key role of AIF in the LPS-induced death of DA cells. Finally we also observed nuclear translocation of AIF in the ventral mesencephalon of Parkinson's disease subjects.

Role of FADD silencing in neural apoptosis

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FADD (Fas-associated protein with Death Domain)/Mort1 is an adaptor protein with a key role in cell death and inflammatory pathways. One of the most prominent death domain complexes described in the initiation of apoptosis is formed by the transmembrane receptor Fas/CD95, the cytosolic adaptor protein FADD/Mort1, and caspase-8 and is referred to as the death inducing signalling complex (DISC). Furthermore, FADD/Mort1 enhances the apoptotic pathway of TNFRI, recruiting caspase-8 to cell membrane, triggering caspase cascade. Degenerative and traumatic neuronal diseases implicate upregulated apoptotic and inflammatory pathways, in which FADD/Mort1 is involved. It has been described that downregulation of FADD/Mort1 reduce inflammation and apoptosis. Therefore, FADD/Mort1 silencing is an interesting therapeutic approach when apoptosis is not desired. The main aim of this work is to reduce the activity of apoptotic pathways TNFR and Fas/CD95 by downregulation of FADD/Mort1 in neural tissue following traumatic injury. Our laboratory has developed a neuroprotective tool consisting of the combination of FADD-siRNA with a neuronal-specific delivery peptide in order to silence FADD expression in neural cell populations. For this study, we employ cell lines, as well as fibroblast and neuronal primary cultures fromnewborn mice to evaluate the specific translocation of FADD-siRNA, silencing efficiency and cell viability after apoptotic stimulation. Preliminary results show specifity and efficiency of our neuroprotective tool. In order to assess the functional implication of FADD silencing In vivo, we use a contusive spinal cord injury model. Injured animals are treated with FADDsiRNA/peptide by i.v. injection after 1st, 3rd and 7th days post-injury. Function motor recovery is evaluated with the Bms locomotor rating scalecomplemented with other tests such as the open field activity test or rotarod device. The main goal of this work is to establish this neuroprotective tool as a potential non-viral therapy in traumatisms and other neurodegenerative disorders.

A novel SIRT inhibitor with anticancer action in vitro, ex vivo and in vivo

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The development and progression of cancer involves both epigenetic and genetic changes leading to the alteration of gene expression and cell phenotype. Emerging evidences suggest the role of acetylation as post-translational modification that plays a critical role in cell fate. Sirtuins, are NAD-positive–dependent class III HDACs that share extensive homologies with the yeast HDAC Silent Information Regulator 2 [1]. In mammals, seven Sir2 homologues (Sirtuin 1-7 orSIRT1-7) have been identified, which target histone and various non histone proteins in distinct subcellularlocations. Sirtuins catalyze the removal o fan acetyl moiety from the ε-ammino group of lysin residues on histone substrate (H1, H3, H4) and non-histone substrate (FoxO1,4; NF-Kβ; p53; p73; p300; tubulin) [2]. SIRT1, the sirtuins member with highest sequence similarity with yeast Sir2, has been found to be involved in many disease, like cancer, and can modulate cell survival by regulating the transcriptional activities of many different targets [3]. Thus SIRT inhibitors represent attractive therapeutic targets for anticancer drug development [4]. However, despite the development of several effective HDAC inhibitors, little is known about SIRT inhibitors, their mechanism(s) of action and targets. By screening of a small compound library, we identified a novel SIRT1 and 2 inhibitor, MC2494, able to induce strong cell death in many cancer cell types, activating the capsase cascade and inducing both apoptotic and necroptotic pathways. Gene expression analyses highlighted the induction of the death-receptor pathway, confirmed by the activation of both TRAIL and DR5 promoters. That both caspase 8 and RIP blockage can revert the induction of MC2494 cell death indicated the induction of apoptosis via RIP1/caspase8/FADD complexes activation. PK and PD studies carried out in mice supported MC2494 potential use as anticancer drug in vivo. Indeed, when tested in xenograft models of breast cancer or in mice solid tumors transplantation models, the MC2494 displayed anticancer action mediated by SIRT inhibition detectable within the tumors. Excitingly, when MC2494 was administered with electric delivery in vivo, the anticancer effects were maximized, strongly suggesting its potential application also in the treatment of cutaneous cancers.

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Nerve Growth Factor mediates active caspase-3 turnover in apoptotic PC12 cells

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Nerve Growth Factor (NGF) is an important pro-survival factor for neuronal cells and has been suggested as a possible therapy for certain neurodegenerative diseases such as Alzheimer's disease. In the aged brain and after neuronal damage there is increased stress on the endoplasmic reticulum (ER) to degrade misfolded proteins. If this stress is prolonged or too severe the cells undergo apoptosis. The NGF responsive rat phaeochromocytoma 12 (PC12) cell line, which expresses both NGF receptors TrkA and p75 and is a good in-vitro model to examine the pro-survival effects of NGF, was used in this study. Using the PC12 cells it has previously been shown that pre-treatment with NGF can protect the cells from ER stress-induced apoptosis due to the ER stressor thapsigargin (TG) [1]. NGF was also shown to exert pro-survival effects up to 18 hours after TG treatment (including increased survival in a clonogenic assay, $p<0.05$). This pro-survival effect was due to the reduction of active caspase-3 (p17 subunit) in the cells. Caspase-3 is known as the executioner of apoptosis and there is general consensus that activation of caspase-3 represents the point-of-no-return after which a cell is committed to undergoing apoptosis. This effect of NGF on p17 is dependent on its binding to the TrkA receptor, activation of ERK1/2 signalling and on novel protein synthesis. It was also shown that inhibitor of apoptosis proteins (IAPs) are not involved. Most recently we have shown biochemically that this NGF-mediated loss of p17 is dependent on lysosomes. These findings suggest that NGF induces a novel pathway of lysosomal degradation to reduce the levels of active caspase-3 in the cell, thus prolonging the survival of the cell during conditions of stress.

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Lack of collagen VI affects apoptosis and autophagy in primary neural cell cultures

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Collagen VI is an extracellular matrix protein expressed in different tissues such as skin, peripheral nerves, cartilages and skeletal muscle. It consists of three α chains encoded by separate genes. Mutations in human COL6 genes cause muscle diseases, including Bethlem myopathy and Ullrich congenital muscular dystrophy. Collagen VI null (Col6a1-/-) mice display an early onset myopathic phenotype characterized by organelle defects, mitochondrial dysfunction and spontaneous apoptosis. We recently found that the persistence of altered organelles and apoptosis is due to defective regulation of autophagy in muscle fibers. Impairment of the autophagic clearance machinery has detrimental effects in several organs, particularly in the central nervous system (CNS). Little is known about collagen VI in the CNS, but recent findings showed that this protein protects neurons from the toxicity of Ab peptides, a critical step in the pathogenesis of Alzheimer's disease, and from UV induced damage. To investigate further the role of collagen VI in CNS, we established cortical and hippocampal primary cultures from brains of neonatal wild-type and Col6a1-/- mice. In wild-type cultures collagen VI is localized on the surface of both neurons and glial cells. Col6a1-/- neural cultures show a significant increase of spontaneous apoptosis, and plating onto purified collagen VI reduces apoptosis. Biochemical analysis revealed a noticeable increase of p62 levels in Col6a1-/- neural culture extracts. In agreement with this, Col6a1-/- neural cultures show a reduced level of LC3-II, the lipidated form of the autophagic marker LC3. These in vitro observations suggest that lack of collagen VI in CNS leads to cell death and defective autophagic regulation in neurons and glial cells.

UNBS1450, a steroid cardiac glycoside inducing apoptotic cell death in human leukemia cells

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Cardiac steroids are currently used in clinics in the treatment of congestive heart failure and cardiovascular diseases. More recently, anti-cancer potentials have been also ascribed for these compounds [1]. The aim of this study was to investigate the anti-leukemic activity of UNBS1450, a semisynthetic cardenolide belonging to the cardiac steroid glycoside family and derived from the plant Calotropis procera [2]. Here, we report that, at low nanomolar concentrations, UNBS1450 strongly exerts cytostatic effects and induces apoptotic cell death in different hematopoietic cancer cell models. Remarkably, we did not detect any toxic effects in healthy blood cells. The accomplishment of the apoptotic program was accompanied by cleavage of pro-caspases 8, 9 and 3/7 and, more upstream by activation/recruitment of the Bcl-2 pro-apoptotic family members Bak and Bax. Searching for the molecular mechanisms implicated, we found that UNBS1450 targeted Mcl-1 protein levels at very early times of treatment, prior to the caspase cleavage/activation. This effect was specific for Mcl-1, since no alterations in the protein level of the most ubiquitous member of the family Bcl-2 could be detected even at late times of incubation with the compound. The potential specific effects of UNBS1450 on cancer vs. normal cells; the low concentrations required to affect cancer cells (in the range of nanomolar concentrations); and the fact that UNBS1450 appears to be particularly active on Mcl-1 expression, suggest a potential use of this compound in future anti-cancer therapies.

Microbial Harakiri: “Lysosome” Mediated PCD in the Malaria Parasite

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The increasing prevalence of drug-resistant Plasmodium falciparum malaria parasites has given impetus for the development of novel antimalarials. One unexplored option is in the triggering of the parasite’s programmed cell death (PCD) pathway. Our findings have already been published showing evidence for a PCD pathway induced by high levels of the antimalarial chloroquine (CQ) in both CQ-susceptible and –resistant parasites, albeit to lesser degrees in the latter (1). This pathway involves mitochondrial depolarization, the activation of clan CA cysteine proteases and results in DNA fragmentation, and has been shown to be discrete from unregulated necrosis. Interestingly, this form of cell death has been shown to require micromolar concentrations of CQ and its localization to the parasite’s equivalent of a lysosome – its digestive vacuole (DV). Notably, this excessive localization of the lysosomotropic drug leads to a destabilization of the DV membrane and an abrupt redistribution of Fluo-4-AM (confocal live-cell imaging) but without ultrastructural compromise of the DV membrane (transmission electron microscopy). This could potentially lead to the leakage of clan CA proteases out of the DV to mediate PCD. This finding is reminiscent of lysosome-mediated PCD in metazoan cells, wherein the regulated leakage of lysosome cathepsin proteases occurs to trigger PCD without compromising lysosome morphology. The effectiveness of other non-antimalarial lysosomotropic compounds in permeabilizing the DV and triggering PCD suggests that the DV destabilization is sufficient to initiate the pathway in the parasite. Moreover, the similar/increased extent of PCD features in drug-resistant strains compared to drug-sensitive parasites suggests that this DV-mediated PCD pathway may be exploited in the development of novel and potent antimalarials.

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p53 and isoforms turn WWOX to tumor promoting and TIAF1 suppresses the effect

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We have recently determined that transforming growth factor beta (TGF-beta) induces self-aggregation of TIAF1 (TGF-beta1-induced antiapoptotic factor), and this may cause generation of amyloid beta and fibrils and apoptosis in cells. Here, TIAF1 was found to be significantly upregulated in many solid tumors, but was downregulated in metastatic cancer. TIAF1 aggregates, which colocalize with Smad4, are found in the stromal tissues of tumors, peritumor coats, and the interface between metastatic tumors and the brain tissue. In vitro induction of TIAF1 self-aggregation upregulated Smad4 and tumor suppressor WOX1 (also named WWOX or FOR). At non-apoptosis-inducing levels, TIAF1 synergistically acted with p53 and WOX1 to block anchorage-independent growth and cell migration and cause apoptosis. Knockdown of TIAF1 caused resistance to apoptosis by stress stimuli, and overexpressed p53 and WOX1. Intriguingly, at low levels, wild type p53 and isoforms converted WOX1 for tumor promoting, and TIAF1 blocked the effect. While cancer cells are normally devoid of wild type p53 and WOX1, increased TIAF1 self-aggregation is likely to limit cancer progression.

Loss of a core apoptotic component in melanoma predicts chemosensitivity

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Metastatic melanoma is a highly intractable disease that accounts for the majority of skin cancer related deaths. Patients with metastatic melanoma usually do not respond to chemotherapeutics; likewise, cells derived from metastatic melanoma are insensitive to chemotherapy-induced apoptosis. To identify a mechanism for chemoresistance, we analyzed a panel of melanoma cell lines for dysfunctional core components of the apoptotic cascade: pre-mitochondria signaling (e.g., stress sensing, BCL-2 family regulation), mitochondrial contribution (e.g., intermembrane protein release), and post-mitochondrial events (e.g., apoptosome formation, caspase activity). From these studies, we identified a consistent phenotype in cells derived from metastatic melanoma that is epigenetically regulated, and causes a substantial shift in the metabolic regulation of tumor cell survival. We are currently investigating these experimental findings with primary tissue samples derived from patients to determine the pertinence within the clinic. We anticipate these discoveries will be prognostic for which chemotherapeutic regimens will yield the best success in patients with metastatic melanoma.

Cyclosporine A-induced endoplasmic reticulum stress triggers autophagy of malignant glioma cells

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Autophagy is a self-digestion process allowing cell survival during starvation but functions also as an alternative death mechanism under certain conditions. Autophagy is accompanied by the progressive formation of vesicle structures from autophagosomes to autophagolysosomes, and involves both autophagy effectors (Atg proteins) and regulators (i.e. mTOR - mammalian target of rapamycin is a negative regulator). Malignant gliomas are highly resistant to available therapies which induce apoptosis, thus induction of the alternative cell death is an attractive strategy. We demonstrate that cyclosporine A (CsA, an immunophilins/calcineurin inhibitor) induces cell death with some apoptotic features. Additionally we observe the appearance of numerous cytoplasmic vacuoles, immunostained for endoplasmic reticulum (ER) stress and autophagy markers detected using fluorescence microscopy. The induction of ER stress in glioma cells by CsA was evidenced by detection of unfolded protein response activation (phosphorylation of PERK) and accumulation of ER stress associated proteins (BIP and CHOP). Upon CsA treatment several autophagy features were observed: formation of the acidic vesicular organelles, increase in punctuate GFP-LC3 (microtubule-associated protein light-chain 3) and LC3-II accumulation. Decrease of phosphorylation 4E-BP1, p70-S6K1, and its downstream target molecule S6 ribosomal protein suggests that CsA affects mTOR signaling. Salubrinal, which protect cells from ER stress, partially blocked CsA-induced decrease of p70-S6K1 and 4E-BP1 phosphorylation, and accumulation of LC3-II. It suggests that ER stress was primary to CsA-induced autophagy. Surprisingly, selective silencing of Atg1, Atg5 or Atg7 increased the level of active caspases 3, 7 and PARP degradation in CsA-treated cells. Our results demonstrate that CsA induces both apoptosis and autophagy in malignant glioma cells via induction of ER stress and inhibition of mTOR/p70-S6K1 pathway, however autophagy is cytoprotective in this context.

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Bortezomib induces caspase-9-mediated apoptosis of Anaplastic Large Cell Lymphoma cells through Noxa

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Outcome in patients with Anaplastic Large Cell Lymphoma (ALCL) is often fatal and therefore new treatment modalities are urgently needed. Recent studies in primary ALCL samples have demonstrated an increased expression of NF-κB regulated anti-apoptotic proteins. NF-κB activity can be inhibited by the proteasome inhibitor bortezomib resulting in induction of apoptosis. In this study, we investigated if bortezomib can induce apoptosis of cultured lymphoma cells of systemic ALK-positive and ALK-negative ALCL patients and ALCL cell lines and we examined the mechanisms by which bortezomib induced cytotoxicity in these ALCL cells. Treatment with bortezomib resulted in induction of apoptosis in all ALK-positive and ALK-negative ALCL patient samples and ALCL cell lines tested. The lethal dose (LD50) varied between 54nM and more than 100nM after 24 hours and varied between 21nM and 52nM after 48 hours of exposure. ALK-negative ALCL cases were more sensitive to bortezomib and showed significant lower LD50 values than ALK-positive ALCL cells. We demonstrated that bortezomib-induced cell death in both types of ALCL is dependent on caspase-9-mediated apoptosis and that bortezomib induces depolarization of the mitochondrial membrane. mRNA-expression and protein analysis revealed remarkable upregulation of the BH3-only protein Noxa. Inhibition of Noxa by si-RNA analysis resulted in a clear decrease of bortezomib-induced cell death and mitochondrial depolarization, indicating that Noxa play a key role in bortezomib sensitivity in ALCL cells. Noxa interacts with the anti-apoptotic proteins MCL-1 and Bcl-2 resulting in Bak and Bax release. Our preclinical data support the therapeutic application of bortezomib as potential drug in the treatment of ALCL, especially ALK-negative ALCL patients to improve their prognosis.

Smac mimetic increases glioblastoma response to agonistic TRAIL-R2 antibody in a NFκB and RIP1-dependent manner

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Glioblastoma (GBM) is the most common primary brain tumor and bears a very poor prognosis, thus highlighting the need to develop novel treatment strategies. We previously reported that small molecule Smac mimetics present a promising approach to prime cancer cells for apoptosis. However, the molecular mechanisms of this sensitization to apoptosis have not been fully elucidated. Here, we report that Smac mimetic profoundly sensitize a panel of GBM cell lines to TRAIL-induced apoptosis. Calculation of combination index revealed that this cooperative induction of apoptosis is highly synergistic. Interestingly, GBM cell lines turned out to be responsive to an agonistic antibody directed against TRAIL receptor 2 (TRAIL-R2) in combination with Smac mimetic. Also, Smac mimetic acts together with TRAIL-R2 antibody to induce apoptosis in glioblastoma-initiating stem cells isolated from primary tumor specimens, which are considered to be particularly resistant to apoptosis. Mechanistically, Smac mimetic-mediated enhancement of TRAIL-induced apoptosis critically depends on RIP1, since RNA interference-mediated silencing of RIP1 almost completely abolishes the Smac mimetic-conferred increase in TRAIL-induced apoptosis. Smac mimetic cooperates with TRAIL-R2 antibody to form a RIP1/caspase-8/FADD-containing cytosolic complex. Also, Smac mimetic acts in concert with TRAIL to trigger caspase-8 and -3 activation. Smac mimetic- and TRAIL-induced apoptosis was blocked by the addition of zVAD.fmk pointing to RIP1- and caspase-dependent apoptosis. Of note, overexpression of a non-degrading IκBα superrepressor profoundly inhibits apoptosis upon treatment with Smac mimetic and TRAIL-R2 antibody, demonstrating that NF-κB exerts a proapoptotic role in this model of apoptosis. Interestingly, the addition of Enbrel, a TNFα blocking antibody, does not interfere with Smac mimetic- and TRAIL-induced apoptosis, demonstrating that apoptosis occurs in a NF-κB-dependent, yet TNFα-independent manner. By demonstrating that Smac mimetic primes glioblastoma cells, including glioblastoma-initiating stem cells, to TRAIL-R2 antibody our findings have important implications for the development of novel strategies for the treatment of glioblastoma.

Transcriptional regulation of autophagy receptors by the Unfolded Protein Response

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Autophagy has been well established as an essential protective response during ER stress and has been described to exert its pro-survival functions through the non-selective bulk degradation of unfolded proteins and damaged organelles. Recent research has identified and characterised the autophagy receptors, Nix/Bnip3L, p62 and Nbr1, which specifically target damaged organelles and ubiquitinated proteins and aggregates to the autophagosome. Despite of these advances, very little is known about their regulation and the functional consequence of the absence of these receptors during different stress responses. Microarray analysis performed in colon cancer cells subjected to ER stress inducers revealed the transcriptional upregulation of known autophagy receptor genes which was further validated by real time PCR. Here we evaluate the functional relevance of these proteins during ER stress and identify which arm of the UPR is involved in their transcriptional upregulation.

Measurements of caspase-2-like activities in intact living cells

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Caspase-2 is one of the most conserved caspases across species and presents structural and functional features of both initiator and effector caspases. Numerous reports have been published that either described or rejected a role of caspase-2 as an apoptosis initiator in response to diverse stimuli such as DNA damage, cellular stress, heat shock, or death receptor activation. We, therefore, devised an approach that allows to determine caspase-2-like activity for the first time within the complexity of intact living cells. To this end we designed a Förster resonance energy transfer (FRET) probe containing the optimal caspase-2 cleavage site VDVAD. Following successful in vitro characterisation we recombinantly expressed the FRET probe to screen for conditions at which caspase-2 is activated as an initiator caspase. We measured VDVAD probe cleavage in response to 7 different stimuli (cisplatin, doxorubicin, 5-fluorouracil, TRAIL, TNF-α, FasL, heat shock) in MCF-7 cells overexpressing Bcl-xL. These cells fail to activate the mitochondrial apoptosis pathway, preventing the contribution of effector caspases to probe cleavage. VDVAD probe cleavage in MCF-7 Bcl-xL cells was insignificant at all conditions tested. In contrast, the presence of caspase-7 in parental MCF-7 cells was sufficient to cleave the entire pool of FRET substrate. Furthermore, a FRET probe containing the caspase-8/-10 preferred sequence IETD was fully cleaved in response to death receptor ligands in MCF-7 Bcl-xL cells. Preliminary data acquired in HeLa cells overexpressing Bcl-2 or Bax/Bak-deficient mouse embryonic fibroblasts so far likewise do not suggest caspase-2 activation upstream of apoptotic mitochondrial engagement. Our current findings, therefore, suggest that, despite presenting structural features of an initiator caspase, caspase-2 may not be an apical caspase in any of the scenarios investigated.

Epigenetic regulation of miR-194 in acute myeloid leukaemia: identification of its new target, BCLAF1, and of its role in differentiation and cell cycle progression.

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MicroRNA are small non coding RNAs about 18 to 25 nucleotides long able to regulate the expression of their target genes by specifically binding and cleaving mRNAs, inhibiting translation and deadenylating mRNA tails. Has been shown that miRNAs are clearly involved in the control of different pathways, such as cell proliferation, differentiation and apoptosis and the alteration of these processes are involved in cancer initiation and progression. In fact the disturbance of miRNAs expression and function may contribute to initiation and maintenance of tumors, so they can behave as tumor-suppressors and oncogenes.
Among all multiple mechanisms altering miRNAs expression and/or function in human cancer, there are the epigenetic deregulations. 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, including leukaemia. Self renewal and hematopoietic differentiation are defined as a dynamic interplay between transcriptional and post-transcriptional regulators, like miRNAs. Based on these evidence in last years are emerging possible applications of miRNAs 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 the role of the couple miR-194-BCLAF1 in tumorigenesis and their epigenetic regulation. It appears that miR-194 is involved in cell proliferation and differentiation in acute myeloid leukaemia. These data will be further discussed.
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Induction of Bim-dependent apoptosis in chemoresistant ovarian carcinoma cells by miR-491-5p-targeted Bcl-xL

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In ovarian cancers, overcoming chemoresistance remains a major hurdle for successful treatment. Protection against apoptotic cell death is particularly involved in tumor chemoresistance, and more precisely Bcl-xL and Mcl-1 are gateway proteins guarding together against apoptosis in ovarian cancer cells. Therefore, new agents targeting Bcl-xL may be useful in combination with pharmacological agents inhibiting Mcl-1 (i.e. chemotherapy) to kill efficiently chemoresistant cancer cells. miRNAs has emerged to play important roles in tumorigenesis and drug resistance. We thus attempted to identify miRNA(s) that may regulate Bcl-xL using both already published "apoptomiRs" and in silico prediction algorithms. We then analyzed whether, by restoring functional apoptotic pathways, they could improve apoptotic cell death, and therefore overcome drug resistance to cancer chemotherapy.
Among selected miRNAs, only miR-491-5p or let-7c reduced Bcl-xL protein expression without modifying mRNAs levels in two ovarian chemoresistant carcinoma cell lines (IGROV1-R10 and SKOV3). No evident phenotype changes were observed with let-7c on both cell lines. In contrast, miR-491-5p induced apoptosis in IGROV1-R10 cells whereas they only slowed-down SKOV3 cell proliferation without apoptosis associated. We focused our attention on the highly cytotoxic miR-491-5p and further investigated the mechanisms of miR-491-5p-mediated apoptosis in IGROV1-R10 cells. Luciferase assays showed that miR-491-5p targets directly 3'UTR of Bcl-xL. A higher level of apoptosis was observed in miRNA transfected cells in comparison to those treated with a siRNA targeting Bcl-xL (siXL1). Interestingly, the BH3-only protein Bim was strongly induced by miR-491-5p in IGROV1-R10 cells and siRNA-mediated Bim inhibition abrogated miR-induced apoptosis. In contrast, Bim induction was not observed neither in response to let-7c or siXL1 in IGROV1-R10 cells nor in SKOV3 cells that resist to miR-491-5p-induced apoptosis.
In summary, in cells in which survival is highly dependent on both anti-apoptotic Bcl-xL and Mcl-1 proteins, the inhibition of Bcl-xL directly by miR-491-5p, and of Mcl-1 via miR-491-5p-induced Bim up regulation, is sufficient for triggering apoptosis. The "licence to kill" of miR-491-5p is related to its ability to induce Bim. We are currently investigating the mechanisms involved in this direct or indirect modulation of Bim expression.This work constitutes the rationale of future pre-clinical in vivo investigations. In regards to recent successes in "miRNA replacement therapy", we are cautiously optimistic that such "killer" miRNAs could be of a particular interest in pro apoptotic based strategies for chemoresistant cancer.

Bax homodimerizes via a BH3:groove interface during apoptosis

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During apoptotic cell death, Bax and Bak change conformation and homo-oligomerize to permeabilize mitochondria. However, the structures of activated Bax and Bak and how these proteins self-associate to form the permeabilizing complex is unknown. We report that after apoptotic signalling, Bax homodimerizes involving an interaction between the BH3 domain and the hydrophobic groove of a partner Bax molecule. Disulphide-linkage analysis indicated that BH3:groove dimers of Bax were symmetric. The BH3:groove interaction was evident in the majority of Bax after apoptotic signalling, and correlated strongly with cytochrome c release, supporting its central role in Bax apoptotic function. A second interface between the Bax α;6-helices is implicated by cysteine linkage studies. We also found that Bax and Bak could heterodimerize during apoptosis via a BH3:groove interaction, further demonstrating that once activated Bax and Bak oligomerize via conserved mechanisms and that they can potentially co-operate to mediate cell death. Our findings identify a key interface that may be targeted to interfere with Bax apoptotic function and highlight the importance of BH3:groove interactions in the regulation of apoptosis by the Bcl-2 protein family.

Basement membrane matrix influence on stable metastatic and surviving MDA-MB-231 breast subpopulation selection.

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The poor efficacy of various anti-cancer treatments against metastatic cells has focused attention on the role of tumor microenvironment in cancer progression. To understand the contribution of the extracellular matrix (ECM) environment to this phenomenon, we isolated ECM surrogate invading cell populations from MDA-MB-231 breast cancer cells and studied their genotype and malignant phenotype. We isolated invasive subpopulations (INV) from non invasive populations (REF) using a 2D-Matrigel assay, a surrogate of basal membrane passage. INV and REF populations were investigated by microarray assay and for their capacities to adhere, invade and transmigrate in vitro, and to form metastases in nude mice. REF and INV subpopulations were stable in culture and present different transcriptome profiles. INV cells were characterized by reduced expression of cell adhesion and cell-cell junction genes (44% of down regulated genes) and by a gain in expression of anti-apoptotic and pro-angiogenic gene sets. Accordingly, in vitro INV cells showed reduced adhesion and increased motility through endothelial monolayers and fibronectin. When injected into the circulation, INV cells induced metastases formation, and reduced injected mice survival by up to 80% as compared to REF cells. In nude mice, INV xenografts grew rapidly inducing vessel formation and displaying resistance to apoptosis. Our findings reveal that the in vitro ECM microenvironment per se was sufficient to select for tumor cells with a stable metastatic phenotype in vivo characterized by loss of adhesion molecules expression and induction of pro-angiogenic and survival factors.

Repressing CYLD promotes neuroprotection against glutamate dependent cell death in vitro and cerebral ischemia in vivo

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The deubiquitinating enzyme CYLD is a regulatory component of the NF-kB pathway. Dysfunctional CYLD has been implicated in tumor genesis, altered immune responses and the regulation of microtubule dynamics. More recently CYLD has been identified as a key player in TNF-induced necrotic cell death. Though increasing evidence emerges on the significance of necrosis in neurodegeneration, the particular impact of CYLD on neuronal viability remains elusive. In this study we used a model of glutamate toxicity in neuronal HT-22 cells to investigate the role of CYLD. In these neurons, glutamate induces glutathione depletion leading to increased ROS production. In response to glutamate HT-22 cells show increased levels of lipidperoxidation and mitochondrial fragmentation, accompanied by an increase of RIP1-RIP3 complex formation. SiRNA-mediated CYLD gene silencing attenuated lipidperoxidation, inhibited formation of the RIP1-RIP3 complex, and prevented mitochondrial fragmentation, thereby promoting neuronal survival. Further, direct targeting of RIP1 or RIP3 by respective siRNA approaches significantly decreased glutamate induced cell death in HT-22 cells. In primary neuronal cultures, glutamate-induced excitotoxicity could not be prevented by CYLD depletion. However, blocking RIP1 kinase by necrostatin exerted neuroprotection against glutamate-induced neuronal death. In an in-vivo model of cerebral ischemia, we found, that CYLD -/- mice exhibit significantly reduced infarction volume compared to control littermates. In conclusion, activation of CYLD and related Rip1/Rip3-dependent mechanisms of necroptosis mediate neuronal cell death after oxidative stress in vitro and promote brain damage after cerebral ischemia in vivo.

Rose Bengal Acetate photodynamic therapy generates multiple cell deaths through caspase -independent and -dependent pathways

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PhotoDynamic Therapy (PDT) is emerging as a promising non invasive treatment for several pathologies, included cancer. Three non toxic elements (photosensitizing drug (PS), visible light matching the absorption spectrum of the PS and tissue oxygen) orchestrate the photodynamic action based on photochemical reactions generating Reactive Oxygen Species (ROS) and subsequent cytotoxicity ending in massive cell deaths. Rose Bengal Acetate (RBAc) (10-5M) stands out against the PSs useful in PDT for its minimal side effects, such as prolonged photosensitivity, facile photocatalytic conversion upon green light irradiation (1.6 J/cm2, 530±15 nm) and its long-term and time-related cytotoxic effects ROS mediated. Indeed, RBAc-PDT, in HeLa cells, induces multiple cell death types, i.e. apoptosis (40% at 12h of recovery post irradiation), autophagy (25% at 8h of recovery post irradiation) and negligible necrosis (<5% at longest times post irradiation). Apoptosis is the first and preferred cell death mechanism triggered through the sequential activation of caspase dependent and independent pathways, under the control of Bcl-2 family members. Particularly, caspase dependent apoptosis is sustained by the cleavage of caspases 9, 8 and 12, respectively at 4 h, from 12 to 72 h and at 18 h post RBAc irradiation, leaders of intrinsic, extrinsic and Endoplasmic Reticulum (ER) stress pathways. The presence of ER stress was confirmed by the increase in Glucose-Regulated protein-78 (GRP78) and phospho-Eukaryotic Initiation Factor 2 α; (P-eIF2α;) proteins. Autophagy occurs at 8h after RBAc photosensitization, as shown by the formation of autophagosomes, acidic vacuoles and increased Light Chain 3B-II (LC3BII) biomarker expression. Our data also suggested that autophagy has a pro-death role, since suppression of autophagy by 3-MethylAdenine (3-MA) significantly augmented cell viability. Interestingly, inhibition of one pathway, i.e. caspase-9 (Z-LEHD-FMK), caspase-8 (Z-IETD-FMK), pan-caspases (Z-VAD-FMK), autophagy (3-MA) and necrosis (Nec-1), did not impair the activation of the others, suggesting the independent onset of the different apoptotic pathways and autophagy. In summary, RBAc is a powerful cytotoxic PDT agent, since it is able to initiate several signalling processes in HeLa cells, ending to rapid, independent and sequential onset of different cell death types. This ensures a high percentage of tumour cell demise when one or more death mechanisms are inhibited.

Loss of PERK sensitises to ER stress-induced apoptosis through upregulation of NOXA

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In mammals, three transmembrane proteins at the endoplasmic reticulum (ER), IRE1, ATF6, and PERK, respond to the accumulation of unfolded proteins in the ER lumen. Activation of these three sensors initiates ER-to-nucleus intracellular signaling cascades collectively termed the Unfolded Protein Response (UPR). Activation of PERK (PKR–like-ER kinase) leads to cellular adaptation to ER stress by multiple mechanisms, including attenuation of protein synthesis and induction of pro-survival genes by transcription factors such as ATF4 and NRF2. Thus, cells lacking PERK are severely compromised in their ability to mount cytoprotective cellular responses to ER stress, leading to a specific sensitivity to ER stress-induced apoptosis. The objectives of this work were to further elucidate PERK's role in the control of cell fate and to better characterise the increased sensitivity of PERK-/- cells to ER stress-mediated cell death. Here we show that PERK-/- MEFs are defective in their induction of several UPR targets and that the expression of genes such as GRP78, spliced XBP1, HERP, ATF4 and CHOP is reduced in PERK-/- cells. Moreover, we identify a novel signalling cascade downstream of PERK which is critical for protection against ER stress-induced cell death. We show that in PERK-/- MEFs phosphorylation of AKT is diminished and that this correlates with diminished phosphorylation of the AKT target FOXO3a. Expression of the FOXO3a target NOXA is elevated in PERK-/- MEFs and knockdown of NOXA expression in PERK-/- MEFs by NOXA shRNA delays the early onset of ER stress-induced caspase activation, loss of mitochondrial membrane potential and apoptosis. These data enhance our understanding of PERK-dependent cytoprotective signalling and describe a novel link between UPR sensors and the AKT pathway which is critical in determining cell fate in response to ER stress.

Klf9 is a key regulator of developmental cell death of Purkinje cells

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During development, neurons pass through a phase of programmed developmental cell death (DCD) during which trophic factors released by their targets are essential for their survival. After this period, neurons can survive without targets. The molecular mechanisms closing this phase remain unknown. In mouse cerebellum, the DCD of Purkinje cells finishes at the end of the first postnatal week. A transcription factor, whose developmental expression increases at the end of the first postnatal week, is a potential candidate for turning off the phase of Purkinje PCD. To find such candidates, we used Affymetrix arrays for transcriptome analysis of cerebellar cortical regions at different ages. From statistical and gene ontology analyses, we selected Klf9 as a candidate gene for turning off the Purkinje DCD. We showed that the number of Purkinje cells in organotypic culture from newborn mice drops between the 3rd and 7th day of culture and remains stable (40% survival) at least until the 14th day. On this new model of Purkinje DCD, we tested the function of Klf9 using lentiviral vector-mediated manipulation of Klf9 expression. Klf9 overexpression multiplies the survival rate of the Purkinje cells by a factor of 2.2 whereas its depletion divides it by 2.6. Klf9-/- Purkinje survival rate is reduced to 23%. The known Purkinje cell trophic factors IGF-1 (insulin growth factor-1) and NT3 (neurotrophin3) were able to rescue these neurons from Klf9 depletion. Altogether, these results show that Klf9 is necessary and sufficient for Purkinje cell survival, suggesting that Klf9 is indeed involved in closing the developmental phase of cell death.

A novel role for lysotropic agents in Glioblastoma therapy

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Glioblastoma is the most common and highly aggressive primary malignant brain tumor, often characterized by MGMT hypermethylation, EGFR receptor amplification and increased activity of the PI3Kinase/Akt – survival cascade. Importantly, the PI3Kinase/Akt signaling pathway is also linked, via the mTOR complex, to the autophagic process, which recently emerged as a key program that is deregulated during cancerogenesis. The role of autophagy in cancer progression is still controversially discussed. While it allows cell survival in nutrient restricted intratumoral environment through lysosomal self-digestion, other mutational studies suggest an anticancer role of autophagy. The key factors which direct autophagy into either a prosurvival or a destructive outcome still remain undiscovered. In order to obtain a better understanding of conditions promoting destructive autophagy, we investigated the possibility of combining an autophagy inducer (PI3K inhibitor) and a lysosome-destabilizing agent (B10, a new derivative of Betulinic Acid) to interfere with late stages of the autophagy process. We treated U87MG cells with different concentrations of PI3K inhibitor and detected an increased LC3-I to LC3-II convertion, a marker of the initial autophagy sequestration step in a time- and dose-dependent manner. Further experiments showed that combination treatment of PI3K inhibitor and B10 in U87MG cells results in enhanced loss of cell viability, increased rate of DNA fragmentation and activation of caspase-3 when compared to single treatments. However, knockdown of Atg7 had no effect on PI3K inhibitor/B10-induced cell death, suggesting that disruption of autophagy at initial stages does not play a role in cell death induction upon PI3K inhibitor/B10 combination treatment. By comparison, inhibition of Cathepsins completely protects against loss of cell viability which is, at least partially, due to prevention of caspase-3 activation by lysosomal enzymes. We discovered that treatment with PI3K inhibitors lead to an increase lysosomal compartment volume, which is accompanied by an increased maturation of lysosomal degradation enzymes and structural proteins. These results argue that lysosomal permeabilization is the central event in PI3K inhibitor/B10-induced cell death and suggest that PI3K inhibitors might contribute to cell death induced by lysotropic agents independently of their effects on autophagy.

Study of Biological Effects of Some Boron Compounds on the Rat Glioma (C6) Cells

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Boron is a natural and essential element in the human food chain. The salty waters and the soil contain large amounts of boron. Biologically, the boron plays an important role in the regulation of the bacterial gene expression, the plant growth and the inhibition of the proliferation in the different cancer cell. Recent studies have reported that the boron-containing compounds is using as preventive and therapeutic agents for some forms of cancer. In this study, the cytotoxity and apoptotic effects of anhydrous borax, sodium perborate and potassium tetraborate were analyzed with cultured rat glioma (C6) cells. The C6 cells were incubated in the ranges of the 0,5-25 mM concentration of the boron compounds. After the 24, 48, 72 h incubations, the cell proliferation and cytotoxicity were measured by using MTT and Neutral Red (NR) assays. Mitocapture assay was applied to measure of mitochondrial membrane permeability. Caspase 3 and 8 enzyme activations and DNA Fragmentation methods were used for determine to the beginning of the apoptosis and breaks of the DNA, respectively. The results indicated that the cytotoxicity occurred at 0,5 mM of anhydrous borax, 5 mM of sodium perborate and 1 mM of potassium perborat concentrations All of the boron compounds reduced the mitochondrial membrane permeability but increased the caspase enzyme activations. Finally, boron compounds caused apoptotic cell death in rat glioma (C6) cells.

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Development of DR5 Virus-Like Particles based on murine pneumotropic virus for vaccination of BALB/c mice as a mean to induce apoptosis and cell death in DR5 positive tumors

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The aim of this study was to produce murine pneumotropic virus (MPtV) Virus-Like Particles (VLPs) expressing mouse death receptor 5 (DR5) epitopes (DR5MPtVLPs), to vaccinate mice, and obtain anti-DR5 antibodies, potentially able to induce death/rejection of DR5 positive tumors.
Background: VLPs have been made with viral capsid proteins from different viruses, for example murine polyomavirus (MPyV) or MPtV, and these VLPs can be used as vectors for cancer immunotherapy, by fusing tumor antigens to different parts of the VLPs, e.g. either on the inside or on the surface of the particles. We have previously successfully used MPyVLPs and MPtVLPs expressing Her2 or PSA antigens, on the inside of the VLPs, to vaccinate against, and inhibit, outgrowth of Her2 or PSA expressing tumors in mice. DR5 is a transmembrane receptor, which is present on the surface of cells of a variety of tumors. It promotes apoptosis when bound by its ligand TRAIL. In this context we have attempted to produce MPtVLPs expressing various DR5 epitopes, on the surface of the VLPs, in order to use these DR5MPtVLPs to immunize mice and induce an anti-DR5 antibody response that potentially will bind to DR5 expressing tumor cells and promote their apoptosis.
Material and Methods: VLPs were produced in insect cells using a baculovirus expression vector system. BALB/c mice are immunized three times, with or without Freunds Incomplete Adjuvant and their sera are examined for presence of antibodies against DR5 using ELISA, and the ability to cause apoptosis in tumor cells in vitro using Annexin V/PI staining and FACS evaluation. BALB/c mice will be immunized with DR5MPtVLPs and challenged with DR5-expressing mouse mammary tumor cells (D2F2). The immunization effect against tumor outgrowth of our DR5MPtVLPs will be compared to that by injection of anti-DR5 antibody MD5-1 described by others.
Results: Seven DR5MPtVLPs have been designed and produced and three DR5MPtVLPs have been successfully characterized as forming VLPs. In addition mice have been immunized once without signs of adverse effects and we are now about to test their sera for functional antibodies, and for in vivo protection against outgrowth of DR5 expressing tumors.
Conclusion: It is possible to design and produce DR5MPtVLPs and they do not induce adverse effects upon immunization of mice. Their functional activity is presently being tested for and will be presented.

Melatonin reduces TNF-α-induced apoptosis in human leucocytes

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The pro-apoptotic signalling cascades induced by tumour necrosis factor-α (TNF-α) have intensively been studied in multiple cellular systems. Moreover, emerging evidence suggests that melatonin may be involved in the protection of different cell types against damage-induced apoptosis. Thus, the objective of this study was to evaluate the effect of melatonin on TNF-α-induced apoptosis in human leucocytes. Peripheral blood leucocytes were purified from healthy individuals by Ficoll-Histopaque centrifugation. Cells were treated with TNF-α alone (100 ng/ml, 2 hours) or in the presence of cycloheximide (10 micrograms/ml), which promotes caspase-8 activation by eliminating endogenous caspase-8 inhibitor, c-FLIP. Caspase-8, caspase-3 and caspase-9 activities were determined from cleavage of their respective specific fluorogenic substrate. Reactive oxygen species (ROS) generation was quantified with the non-fluorescent, cell-permeable probe dihydrorhodamine-123 (DHR-123). Also, apoptotic cell death were analysed by redistribution of phosphatidylserine (PS) in the presence of propidium iodide (PI). Cleavage of different specific fluorogenic substrate showed activation of caspase-8, caspase-3 and caspase-9 in TNF-α-treated leucocytes, while the treatment with TNF-α plus cycloheximide resulted in further caspase activation. Moreover, activation of caspases induced by the treatment with TNF-α plus cycloheximide was accompanied by an increase in ROS production and, subsequently, led to apoptotic cell death. Conversely, pre-treatment with melatonin (1 mM) for 1 hour inhibited TNF-α plus cycloheximide-evoked activation of caspases, ROS production and apoptotic cell death. Likewise, pre-treatment with the wellknown antioxidant, N-acetyl-L-cysteine (NAC, 1 mM) for 1 hour was also able to reduce TNF-α plus cycloheximide-induced caspase-9 and -3 activities, but not caspase-8 activity, thus indicating that caspase-8 activation may be independent of endogenous ROS production. As expected, pre-treatment with NAC forestalled ROS production and partially prevented apoptosis produced by TNF-α plus cycloheximide. In conclusion, our results suggest that melatonin inhibits caspase activation, prevents ROS generation and reduces apoptotic cell death induced by TNF-α (in the presence of cycloheximide) in human leucocytes.

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The role of c-FLIP splice variants in urothelial tumours

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Apoptosis plays an important role in maintaining tissue homeostasis. Deregulation of this mechanism can cause uncontrolled cell proliferation, which eventually may lead to cancer. Various cancer types have been shown to up-regulate cellular FLICE inhibitory proteins (c-FLIP). These proteins are known to inhibit death receptor-mediated apoptosis and the high expression of c-FLIP proteins is thought to be a mechanism for escaping immune surveillance. The functional role of c-FLIP splice variants in urothelial carcinoma has not yet been investigated. Six urothelial carcinoma cell lines were characterized based on the death receptor expression, sensitivity towards CD95L-induced apoptosis, c-FLIP expression as well as the expression of the proteins involved in the 'extrinsic' and 'intrinsic' apoptotic pathways. The cell lines VmCub1 and SD were chosen for further studies. Both cell lines could be sensitized towards CD95L-induced as well as TRAIL-induced apoptosis by the protein translation inhibitor cycloheximide (CHX). A CHX-kinetic showed that both the long and the short isoforms of c-FLIP (c-FLIP-L and c-FLIP-S) are short-lived proteins and these could therefore be responsible for the CHX-sensitization of the urothelial carcinoma cell lines. Overexpression of the splice variants c-FLIP-L, c-FLIP-S and c-FLIP-R all protected the VmCub1 and SD cells against CD95L-induced apoptosis. Knock-down urothelial carcinoma cells were generated by lentiviral delivery of shRNAs targeting either c-FLIP-L, c-FLIP-S or both isoforms. The double knock-downs of both the VmCub1 and SD cells were sensitive for CD95L-induced as well as TRAIL-induced apoptosis, this shows that c-FLIP proteins are important for protecting urothelial carcinoma cells against apoptosis. At the moment patient material is being examined by qPCR to investigate which of the c FLIP isoforms is differentially expressed in urothelial tumours.

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

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Apoptosis is an efficient method for preventing malignant transformation. Dysregulation of apoptosis cause signifecant human diseases and promote cancer development. Fucosing on identifying of natural apoptotic factors and understanding their molecular mechanisms may inhance our understanding on the oncology and on the biology of human cancer. Moreover, this may offer new therapeutic stratrgies to exploit agents that inactivate oncogens or activate tumor suppressor genes which could lead to apoptosis in cancer cells.
Human cancer cell lines of breas, prostate and pancrease were exposed to different dietary indole derivatives [indol-3-carbinol or to 3-3' diindolylmethane (DIM)] or natural antioxidents (NAOs) extracted from the leaves of Spinach (S), Inulla viscosae (IV) and Citrullus Colocynyhis (CC)or to extracts from medicinal mushrooms. Our results indicated that these factors inhibit proliferation and DNA synthesis of 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 animals with DIM or with IV three times a week for five weeks, caused a significant deceleration in the volumes and weights of tumors which were induced in C57BL/6 mice, by transplanting the TRAMP-C2 prostate cell line subcunaneously. This effest was found to be mediated by inducing apoptosis as it was detected by morphological and staining studies. Moreover, pre-treatment of animals with he pre-apoptotic facors, DIM or IV, for five weeks before transplanting the TRAMP-C2 cells, significantly reduced tumor development as compared to controls. Tumors were developed in 90% of controls 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. 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.

Triggering of Transient Potential Receptor Vanilloid 1 (TRPV1) induces autophagy that delays apoptotic cell death in murine thymocytes.

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We have previously reported (Amantini et al., 2004), that activation of TRPV1 triggers apoptotic cell death of rat thymocytes. Recent findings demonstrate that autophagy is an early event related to promotion or inhibition of cell survival (Ferraro et al., 2007); however, at present, no data on the interplay between TRPV1-induced apoptosis and autophagy has been provided. Aim of this study was to investigate the relationship between these processes in thymocytes treated with low-dose (10μM) of capsaicin (CPS), a full agonist of TRPV1. We firstly demonstrated that mouse thymocytes express the receptor both at mRNA and protein levels. Treatment of cells with CPS for 8 h reduced the viability by inducing apoptotic cell death. Time-course analysis evidenced that exposure of thymocytes to CPS up to 2-4 h induces autophagy: the process was dependent on ROS generation, resulting in the modulation of AMPK, Atg4C, and Beclin-1 proteins. Interestingly, the use of the autophagic inhibitor 3-methyladenine (3-MA) induced apoptosis of thymocytes, indicating that autophagy delays apoptotic cell death. Moreover, inhibition of autophagy induced down-regulation of the autophagic protein Beclin-1 and the anti-apoptotic protein Bcl-XL, and triggered caspase-3 activation. In addition, the pancaspase inhibitor, z-VAD, completely reverted the 3-MA-induced Beclin-1 down-regulation, indicating that Beclin-1 is directly cleaved by caspase. CPS treatment affected CD4 and CD8 α expression in double positive (DP) cells with the generation of a distinct subpopulation, namely DPdull, expressing lower levels of both antigens as respect to the majority of DP thymocytes. Interestingly, we found that DPdull cells overlaps with the autophagic thymocytes and that inhibition of autophagy induces DNA fragmentation. Overall, our findings extend the previous observations (Vasquez NJ et al., 1992) demonstrating that DPdull thymocytes can delay apoptotic cell death by activating a pro-survival autophagic process.

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The antiapoptotic protein AAC-11 as a novel exploitable therapeutic target in human malignancies

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Defects in apoptosis contribute to a number of human diseases, ranging to tumour formation, development and treatment failure to neurodegenerative and autoimmune disorders. The expression of genes that regulate apoptotic cell death has an important function in malignancy and in determining the sensitivity of tumour to chemotherapy. Therefore, selective inhibition of antiapoptotic pathways represents an attractive therapeutic approach in cancer and has stimulated intensive efforts to restore apoptosis in cancer cells in order to combat them. We report that AAC-11 (antiapoptosis clone 11), an antiapoptotic factor that we have demonstrated to be involved in cancer cells sensitivity to anticancer drugs, possesses a major role in both invasiveness and adhesiveness. Whereas AAC-11 expression dramatically increased growth as well as invasiveness and adhesiveness, its depletion markedly decreased the invasive and adhesive properties of an array of cancer cell lines. AAC-11 possesses a leucine zipper motif that is mandatory for its antiapoptotic function. Based on this motif, we have developed a cell permeable peptide able to drastically increase drug-induced apoptosis in cancer cells, but not in normal cells. Interestingly, this peptide was also able to decrease both mobility and invasiveness of a panel of cancer cells. Therefore, these results raise the possibility that AAC-11 contributes to the development of cancer invasion and that AAC-11 might be an exploitable therapeutic target in human malignancies.

Natural killer cell-induced cleavage of HAX-1 in multiple myeloma cell lines is granzyme B-dependent

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HS-1-associated protein X-1 (HAX-1) was originally identified in B cells as a 35kD protein that interacts with HS-1, a src kinase substrate. Multiple functions of HAX-1 protein have been reported, including regulation of cell apoptosis, cell migration and interaction with mRNA. It has been shown that HAX-1 is critical for maintaining the inner mitochondrial membrane potential in myeloid progenitor cells, thus supporting the role of this protein in protection against apoptosis. Patients with severe congenital neutropenia (SCN) harbor homozygous mutations in the HAX1 gene and display a maturation arrest in the bone marrow and lack of mature neutrophils in peripheral blood. Natural killer (NK) cells deliver cytotoxic granules which contain granzymes and perforin, leading to apoptosis of virus-infected or malignant cells. Our examination of public cDNA microarray databases has revealed high expression of HAX1 mRNA in hematopoietic malignancies including multiple myeloma (MM), a B cell malignancy. We also noted high expression of HAX-1 protein in bone marrow samples from MM patients. Studies of primary NK cells and MM cell lines demonstrated that NK cells can cleave HAX-1 in MM cells in a granzyme B-dependent manner. We also provided evidence that the introduction of recombinant granzyme B to MM cells results in HAX-1 cleavage. We propose that HAX-1 cleavage could serve as mechanism to potentiate NK-mediated killing of susceptible cancer cells.

Increased Expression of miR-34 in NAFLD Patients and Targeting by Ursodeoxycholic Acid in Rat Liver

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Background and aims: Non-alcoholic fatty liver disease (NAFLD) comprises a spectrum of stages ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and represents a major cause of liver-related morbidity and mortality when progressing to cirrhosis. However, disease pathogenesis remains largely unknown. Recently, microRNA (miRNA or miR) expression in the liver was found to be altered in human NASH, and ursodeoxycholic acid (UDCA) was shown to modulate miRNA expression in rat liver. Therefore, our aim was to evaluate miR-34 family expression in human NAFLD liver, and to elucidate its functional role in both rat liver and primary rat hepatocytes exposed to UDCA. Methods: Liver biopsies were obtained from NAFLD morbid obese patients undergoing bariatric surgery, and classified as steatosis (n= 15), less severe (n= 5) and more severe (n= 6) NASH. Rat livers were collected from animals fed diets containing 0.4% UDCA. Primary rat hepatocytes were incubated with UDCA or no addition, for 16 to 64 h, and transfected with a specific miRNA-34a precursor. miR-34 family expression was analyzed by qRT-PCR. miR-34 target Sirtuin-1 (Sirt-1) was analyzed by immunoblotting. Cell viability and apoptosis were evaluated by LDH, Hoechst and TUNEL assays. Results: Human liver tissue exhibited reduced expression of miR-122, -143 and -451 from steatosis to severe NASH ($p < 0.05$). In contrast, miR-34a expression and apoptosis increased from steatosis to less severe NASH ($p < 0.05$), and further to more severe NASH ($p < 0.01$). miR-34b and 34c were similarly increased, and Sirt-1 protein levels significantly decreased ($p < 0.05$). In rat liver in vivo and in primary rat hepatocytes in vitro, UDCA treatment resulted in diminished miR-34a expression and enhanced Sirt-1 protein levels($p < 0.05$). miR-34a overexpression in vitro confirmed targeting by UDCA., which prevented miR-34a-induction of apoptosis and increased cell viability ($p < 0.05$). Conclusions: Our results support a link between liver cell apoptosis, miR-34a and NAFLDseverity. Importantly, UDCA specifically targets the miR-34/Sirt-1 pathway in rat hepatocytes, thus preventing cell death. A further understanding of NAFLD pathogenesis and potential endogenous modulators may ultimately provide new targets for therapeutic intervention.

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Increased Fas-expression and increased caspase 8 activity suggests enhanced Fas-mediated apoptosis of peripheral T cells in HCV mono-infected and HIV/HCV coinfected patients

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Background: Recent studies have shown increased caspase-3 activity in T cells of chronic HCV infected patients, indicating that T cell apoptosis is enhanced in chronic HCV, which may contribute to viral persistence. Since Fas-mediated apoptosis pathways are considered to contribute to this phenomenon, we analyzed various markers of these pathways to elucidate their role in chronic HCV with or without HIV infection.
Methods: Peripheral blood mononuclear cells (PBMC) were drawn and freshly analyzed using 8-colour flow cytometry for CD3, CD4 and CD8, Fas (CD95), Flica caspase-8, Flica caspase-3/7 and annexin V. Included patient groups were: 1) chronic, presently untreated, HCV genotype 1 (n=15); 2) HIV/ HCV coinfected patients on HAART (n=12); and 3) healthy controls (HC, n=10).
Results: Since Fas-mediated T cell apoptosis requires activation of initiator caspase-8 and subsequent activation of executioner caspases 3 and 7, we investigated the expression of these caspases as well as annexin V on peripheral T cells. Compared to HC (41%), expression of Fas was significantly higher on CD4+ T cells of HCV-infected patients (62%, p=0.02) and HIV/ HCV-coinfected patients (70%, p=0.02). Similarly, in CD8 T cells higher Fas expression was seen. Compared to HC (4.3% and 2.9%), initiator caspase-8 activation was also more present on CD4+ and CD8+ T cells in HCV mono-infected (5.1% and 3.5%; p=0.15 and p=0.07) and HIV/ HCV coinfected (8.5% and 7.7%; p=0.04 and p=0.02) patients. Further downstream, activated executioner caspases 3 and 7 and annexin V in CD4+ and CD8+ T cells did not differ among the three groups.
Conclusions: Compared to healthy controls, expression of Fas and caspase-8 by CD4+ and CD8+ T cells is higher in HCV mono-infected and HIV/ HCV coinfected patients. Although no difference of downstream caspase 3 and 7 or annexin V were observed, our findings are suggestive for enhanced Fas-mediated apoptosis of T cells in chronic HCV with or without HIV co-infection.

Attenuation of ERK phosphorylation and down-regulation of c-Myc expression during BHV-1-induced apoptosis

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Bovine Herpesvirus 1 (BHV-1) is an important viral pathogen that, in cattle, can cause infectious rhinotracheitis, conjiunctivitis, genital disorders, abortions and shipping fever, which is a complicated infection of the upper respiratory tract. Apoptosis is a coordinated and often energy-dependent process that involves the activation of a group of cysteine proteases called "caspases" and a complex cascade of events that link the initiating stimuli to the final demise of the cell. In cultured cells or in calves BHV-1 infection leads to cell death, partially through induction of apoptosis by activation of caspases, through an intrinsic pathway via members of the Bcl-2 family. Viruses depend on cells for their replication and can differentially affect various signaling pathways. Signal transduction pathways are linked to the apoptotic machinery. The extracellular regulated kinase (ERK) survival pathway overrides the effects of apoptotic signals, by up-regulating antiapoptotic Bcl-2 proteins. Phosphorylation of ERK1/2 is upstream of the transcription factor NF-kB which, found essentially in all mammalian cell types, regulates a wide range of genes important in inflammation, immunity and prevention of apoptosis. Normally, NF-kB is sequestered within cytoplasm and traslocates into nucleus upon activation. Based on these observations, in the present study our results indicated that, following infection of BHV-1 in bovine cells (MDBK), nuclear fraction of infected cells showed NF-kB protein levels starting from 8 h p.i., that increased reaching the peak at 36 h p.i., before detection of BHV-1-induced apoptosis. The functional interaction between c-Myc and p53 resulted in the up-regulation of expression levels of key tumor-suppressor proteins as p53, p-p53 and p21Waf1. Whereas, simultaneously, the protein levels of protooncogene c-Myc decreased. Furthermore, western blot analysis of ERK1/2 showed that BHV-1 infection occurred through attenuation of the phosphorylation of ERK1/2. Taken together, these results support the idea that, in MDBK cells, BHV-1 inhibited cell proliferation, by attenuating the phosphorylation of ERK1/2 and down-regulating the expression of c-Myc, which significantly intensified BHV-1-induced apoptosis.

Recombinant expressed Bax induces cytochrome c release in isolated Neurospora crassa mitochondria

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As a highly conserved feature of multicellular organisms, the process of apoptotic cell death is essentially contributing to developmental processes, cell homeostasis and the elimination of redundant, genetically altered and unhealthy cells. The Bcl-2 protein family consists of an anti-apoptotic and a pro-apoptotic fraction, which are both known to be keyplayers in apoptosis related signal transduction and regulation. The pro-apoptotic Bcl-2 associated X protein (Bax) is known to translocate subsequently to an apoptotic stimulus to the mitochondria and inserts into the outer mitochondrial membrane causing the release of cytochrome c and apoptogenic factors, finally leading to the cellular self-digestion of the cell. Apoptotic-like cell death occurs also in some fungi and unicellular organisms, although the complexity of interaction seems not be as sophisticated as in higher eukaryotes (reviewed by Sharon et al., 2009). Here we show that recombinant expressed pro-apoptotic protein Bax is capable of inserting into isolated Neurospora crassa mitochondria finally leading to the permeabilization of the outer mitochondrial membrane and the release of cytochrome c.

Histone onco-modifications

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Post-translational modifications of histones provide an important regulatory platform for processes such as gene expression, DNA replication and repair, chromosome condensation and segregation and apoptosis. Disruption of these processes has been linked to the multistep process of carcinogenesis. Work on histone modifications and regulation of gene expression have coalesced into the "histone code" hypothesis, initially proposed by Allis and Turner, that encapsulates the function of histone modifications in chromatin structure and in the regulation of nuclear functions. Interestingly, alterations in histone modifiying enzymes, can contribute to the development of a variety of human cancers. As a conclusion, a new terminology "histone onco-modifications" is proposed to describe post-translational modifications of histones which have been linked to cancer. Additionally, loss or gain of certain modifications like the loss of acetylation of histone 4 lysine 16 (H4K16) have not only been strongly correlated to cancer but also to therapy resistance. Thus we have shown that both HDAC inhibitors 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 histone H3 and H4, including H4K16. Our recent publications and ongoing work aim to establish a network of cancer and cell death associated histone modifications to better understand the epigenetic role of cancerogenesis.

Crosstalk between Akt phosphatases in prostate cancer cells.

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Akt or protein kinase B plays a key role in multiple cellular processes, includ ing apoptosis and cell proliferation and it is aberrantly activated in many cancer cells. There are several protein phosphatases that may dephosphorylate Akt. We have previously shown that cholesterol-lowering drugs, statins, or extracellular ATP, induce a complex and rapid response in insulin-stimulated non-small cell lung cancer, A549. ATP depletes nuclear pAkt within minutes, and this response is associated with cell cycle stop or apoptosis. The pAkt depletion is mediated by the purinerigic P2X7 receptor (J. Biol Chem 2010, 285, 27900-27910), previously implicated in e.g. NALP3 inflammasome activation. We now report similar effects of ATP or statins in prostatic cancer DU145 and 22RV1 cells. P2X7 was activated by extracellular purinergic nucleotides such as ATP or BenzoATP. However, the level of pAkt in PTEN deficient prostate LNCap and PC3 cells were resistant to this treatment. This suggested that PTEN was necessary for pAkt depletion, and after PTEN transfection AT P or statin decreased the level of pAkt in LNCap cells, but not PC3 cells. Western blots revealed that in PC3 cells the level of PHLPP1 and PHLPP2 were low, while LNCAP cells overexpress these proteins. PHLPP1 and PHLPP2 can dephosphorylate Akt by direct binding to the hydrophobic motif, and have been shown to induce apoptosis in cancer cells. After double-transfection with both PTEN and PHLPP2, ATP induced changes in nuclear pAkt levels also in PC3 cells. While doing these experiments we also observed that the expression of phosphatases affected each other. For example, we saw that transfection of PC3 cells with PTEN suppressed the level of PHLPP2, and vice versa. The same crosstalk was seen in 22RV1 cells. Furthermore, transfection of 22RV1 cells with PHLPP1 decreased the level of PHLPP2, and vice versa. The expression and subcellular localization of phosphatases involved in nuclear pAkt depletion were analyzed by western blotting and immunostaining. Our results demons trate that the level of at least three pAkt phosphatases was crucial for their functional effect. In additional experiments we used PIK3CB (p110beta -/- or WT MEF cells (a generous gift from J.J. Zhao, Harvard, USA). The results indicate that also the p110beta nuclear protein was necessary for pAkt depletion. We conclude that the downstream effect of extracellular ATP on prostatic cancer cells is depended of the complex formation between phosphatases and additional proteins, perhaps partially with scaffolding function. Our results suggest that phosphatases governing nuclear pAkt levels may form multi-protein complexes that are necessary for ATP-induced and P2X7 mediated nuclear pAkt depletion. Disturbances in the stoichiometry in these complexes may lead to increased Akt signaling and inhibited apoptosis.

Involvement of caspase-2 and -8 in citrate-induced apoptosis

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Despite the heterogeneity of tumors, which dictates an individual approach to anticancer treatment, almost all tumor cells demonstrate enhanced uptake and utilization of glucose, a phenomenon known as the Warburg effect. Considering glycolytic phenotype of tumor cells, inhibition of glycolysis can be suggested as a therapeutic approach. Indeed, suppressors of glycolytic steps such as 2-deoxyglucose or 3-bromopyruvate provide a promising therapeutic strategy to overcome drug resistance in rapidly growing tumor cells. Among the agents, which can suppress glycolysis, is citrate, a member of Krebs cycle and a compound involved in a variety of reactions essential for cell physiology. Thus, citrate is a precursor of fatty acids in rapidly proliferating cells; inhibition of citrate ATP lyase, an enzyme that represents an important step in fatty acid biosynthesis, was shown to suppress tumor cell growth. We show that citrate-induced cell death is based not only on glycolysis suppression or moderate chelation of Ca2+, but involves stimulation of key steps in apoptotic pathways. Analysis of sensitivity of different neuroblastoma cells (Tet21N, SK-N-AS, SK-N-SH, and SK-N-BE(2)) as well as non-small-cell lung cancer cells (U1810) revealed that citrate induces typical apoptosis manifestations such as detachment of cells, stimulation of caspase-3-like activity, appearance of apoptotic nuclei and release of cytochrome c in a dose-dependent manner. Remarkably, apoptotic response was found to be dependent on the expression level of caspase-8. In accordance with this finding we observed caspase-8 processing and activation after treatment with citrate. Downregulation of caspase-8 using siRNA technique significantly suppressed apoptosis. Apoptosis was also markedly suppressed in Jurkat cells lacking caspase-8 as compared to wild type cells. Surprisingly, citrate also caused processing and activation of caspase-2. In conclusion, the results that we present here show that citrate can induce apoptotic cell death not only through caspase-8 but also caspase-2 processing and activation. This can be explained by the ability of citrate to act as a kosmotropic factor stabilizing initiator caspases, facilitating their autocleavage and activation, which was described previously in vitro [1].

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Mediators of cell death in Neurospora crassa: the transcription factor NCU09974

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The protein kinase inhibitor staurosporine induces cell death in the filamentous fungus Neurospora crassa. A study of the transcriptional profile of N. crassa upon exposure to staurosporine revealed a differential expression of several genes, pointing to candidate molecules for intervening in the cell death process. NCU09141 and NCU09975 are the two mainly staurosporine-induced genes, as seen by microarrays analysis and validated by real-time PCR. Another highly induced gene is NCU09974 and a knockout mutant lacking this gene (ΔNCU09974) is very sensitive to staurosporine when compared to the wild type strain, strongly indicating that the encoded protein is a mediator of cell death in N. crassa. The protein sequence of NCU09974 includes a specific domain whose function is not well understood, though it is conserved in several fungal transcription factors. Corroborating this predicted function of NCU09974 as a transcriptional regulator, the induction of the expression of NCU09141 and NCU09975 upon staurosporine treatment is severely impaired in the ΔNCU09974 background. Our data suggest that this new transcription factor NCU09974 is crucial for N. crassa cells to set up a genetic response to staurosporine.

DNA damage Response in Female Germ Cells: c-Abl as a regulatory switch?

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Cells experiencing DNA damage undergo a complex response entailing cell cycle arrest, DNA repair and apoptosis, the relative importance of the three being modulated by the extent of the lesions. The observation that c-Abl interacts in the nucleus with several proteins involved in different aspects of DNA repair has lead to propose that this kinase is part of the damage-sensing mechanism. However, the mechanistic details underlying the role of c-Abl in DNA repair are largely unclear. Here, we focus on the relevance of such mechanisms in protecting DNA injured female germ cells. Intraperitoneal injection of cisplatin in newborn female mice leads to depletion of the follicle reserve and to long-term infertility. Recent findings have implicated c-Abl in a cisplatin-induced signaling pathway eliciting death of immature oocytes (1). A p53-related protein, TAp63, is an important immediate downstream effector of this pathway. Pharmacological inhibition of c-Abl by Imatinib (STI571) protects the ovarian reserve from the toxic effect of cisplatin. This suggests that the extent of c-Abl catalytic outputs may shift the balance between survival (likely through DNA repair) and activation of a death response (2). We investigate in vivo the effects of different dosages of Imatinib in tandem with cisplatin. Our studies will be further validated through the use of different types of the c-Abl inhibitors. Lastly, we are in the process of testing whether the protective effect of the c-Abl inhibitors can be extended towards other types of chemotherapeutic agents. In short, we have recently shown that co-treatment with Imatinib has a protecting effect on the ovarian reserve. We need to clarify the mechanisms underlying such an effect. A fine-tuning of nuclear outcomes, through pharmacological inhibition of c-Abl, will certainly provide the basis for the development of effective adjuvant (3) to protect oocytes from the damaging effects of cancer treatment.

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Alteration by nitric oxide of CD95, TNF-R1 and TRAIL-R1 cell death pathways in hepatoma cells

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Background: The regulation of intracellular nitric oxide (NO) production may be a relevant antitumor strategy in liver cancer cells. Objectives: The aim of the study was the identification of the alteration of different cell death pathways (CD95, TNF-R1 and TRAIL-R1) by NO in HeG2 cells. Methods: The intracellular NO concentration was increased either by treatment with NO donor (NONOate), or by stable transfection with pcDNA4/TO overexpression plasmid including nitric oxide synthase-3 (NOS-3) cDNA generating 4TO-NOS cell line. The activity of NOS-3, caspase-3, -8 and -9 were assessed by different procedures. The expression of p53, CD95, TNF-R1, TRAIL-R1, cFLIPS, cFLIPL and nitrated proteins was assessed by Western-blot analysis. CD95, TNF-R1 and TRAIL-R1 immunoprecipitation allowed the evaluation of cFLIP expression bound or unbound to cell death receptor. Results: NOS-3 overexpression increased p53, CD95, TNF-R1 and TRAIL-R1 expression, oxidative stress and reduced cell proliferation in HepG2 cells. These effects of NOS-3 overexpression were related to increased protein nitration, caspase-3 and -8 activities, and lactate dehydrogenase (LDH) release. The administration of Trail induced caspase-3 (605 %) and lactate dehydrogenase (LDH) release (183 %) more potently than anti-CD95 agonist (187 % and 156 %, respectively) and TNF-α; (163 % and 157 %, respectively) after 12 hours of stimulation in HepG2 cells. However, NOS-3 overexpression or the exogenous administration of NONOate altered cell death induced by Trail, TNF-α; and anti-CD95 agonist in HepG2 cells. In this sense, NOS-3 overexpression increased caspase-3 activity induced by TNF-α; (215 %) and CD95 (175 %), and reduced cell death by Trail (80 %). Interestingly, NOS-3 overexpression induced an alteration of cFLIPS and cFLIPL ratio bound to cell death receptors. Conclusions: The overexpression of NOS-3 increased oxidative and nitrosative stress which was associated with reduced cell proliferation and increased cell death in HepG2. However, the increase of cell death by NO enhanced TNF-α; and CD95 cell death pathways, but reduced Trail-dependent cell death pathway in HepG2. The differential effect of NO may be related to the alteration of cFLIP binding to cell death complexes.

Data-driven mathematical modelling of epigenetic de-regulations in acute myeloid leukaemias

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Epigenetic modifications of gene expression are intensively studied in abnormal events leading to oncogenesis. Aberrant patterns of DNA methylation and histone acetylation/methylation result in silencing or activation of specific genes in malignant blasts. It was suggested that such modifications are dynamically linked and that the disruption of an epigenetic mark will inevitably affect the others. Moreover, de-regulated patterns were shown to be restored by treatment by HDAC inhibitors (HDACi). Our work aims at better understanding the mechanisms by which epigenetic modifications influence gene expression and subsequent major cellular processes in acute myeloid leukaemias (AML). It also aims at defining the epigenetic-dependent mechanisms that are re-established after HDACi-based treatment, and are thereby potentially relevant to clinical response prediction. We developed a dynamical mathematical model based on the logical formalism, which recapitulates the main epigenetic mechanisms affecting the regulation of transcription, and accounts for their de-regulations and HDACi-based restoring in AML. This literature-based model focuses on the relationship between histone acetylation and DNA methylation and is currently being refined thanks to high-throughput data. Our approach consists in a comprehensive analysis of DNA-microarray, ChIP-seq and MethylCap-seq data using a clustering procedure to classify genes that are similar in terms of expression patterns and associated epigenetic modifications. A functional annotation study will assign each group of genes to specific cellular functions, and thus to affected processes. The information retrieved will allow improving and validating our model, which will hopefully be used to make predictions exploitable for diagnostic and clinical response tools.

Regulation of FasL-Induced Cell Death in Murine Neutrophils

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We are interested in the regulation of apoptosis by Bcl-2 family members, which regulate the mitochondrial (stress-induced) apoptotic pathway. A special focus is laid on the crosstalk between the death receptor-induced and the mitochondrial apoptotic pathway. For apoptosis induced by the death receptor Fas (CD95) such a crosstalk has been shown to be crucial in some (called type II cells) but not in other cell types (type I cells). The crosstalk is mediated by the BH3-only protein Bid, which is cleaved and activated by caspase-8, the apical caspase downstream of Fas. To verify whether a Fas ligand (FasL) sensitive cell type behaves like 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. By such approaches it has been shown that mouse hepatocytes are type II like cells, whereas lymphocytes undergo type I Fas-induced death. Here, we investigate whether murine neutrophils, which are highly sensitive to FasL-induced killing, are type I or type II cells. In a first approach we compared FasL-induced killing in primary mature neutrophils (Gr1+) isolated from wildtype and bid-/- mice. Our data suggests that FasL-induced death is delayed in bid-/- neutrophils, proving a type II like signalling pathway. 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, including Fas and TNF-R1, have been described to also induce necroptosis (programmed necrosis). A drawback of using primary murine neutrophils is the relatively low number of cells that can be isolated from a mouse, making biochemical analyses difficult. In a second approach, using conditional Hoxb8, we are differentiating Hoxb8-immortalized myeloid progenitor cells into mature neutrophils in vitro. Using this approach, progenitor cell lines can be generated from any desired genetically modified mouse strain and mature neutrophils can be obtained in near unlimited numbers. To investigate the pathophysiological role of Bid and type II Fas signalling in neutrophils, we are using the dextrane sulfate sodium (DSS) model of acute colitis in the mouse; an inflammatory model largely dependent on neutrophils and macrophages. Preliminary data indicate that bid-/- mice are more protected from DSS induced colitis compared to WT mice.

MiR-200c Regulates Noxa Expression and Sensitivity to Proteasomal Inhibitors

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The pro-apoptotic p53 target Noxa is a BH3-only protein that antagonizes the function of selected anti-apoptotic Bcl-2 family members. While the transcriptional regulation of Noxa has been studied extensively, little is known about its posttranscriptional regulation. We therefore set out to investigate, whether Noxa is regulated by microRNAs. Using a screen combining luciferase reporters, bioinformatic target prediction analysis and microRNA expression profiling, we identified miR-200c as a potential negative regulator of Noxa expression. Subsequently, miR-200c was shown to repress basal expression of Noxa, as well as Noxa expression induced by various stimuli, including proteasomal inhibition. Luciferase reporter experiments furthermore defined one miR-200c target site in the Noxa 3'UTR that is needed for this regulation. In spite of the miR-200c:Noxa interaction, miR-200c overexpression led to increased sensitivity to the clinically used proteasomal inhibitor bortezomib in several cell lines. This apparently contradictory finding was reconciled by the fact that upon siRNA-mediated knockdown of Noxa, miR-200c overexpression had an even more pronounced positive effect on apoptosis induced by proteasomal inhibition. Together, our data define miR-200c as a potentiator of bortezomib-induced cell death. At the same time, we show that miR-200c negatively regulates the pro-apoptotic Bcl-2 family member Noxa.

Knock-out of Bcl-2 interacting mediator of cell death (Bim) decreases apoptosis in intestinal epithelial cells and ameliorates acute DSS colitis

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BACKGROUND: In non-apoptotic cells Bcl-2 interacting mediator of cell death (Bim) is bound to the microtubule-associated dynein motor complex and therefore separated from anti-apoptotic B-cell lymphoma-2 (Bcl-2). Upon stress stimuli, translocation to the mitochondrial surface is initiated, action of Bcl-2 is neutralized and apoptosis is initiated via the release of cytochrome c. As increased apoptosis of intestinal epithelial cells (IEC) is considered to impair the function of the gut mucosa in intestinal bowel diseases, we investigated whether the knock-out of Bim might prolong the life span of IEC and therefore prevent mice from dextran sulfate sodium- (DSS-) induced colitis.

METHODS: Acute colitis was induced in female B6.129S1-Bcl2l11tm1.1Ast (Bim-/-) mice weighing 20-26g with 2% DSS as well as in male Bim-/- mice weighing 27-37g with 3.5% DSS for seven days. Animals were killed on day eight. Percental loss of body weight, spleen weight and colon length were recorded. Colonoscopy was performed and the inflammatory status was evaluated by murine endoscopic index of colitis severity (MEICS). Additionally, a histological score was determined. After ex vivo isolation of IEC, single cells were stained with propidium iodide and underwent flow cytometric cell cycle analysis to determine cell death in the sub-G1 fraction.

RESULTS: Upon DSS weight loss in general was higher in controls as compared to Bim-/- mice. Weight loss of male Bim-/- mice was significantly less as compared to wild type (wt) animals ($p<0.05$) from day six to eight. In female Bim-/- mice it was significantly less on day eight ($p<0.05$). Shortening of colon length during acute colitis was clearly less pronounced for both genders. MEICS scores were lower in DSS-receiving Bim-/- mice of both genders (female animals: $p<0.05$) and also DSS-induced histological changes were significantly lower in Bim-/- animals when compared to wt controls ($p<0.05$). Flow cytometric analysis of isolated IEC revealed that the fraction of apoptotic cells was significantly smaller in female ($p<0.01$) and male ($p=0.05$) Bim-/- mice as compared to wt controls.

CONCLUSION: Induction of acute colitis in Bim-/- mice results in less severe inflammation as compared to wt animals. The epithelial barrier is disturbed and damaged upon DSS which is, amongst others, due to an increased number of apoptotic IEC. As in Bim-/- mice apoptosis of IEC during colitis is reduced, we suggest an improved barrier function in those animals.

Transcriptional regulation of Atg5 expression

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Many cellular stress factors, such as, starvation, oxidative stress, or accumulation of protein aggregates, are capable to induce autophagy, a process, in which parts of the cytoplasm or organelles are sequestered by a double-membraned autophagosome and subsequently degradated by the lysosomal enzymes. Low level of autophagy can rescue the cells by reestablish homeostasis, whereas massive autophgy triggers type II cell death, the so-called autophagic cell death. As a key molecule of autophagy, Atg5 contributes on the double-membraned vesicle expansion and completion by forming the complex with Atg12 and Atg16. Furthermore, Atg5 can also be proteolytically activated to become a pro-apoptotic molecule that translocates to mitochondria and triggers apoptosis. Because of the multiple fuctions of Atg5, we are interested in how its expression is regulated. Based on the results of luciferase assay, we could find that numbers of transcription factors can regulate Atg5 expression including p53 family members. We are now investigating whether and how p53 family members regulate Atg5 expression.

Regulation of mitochondria-ER interface proteins in Alzheimer's disease models

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The metabolism of glucose, lipids and cholesterol and the regulation of calcium homeostasis are processes that are impaired in Alzheimer's disease (AD). These processes are all controlled in lipid raft-like specialized regions of the endoplasmatic reticulum (ER) called mitochondria-associated ER membranes (MAM). MAM are connected to mitochondria and the interaction between ER and mitochondria at these contact points are crucial for proper cell function. Here we have investigated the role of the MAM region in relation to neuronal degeneration and AD. Our results show that the MAM proteins PACS-2, sigma-1R and IP3R3 colocalize with the outer mitochondrial membrane protein VDAC1 in hippocampal neurons. Silencing siRNA against sigma-1R and PACS-2 resulted in neuronal degeneration demonstrating the important role of these MAM proteins for cell survival. In other cell models sigma-1R has been shown to prolong calcium signaling from ER to mitochondria by stabilizing IP3R3 receptors, while PACS-2 is controlling the ER–mitochondria contact and is also a cargo protein bringing Bid to mitochondria upon apoptotic stimuli. When hippocampal neurons were exposed to amyloid-beta peptide (Abeta) secreted from CHO cells over-expressing the APP Val717Phe mutation we detected a transient increase in sigma-1R and PACS-2 expression, suggesting a type of stress response. Furthermore, a change in sigma-1R and PACS-2 expression levels was also detected in cortex and hippocampus derived from mouse brain over-expressing APPswe/lon mutation. Experiments investigating the neuroprotective effect of sigma-1R agonists are underway. In summary we show that MAM proteins i) locate to distal parts of neurons ii) are important for neuronal survival iii) expression levels are changed in cellular and animal models of AD.

minocycline inhibits cell death and decreases mutant Huntingtin aggregation by targeting Apaf-1

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Minocycline (7-dimethylamino-6-dimethyl-6-deoxytetracycline) is a second-generation tetracycline that can cross the blood-brain barrier and has anti-inflammatory and neuroprotective effects. The potential of minocycline as a drug for treating Huntington's disease (HD) has been studied however, the molecular mechanism underlying the neuroprotective properties of minocycline remains elusive. In this study, we tested the hypothesis that a principal cellular target of minocycline is Apaf-1, a key protein in the formation of the apoptosome, a multiprotein complex involved in caspase activation. Minocycline binds to Apaf-1, as shown by nuclear magnetic resonance spectroscopy, and inhibits apoptosome activity in vitro and in ex vivo models. As a consequence minocycline-treated cells as well as Apaf-1 knock-out cells are resistant to the development of mutant huntingtin-dependent protein aggregation.

RACK1, a novel binding partner for c-FLIP

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c-FLIP is an inhibitor of apoptosis mediated by the death receptors Fas, DR4, and DR5 and is expressed both as long (c-FLIPL) and short (c-FLIPS) splice forms. Previous studies by this group have shown that c-FLIP is an important regulator of colorectal cancer cell death and that silencing of c-FLIPL can overcome resistance to chemotherapy in a variety of disease models. In order to learn more about the biology of c-FLIP a yeast two hybrid screen was carried out to identify novel binding partners of c-FLIPL.
Receptor for activated protein kinase C1 (RACK1) was identified as an interacting partner of c-FLIPL. RACK1 is a seven-WD-domain-containing protein with numerous downstream effectors regulating various cellular functions such as growth and migration. The RACK1-FLIPL interaction was verified in mammalian cells by co-immunoprecipitation. Co-immunoprecipitation studies also revealed an interaction between FLIPS and RACK1. In addition, the silencing of RACK1 by RNA interference enhanced apoptosis induced by the death ligand TRAIL. TRAIL treatment was also shown to attenuate the binding of FLIPL and RACK1. These results suggest that RACK1 may act as a key regulator of death receptor signalling. Peptide array studies further verified the RACK1-c-FLIPL interaction and localised the c-FLIPL binding interface to specific sites on the RACK1 propeller. Site-directed mutagenesis has been carried out on these residues and current studies are ongoing using the RACK1 mutants to determine the functional significance of this novel partnership.

Redox environment and cell death: Probing subcellular redox state in yeast S. cerevisiae cells

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Redox homeostasis by multiple dynamic equilibrium adjustment and regulation mechanisms is crucial for many cellular functions. Recent studies have shown that alterations in the redox environment are important regulators of the initiation and the progression of programmed cell death signaling. In order to estimate the intracellular redox environment, it is necessary to monitor the concentration of oxidized and reduced species from a main redox couple. However, informative measurements of cellular redox states remain technically challenging and have been a limitation for research in this vast field. Conventional approaches, such as using redox-sensitive fluorescent dyes or quantifying reduced and oxidized glutathione, either lack well-defined specificity or disrupt cellular as well as organelle integrity, making estimations prone to artifacts. Understanding the relevance and specificity of redox changes will require nondisruptive measurements of defined redox couples in defined subcellular locations. An alternative approach to overcome the limitations of conventional redox measurements includes redox Western blot methods to quantify redox changes in thiols of endogenous redox proteins such as peroxiredoxins/thioredoxins or in redox-sensitive fluorescent proteins (rxYFP and roGFP) targeted to different subcellular locations. We apply these new tools to monitor dynamic redox changes in vivo. Quantitative, dynamic and compartment-specific observations have been made in yeast cells in response to exogenous oxidant treatments and in yeast strains with defective redox regulatory systems. The detail results of this work will be presented. A better knowledge on redox environment will allow us to better understand the regulation of cell death signaling pathways.

GADD34 mediates cytoprotective autophagy in mutant huntingtin expressing cells via the mTOR pathway

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Increased protein aggregation and altered cell signaling accompany many neurodegenerative diseases including Huntington's disease (HD). Cell stress is counterbalanced by signals mediating cell repair but the identity of these are not fully understood. We show here that the mTOR pathway is inhibited and cytoprotective autophagy is activated in neuronal PC6.3 cells at early time points after expression of mutant huntingtin proteins. The TSC1 protein acting upstream of mTOR was increased in mutant huntingtin expressing cells and interacted with the GADD34 stimulating autophagy. However, GADD34 and autophagy decreased at later time points with the concomitant increase in mTOR activity. Overexpression of GADD34 counteracted these effects and increased cytoprotective autophagy and cell survival. These results show that GADD34 plays an important role in cell protection in mutant huntingtin expressing cells. Modulation of GADD34 and the TSC pathway may prove useful in counteracting cell degeneration accompanying HD and other neurodegenerative diseases.

Genetic Inhibition of Caspase-2 reduces Hypoxic-Ischemic and Excitotoxic Neonatal Brain Injury

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Objective: Perinatal brain injury is a major cause of neurodevelopmental handicaps. Multiple pathways of oxidant stress, inflammation, and excitotoxicity lead to cell damage and death, including caspase-dependent apoptosis. Caspase-2 (Casp2; Nedd-2, Ich-1) is a developmentally regulated initiator caspase, which poorly cleaves other caspases but can initiate mitochondrial outer membrane permeabilization. We have investigated if Casp2 could mediate perinatal ischemic brain damage.
Methods: Casp2 expression in human neonatal brains and developmental patterns in rats and mice were evaluated. Casp2-deficient (Casp2-/-), wild-type (wt), and heterozygous (Casp2+/-) newborn C57BL/6 mice were subjected to hypoxia-ischemia (unilateral carotid occlusion + exposure to 10% oxygen for 50 min) or intracerebral injection of the excitotoxic NMDA-receptor agonist ibotenate. In addition, Casp2 specific siRNAs were pre-injected into the brain of wt newborn mice 24h before ibotenate treatment. Brain tissues were examined by immunohistochemical staining (Cresyl-violet, MAP2, NF68, Caspase-2, -3) and Western blotting. Lesion volumes and injury in the cortical plates and white matter were quantified together with activated caspase-3.
Results: Casp2 is highly expressed in the neonatal brain. Casp2-deficient mice subjected to hypoxia-ischemia at postnatal day 9 present significantly lower cerebral infarction, reduced white matter injury, and reduced caspase-3 activation in the thalamus and hippocampus. Both Casp2-/- mice and siRNA-administered wt mice conferred reduction of grey and white matter injury after excitotoxic insult at postnatal day 5. Caspase-3 activation was also found reduced in Casp2-deficient mice subjected to excitotoxicity.
Interpretation: These data suggest for the first time a role of caspase-2 in neonatal brain damage.

Overexpression of Cathepsin D senzitizes breast cancer cells to TRAIL-induced apoptosis

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The classical caspase-dependent apoptosis pathway is often impaired in tumor cells. Targeting lysosomes may therefore represent an effective alternative therapeutic strategy as cancer progression is often associated with dramatic lysosomal changes that sensitize cancer cells to lysosomal membrane permeabilization. Moreover, many human tumors exhibit increased levels of lysosomal proteases as well. The lysosomal aspartyl protease cathepsin D (CTSD) acts as mediator of apoptosis induced by various chemotherapeutics. CTSD-induced pro-apoptotic effects may depend on its enzymatic activity but the role of catallyticaly inactive CTSD in apoptosis induction was also reported. TRAIL (TNF-related apoptosis-inducing ligand) represents an anti-cancer therapeutic agent that can induce both classical and lysosomal apoptotic signaling. In order to clarify the role of CTSD and its enzymatic activity in the TRAIL-induced apoptotic signaling, MDA-MB-231 breast carcinoma cells were transfected with cDNA coding for CTSDwt and its enzymatically inactive counterpart (CTSDmut). The cells overexpressing CTSD wt and mut were exposed to TRAIL and frequency of apoptosis was analysed by chromatin condensation, nuclear fragmentation, cleavage of PARP, and externalization of phosphatidylserine. An increased frequency of apoptosis in cells over-expressing wt but not mut CTSD was clearly demonstrated. We also found that increased frequency of apoptosis observed in wt CTSD cells corresponded to cleavage of the pro-apoptotic Bcl-2 family member, the Bid protein, that was earlier identified as one of the targets of CTSD in cancer cells. We conclude that CTSD sensitizes MDA-MB-231 cells to the TRAIL-induced apoptosis in enzymatic activity-dependent manner and that the TRAIL-induced apoptosis is potentiated by the CTSD-mediated activation of Bid, the pro-apoptotic member of the Bcl-2 family.

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c-Myc is indispensable for colon cancer cell apoptosis induced by combination of platinum drugs and TRAIL

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TRAIL (tumor necrosis factor-related apoptosis inducing ligand), an interesting cytokine belonging to the TNF family, is a potent and promising agent that triggers apoptosis in many cancer but not normal cells. However, in a number of tumors, the successful application of TRAIL is hampered due to their inherited or acquired resistance. In such cases, combined treatments seem to be the most efficient way to overcome it. We and others reported previously that platinum-based chemotherapeutic drugs are very effective in potentiation of TRAIL-induced apoptosis in human cancer cells. Importantly, we showed that the novel platinum(IV) adamantylamine ligand-containing complex (LA-12) exerts potent TRAIL-sensitising effects in colon cancer cells even in twenty-fold lower dose compared to the conventionally used cisplatin. Molecular mechanisms involved in modulation of cytotoxic effects of the drug combinations were further investigated. The c-Myc is a well known regulator of oncogenesis and an important factor modulating the cancer cell sensitivity to the cytotoxic effects of various chemotherapeutic drugs and some death ligands including TRAIL. We observed that colon cancer cells treatment with LA-12 led to an increase in c-Myc protein levels. We therefore investigated its role in regulation of apoptosis induced by combined treatments of LA-12 and TRAIL in our model cell lines. We showed that downregulation of c-Myc expression via siRNA abrogated the potent killing effects exerted by combination of LA-12 and TRAIL. We further studied the possible molecular mechanisms involved, and analyzed activation of caspases, assembly of death-inducing signaling complex, and mitochondria-related pro-apoptotic events. Our results highlighted the importance of c-Myc in regulation of colon cancer cell sensitivity to LA-12 and TRAIL, and pointed to some important intracellular targets for possible therapeutic interventions.

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Preconditioning inhibits myocardial apoptosis through enhanced myocardial substrate uptake: Role of inslulin-stimulated Akt and AMPK activation

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Introduction: We previously found that IPC cardioprotection was markedly attenuated in diabetic rats. The present study was designed to examine the mechanism of IPC-afforded cardioprotection involved in insulin-regulated myocardial substrate metabolism. Methods: Adult male rats were subjected to 30 min of myocardial ischemia and 3 h of reperfusion (MI/R). IPC was achieved by two cycles of 5 min ischemia and 5 min reperfusion. Myocardial glucose and fatty acid (FA) uptake were assessed at the end of 1 h reperfusion by determining 18F-2-deoxy-2-fluoro-D-glucose uptake and fatty acid translocase (FAT)/CD36 translocation, respectively. Results: IPC significantly improved cardiac function with reduced apoptotic cell death, myocardial infarction and blood creatine kinase/lactate dehydrogenase levels following MI/R (all $P<0.05$). Myocardial glucose uptake was markedly elevated after IPC treatment (17.0±1.5 vs. 12.4±1.0 in MI/R, n=10-12, $P<0.05$), as well as translocation of glucose transporter 4 (GLUT4) to plasma membrane (PM) ($P<0.01$). Interestingly, IPC also increased CD36 translocation to PM, a rate-limiting step in long-chain fatty acid uptake. Meanwhile, myocardial PI3K expression, Akt and AMPK phosphorylation were significantly enhanced in IPC group ($P<0.05$). Wortmannin not only abrogated the antiapoptotic effect of IPC, but also inhibited IPC-induced Akt/AMPK phosphorylation and subsequent GLUT4/CD36 translocation. Furthermore, the antiapoptotic effect of IPC was markedly blunted in STZ-induced insulin-deficient diabetic hearts with failure of increase in glucose/FA uptake and impaired IPC-stimulated PI3K-Akt and AMPK signaling activation (n=6, $P<0.05$). Conclusions: These results suggest that IPC increases both glucose and FA uptake during early reperfusion to reduce myocardial apoptotic death via insulin/PI3K-dependent Akt and AMPK activation. Therefore, augmenting insulin signaling may be a potential therapy to improve myocardial substrate uptake and restore the cardioprotection of IPC in diabetic hearts.

Hepatitis B Virus infection triggers the activation of p53 downstream target genes in primary human hepatocytes

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p53, a short-lived transcription factor, is stabilized and activated by post-translational phosphorylation in response to cellular stress, e.g. virus infection. The p53 pathway is affected at multiple levels in hepatocellular carcinoma (HCC), the most common primary malignant liver cancer. One major risk factor for the development of 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 signalling pathway are essential for viral clearance. The present study was designed to investigate the molecular mechanism leading to the apoptosis of HBV-infected primary human hepatocytes. Adenoviral transfer of recombinant HBV constructs (rAd HBV X-, rAd HBV L- and rAd HBVwt) in primary human hepatocytes (PHH) resulted in the accumulation and activation of p53. This activation resulted in the transactivation of the known p53-target gene CD95, as well as the expression of the BH3-only protein Puma and the pro-apoptotic Bcl-2 protein family member Bax. The cleavage of the BH3-only protein Bid and the subsequent appearance of tBid represent the link between the extrinsic and intrinsic apoptosis signalling pathway in HBV-infected PHHs. Induction of apoptosis by HBVwt-infection was accompanied by the cleavage of caspase 3 and poly-(ADP-ribose) polymerase (PARP-1). This led to the appearance of a 19 kDa, respectively a 89 kDa protein fragment, which was not observed in the knock-out constructs. We characterized the apoptosis signaling pathways involved in the elimination of HBV-infected PHHs. Our data suggest that not only the extrinsic but also the intrinsic apoptosis pathway plays a role in the elimination of HBV-infected PHHs. We could identify not only CD95 but also the BH3-only proteins Puma and Noxa as well as the Bcl-2 protein Bax as crucial proteins activated upon HBV-infection, thereby contributing to viral clearance.

Concanavalin A-induced hepatitis is a relevant model for TRAIL (TNF-Related Apoptosis Inducing Ligand)-induced necroptosis in vivo

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The mouse model of Concanavalin A (Con A)-mediated liver injury results in fulminant hepatitis, and is currently considered as an experimental model for human autoimmune or viral hepatitis. Several lines of evidence suggest that Natural Killer T (NKT) cells are critically involved and that TRAIL is directly responsible for mediating hepatic cell death, as TRAIL-deficient mice or mice treated with a blocking TRAIL receptor are resistant to Con A-induced hepatitis. Examination of liver tissues stained with Hematoxylin/Eosin showed that an injection of 20 mg/kg Con A led to a clear liver damage (large diffused perivascular and parenchymal zone of necrosis) observed at 6h or 10h in WT C57Bl/6 mice. These damages were not observed in CD1d KO C57Bl/6 mice, confirming a role for NKT cells in Con A-induced hepatitis. These histological findings were correlated with high levels of aspartate aminotransferase and alanine aminotransferase in serum of WT mice treated with Con A and low levels in serum of CD1d KO mice treated as above. Besides, we reported an increase in TRAIL mRNA level in the liver of Con A- treated WT mice but not in CD1d KO mice, suggesting that Con A treatment might increase TRAIL mRNA level in some immune cells such as NKT cells. Moreover, we also observed an increase in TRAIL-R2 mRNA level, in the liver of Con A- treated WT mice, which could be related to hepatocyte cell death mediated by TRAIL-expressing NKT cells. Then, we showed that Con A injection activated PARP-1 in the liver of WT mice but not of CD1d KO mice. Moreover, high PARP-1 activity was correlated with liver damage characterized by high AST/ALT serum levels in WT mice treated with Con A. These data suggest that PARP-1 activity may serve as a biological marker of liver damage in Con A-induced hepatitis. Finally, a pre-treatment of mice with 125 µg Nec-1, an inhibitor of RIPK1 kinase activity, significantly protected mice from Con A-induced hepatitis by inhibiting both Con A-induced increase in AST/ALT serum levels and Con A-induced PARP-1 activation in the liver, suggesting a role for RIPK1 upstream PARP-1 in necrosis of hepatocytes. All these data suggest that Con A-induced hepatitis may be a relevant in vivo model of TRAIL-induced necroptosis and that RIPK1 and PARP-1 play a major role in this hepatic cell death pathway. As necrosis is mostly associated with pathological conditions including myocardial infarction, cerebral ischemia, acute organ failure and infection, this in vivo model may allow us finding new inhibitors of necroptosis. These drugs would be particularly useful in the treatment of such type of diseases where necrotic pathway is involved.

Inhibition of autophagy delays lung cancer cell growth and leads to sensitization of tumor cells to ROS-dependent apoptosis

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The high levels of basal autophagy in some parts of tumors or its large-scale activation in tumor cells upon chemo- and radiotherapy makes this area of research attractive to take attempt to modulate autophagy in order to increase the sensitivity of cancer cells to treatment. We found that inhibition of autophagy sensitized non-small cell lung carcinoma (NSCLC) cells to cisplatin-induced apoptosis which was partially dependent on caspase activation. Autophagy inhibition stimulated ROS formation and treatment with cisplatin had a synergistic effect on accumulation of ROS. Antioxidant NAC or scavengers of highly reactive hydroxyl radicals, but not scavengers of superoxide or mimetic of MnSOD, reduced the release of cytochrome c and abolished the sensitization of the cells to cisplatin-induced apoptosis by suppressed autophagy. Such inhibition of ROS prevented the processing and release of apoptosis-inducing factor (AIF) from mitochondria. Moreover, scavengers of hydroxyl radicals were able to inhibit processing of pro-cathepsin D and inhibition of cathepsin D activity reduced the sensitization of cells to cisplatin. Furthermore, siRNA-mediated suppression of autophagy inhibited proliferation of NSCLC cells without significant effect on the cell-cycle distribution. Such inhibition of cell proliferation reduced accumulation of cells in the S phase upon treatment with etoposide, suggesting attenuation of the execution stage of the etoposide-induced apoptosis. These findings revealed that autophagy suppression leads to inhibition of NSCLC cell growth and sensitizes them to ROS-mediated apoptosis.

Investigating the Molecular Mechanisms of the Bcl-2 Family Member Bok

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The multi BH-domain Bcl-2 family member Bok (also called Mtd) is able to induce apoptosis when overexpressed in mammalian cells. Although Bok is often referred to as a Bax/Bak homologue, we found several indications pointing towards non-redundant roles for the function of Bok: i) Bax/Bak double-deficient cells are usually fully protected from classical intrinsic apoptosis, even when they express endogenous Bok. ii) On the same line, Bok-induced apoptosis is largely Bax/Bak dependent, as enforced Bok expression fails to resensitise Bax/Bak double-deficient cells towards intrinsic apoptotic stimuli. iii) Unlike Bak or activated Bax, Bok preferentially associates with the ER/nuclear outer membranes, Golgi membranes and vesicle-like structure not colocalising with mitochondrial markers. iv) A C-terminal deletion mutant of Bok still potently induces cell death. v) In certain cell types, Bok-induced cell death cannot be blocked by the pan-caspase inhibitor Q-VD-oph.We are using several cellular systems derived from our recently generated Bok-deficient mouse strain to investigate the molecular functions of this still rather mysterious Bcl-2 family member. Based on the subcellular localisation of Bok, a focus lies on its putative role in regulating ER-stress induced cell death. We hypothesise that Bok is either an ER/secretory system-resident Bax-like protein or that Bok functionally acts more like a BH3-only protein. In the mouse, we find Bok widely expressed throughout most tissues, with high levels in the uterus, ovaries, brain, pancreas, spleen and liver. Interestingly, Beroukhim and colleagues very recently reported that, unlike BAX or BAK, BOK is deleted in human cancers with high frequency (Nature 2010, Vol 463, p889ff). In line with this report, we find very significant reduction (up to total loss) in Bok levels in several human cancer lines. We are currently investigating the nature and the relevance of this downregulation.

p57KIP2 control of actin cytoskeleton dynamics is responsible for its mitochondrial pro-apoptotic effect

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p57 (Kip2, CDKN1C), often found down-regulated in cancer, is reported to hold tumor suppressor properties. Originally described as a cyclin dependent kinase inhibitor, p57KIP2 has since been shown to influence other cellular processes, beyond cell cycle regulation, including cell death and cell migration. Inhibition of cell migration by p57KIP2 is attributed to the stabilization of the actin cytoskeleton through activation of LIM Kinase-1 (LIMK-1). Furthermore, p57KIP2 is able to enhance mitochondrial-mediated apoptosis. Here we report that the cell death promoting effect of p57KIP2 is linked to its effect on the actin cytoskeleton. Indeed, whereas Jasplakinolide, an actin cytoskeleton stabilizing agent, mimicked p57KIP2's pro-apoptotic effect, destabilizing the actin cytoskeleton with cytochalsin D reversed p57KIP2's pro-apoptotic function. Conversely, LIMK-1, the enzyme mediating p57KIP2's effect on the actin cytoskeleton, was required for p57KIP2's death promoting effect. Finally, p57KIP2 mediated stabilization of the actin cytoskeleton was associated with the displacement of hexokinase-1, an inhibitor of the mitochondrial voltage-dependent anion channel, from the mitochondria, providing a possible mechanism for the promotion of the mitochondrial apoptotic cell death pathway. Altogether, our findings reveal that two tumor suppressor properties of p57KIP2, namely its effect on the actin cytoskeleton and on mitochondrial mediated apoptosis, are linked.

Sorafenib alone or in combination with ABT737 shows potent preclinical activity against human Multiple Myeloma

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Multiple Myeloma (MM), an incurable B cell malignancy relies on multiple signaling cascades, including tyrosine kinase activated pathways, to proliferate and evade cell death. Thus, targeting of these signaling cascades by using the multi-tyrosine kinase inhibitor (TKI), Sorafenib, represents a promising approach against MM. In this study we demonstrate that the Raf/MEK/ERK1/2, the PI3K/AKT and the Jak/STAT3 pathways are the downstream targets of Sorafenib. Furthermore, we delineate the molecular mechanisms of Sorafenib-induced cell death in MM cell lines and in CD138+ enriched primary MM patient samples. MM cells undergo autophagy in response to Sorafenib and inhibition of this cytoprotective pathway potentiates the efficacy of this TKI. Mcl-1, a resistance factor in MM, is potently down regulated by Sorafenib, a step required for the execution of cell death since ectopic overexpression of this protein protects MM cells. Concomitant targeting of Mcl-1 by Sorafenib and of Bcl-2/Bcl-xL by ABT737 potentiates the efficacy of Sorafenib in MM cell lines and CD138+ enriched primary cells (newly diagnosed or in relapse), mono- or co-cultured with bone marrow stromal cells. Altogether, our data support the usage of Sorafenib alone or in combination with ABT737 as a therapeutic strategy against MM.

Ubiquitin specific protease 14 (Usp14) in oxidative stress in rat hippocampal neurons

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Ubiquitin proteasomal system is the major proteolytic system in the cell. Proteins targeted for proteasomal degradation are labeled with multiple ubiquitin moieties via a multi-enzyme cascade. Many ubiquitin-dependent events are also regulated by deubiquitination, releasing ubiquitin chains, which involves deubiquitinating enzymes (DUBs). Ubiquitin specific protease 14 (Usp14) is one of the best-characterized DUB. It is mutated in ataxia mice that show reduced free ubiquitin levels and suffer from tremor, ataxia, muscle wasting and paralysis ultimately leading to too early death. Recent data suggest that USP14 inhibition may accelerate degradation of oxidized proteins. Knowing that oxidative stress is involved in many human diseases, we wanted to study closer the role of Usp14 in neuronal cell death caused by oxidative stress. Oxidative stress was induced in cultured rat hippocampal neurons with xanthine and xanthine oxidase. Usp14 protein and mRNA expression were studied using quantitative PCR and western blotting followed by functional studies.

Andes hantavirus inhibits apoptosis and its nucleocapsid protein inhibit human caspase 3 and granzyme b

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Andes hantavirus (ANDV) causes a severe zoonotic disease in the Americas, namely hantavirus cardiopulmonary syndrome, with up to 40% mortality. Hantaviruses do not cause any CPE in vitro, thus we speculated that they might be able to inhibit apoptosis. Here, we treated ANDV-infected and non-infected A549 cells with the apoptosis-inducer staurosporine (STS). Levels of lactate dehydrogenase (a marker of plasma membrane integrity) were lower in supernatant from infected-cells compared to non-infected cells. Importantly, infected cells displayed lower caspase 3-activity, less cleaved poly-ADP ribose polymerase (a hallmark for caspase 3-mediated apoptosis), and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) confirmed that infected cells were less prone to get apoptotic after STS-treatment. These results show that ANDV can inhibit STS-induced apoptosis, demonstrating this effect for the first time for hantavirus. Furthermore, we observed that the ANDV nucleocapsid protein (NP) was cleaved late in STS-treated cells. A similar cleavage pattern as observed in STS-treated cells was seen for NP after co-incubation of lysed cell-free ANDV particles with recombinant human caspase 3, showing that NP is a target for caspase 3. Cytotoxic immune cells are important for killing virus-infected cell via granzyme B (GzmB)-induced apoptosis. Interestingly, inhibition assays showed that NP efficiently inhibited both caspase 3 and GzmB enzymatic activity, showing for the first time that a virus protien can inhibit both these enzymes. In conclusion, we report that hantavirus inhibit signalling pathways used for activation of extrinsically induced apoptosis. We are currently investigating if also other apoptosis-inducing signaling pathways are affected by hantavirus.

Inhibition of Hsp90 induces apoptosis in multiple myeloma cell lines through downregulation of STAT3 activity

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Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that positively regulates cellular processes responsible for cancer development and progression. It is constitutively activated in many cancer types by the upstream tyrosine kinases. Being a convergence point of multiple signalling pathways, STAT3 represents an attractive target for cancer drug development. Recently, it has been shown that IL-6-dependent STAT3 activation depends on a molecular chaperone Hsp90 (heat shock protein 90). Inhibitors of Hsp90 are being currently used in clinical trials for treatment of an incurable malignancy of B cells, multiple myeloma, MM. In this study, we demonstrated that STAT3 form complex with Hsp90 in U266 MM cell line using proximity ligation assay and immunoprecipitation. We also found that it is preferentially a tyrosine phosphorylated form of STAT3 which forms a complex with Hsp90. Hsp90 inhibition by 17-DMAG lead to a prominent ablation of STAT3 tyrosine phosphorylation, which correlated with the activation of caspases 3 and 7 and induction of apoptosis in three MM cell lines with an activated STAT3 pathway. A constitutively active form of STAT3 (STAT3C) protected U266 cells from 17DMAG-induced cell death. These results suggested that interaction of STAT3 with Hsp90 is required for maintaining its activity and that apoptotic cell death induced by Hsp90 inhibitors in a subset of multiple myeloma cell lines occurs through inhibition of STAT3 phosphorylation. These data provide insight into the role played by Hsp90 in maintaining STAT3 activity and suggest the mechanism of action of Hsp90 inhibitors in the induction of MM cell death.

Light Microscopic Examination Of Apoptotic Cells in Cervico-Vaginal Smears: Using Tunel Assay After Decolorization

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Background/Objective: Although TUNEL method is well-known method for detecting cells undergoing apoptosis in histological specimens and cell suspansions, there is no study concerned with the application of TUNEL assay in cervico-vaginal smears. The aim of the study is to detect apoptotic cells in cervico-vaginal smears by using TUNEL method after destaining of slides stained with Papanicolaou tecnique.
Material Methods: Cervico-vaginal smears taken from 200 non-pregnant women were stained with Papanicolaou and examined by light microscopy to identify apoptotical changes in epithelial cells, neutrophil leucocytes and macrophages. After decolorization of 200 slides, TUNEL assay was applied for immunocytochemical detection. According to whether they have positive reaction with TUNEL staining, cells have dark brown nuclei were evaluated as “apoptotic cells”.
Results: According to light microscopic observation of Papanicolaou stained smears, 24 of 200 (12%) smears have apoptotic epithelial cells. After applying to TUNEL assay to destained slides, a hundred epithelial cells and neutrophils were counted in each smear. Fifty-six of (28%) 200 smears have apoptotic epithelial cells. Thirty-two of 56 (57.1%) smears have apoptotic parabasal cells and superficial epithelial cells were seen in 12 of 56 (21%) smears. Sixty-one of 200 (30.5%) smears have apoptotic neutrophil leucocytes. According to TUNEL results, we also observed macrophages containing apoptotic bodies in 25 of (12.5%) 200 smears.
Conclusion: TUNEL assay is a practicable method for detection of apoptotic epithelial cells and neutrophils on cervico-vaginal smears, although applying of TUNEL method on smears has some limitations because of decolorization. It is also concluded that using of different techniques together would be more effective for determination of apoptotic cells in cervico-vaginal smears.

Pim kinases and their novel inhibitors in cancer cell survival and metastatic growth

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The three Pim family proteins are small constitutively active serine/threonine kinases expressed mainly in tissues and cells of hematopoietic origin, but also in the central nervous system and in epithelia. We and others have detected elevated levels of Pim expression in human lymphoid and myeloid malignancies as well as in prostate cancer and radioresistant squamous cell carcinomas (Peltola et al., Neoplasia 2009). Therefore, drugs inhibiting Pim kinase activity may have therapeutic value against several types of human cancer. Since there are no inhibitors against Pim kinases commercially available, we have been searching for small molecule compounds targeting either individual or all Pim family kinases. Two types of Pim-selective inhibitors have been identified and validated both in vitro and in cell-based assays, where we have used cytokine-dependent myeloid cell lines. Since we have shown that overexpression of Pim-1 prolongs survival of these cells after cytokine withdrawal (Aho et al., FEBS Letters 2004), specific inhibitors were expected to abrogate the protective effects of Pim kinases. Indeed, such compounds have now been found that are able to inhibit phosphorylation of known Pim substrates such as Bad and abrogate the anti-apoptotic effects of Pim kinases. In addition, these Pim inhibitors have revealed a a previously unrecognized role for Pim kinases in regulation of cell motility (Santio et al., Molecular Cancer 2010), suggesting that Pim kinases may regulate cancer cell spreading, migration and metastases. Thus, the novel Pim-selective inhibitors can be used not only as efficient research tools, but provide attractive new molecules for cancer drug development to inhibit both survival and motility of cancer cells.

Expression of MICA/B under ethanol-induced cell stress is independent of apoptosis provoked by ethanol

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MICA and MICB (MHC class I-related chain A and B) cell proteins are stress-induced ligands for activating receptor NKG2D expressed by NK cells and CD8-positive T cells. Surface MICA/B expression in stressed, infected or tumor cells serves as “danger signal” for the cytotoxic lymphocytes indicating the cells that should be eliminated. Mechanisms of regulation of MICA expression are still not clear. It is known that ethanol can affect immune response, induce oxidative stress and trigger apoptosis in lymphoid and myeloid cells. We investigated surface expression of MICA/B in human hemopoietic cell lines K562 and THP1 in a model of ethanol-induced cell stress. Surface MIC expression was analyzed by flow cytometry and confocal microscopy. Expression of mica/b genes was registered by RT-PCR. Percentage of apoptotic and necrotic cells was measured by cell staining with annexin V-FITC and propidium iodide using flow cytometry. Spontaneous surface MICA expression determined in both cell lines was higher in THP1 cells comparing with K562 cells. Incubation of cells with ethanol at 80-400 mM concentrations for 24 h resulted in increase of MICA expression with a maximum at 200-300 mM. Further increase of ethanol concentration led to decrease of MICA surface expression and elevation of both apoptotic and necrotic cell death. Shortening of ethanol treatment to 1 h resulted in decrease of apoptotic cell proportion while MIC expression was unaffected. Increase of cell surface MICA/B expression provoked by ethanol may contribute to ethanol-induced immune suppression.

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α;-Tocopheryl succinate kills neuroblastoma cells irrespectively of MycN oncogene expression.

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Amplification of the MycN gene, which is considered as an adverse prognostic factor, is found in a large proportion of neuroblastomas, the most common extracranial solid tumor of childhood. The data on the role and significance of MycN amplification for cell survival are controversial. Amplification of the MycN oncogene characterizes the subset of most aggressive NBs. On the other hand, MycN presumably cooperates with cytotoxic drugs to induce p53 and Bax protein expression. Downregulation of MycN makes cells more resistant towards some DNA damaging drugs such as cisplatin or doxorubicine. Switching off MycN prominently suppressed apoptosis assessed by release of cytochrome c, stimulation of caspase-3 like activity, and the number of floating cells. Dependence of cisplatin-induced apoptosis on MycN expression was confirmed by the analysis of phosphatidyl serine (PS) externalization using staining with Annexin V and PI. In MycN- cells the number of cells exposing PS on the outer surface of plasma membrane noticeably decreased (29.7 vs 8.3%). In addition, downregulation of MycN markedly decreased the number of cisplatin-treated cells with apoptotic morphology. Incubation of MycN+ and MycN- cells with cisplatin stimulated expression of p53, which is capable of launching an apoptotic program that includes direct transcriptional activation of death-inducing genes, such as Noxa, Puma and Bax. Although cisplatin stimulated p53 expression both in MycN+ and MycN- cells, in MycN+ cells the level of p53 was distinctly lower. Further, the expression of the proapoptotic Bcl-2 family protein Bax significantly attenuated in cisplatin treated MycN- cells, which can explain suppression of cytochrome c release, cleavage of caspase 3 and PARP. In contrast to cisplatin and doxorubicin, there was no difference in apoptosis manifestation between MycN+ and MycN- cells upon treatment with α-tocopheryl succinate (α-TOS), a redox silent analog of vitamine E, which was shown to stimulate cell death via interaction with mitochondria. Although downregulation of MycN suppressed α-TOS-induced expression of pro-apoptotic protein Noxa, this event did not attenuate cell death and apparently had a minor contribution to α-TOS-induced apoptosis. Alpha-TOS stimulates mitochondrially-mediated apoptosis via induction of mitochondrial permeability transition (MPT). It causes a rapid entry of Ca2+ into both MycN+ and MycN- cell with subsequent accumulation of these ions in mitochondria – a prerequisite step for MPT induction. Comparison of the Ca2+-buffering ability of mitochondria revealed that MycN+ and MycN- cells are equally susceptible to MPT induction, which is responsible for the release of cytochrome c, subsequent activation of the caspase cascade and cell death. Chelation of intracellular Ca2+ by BAPTA attenuated cell death induced by α-TOS. Thus, steps directed to mitochondrial weakening and facilitating stimulation of permeability transition might overcome protective effect caused by MycN downregulation. Direct mitochondrial targeting seems to be an efficient tool in eliminating cancer cells with suppressed apoptotic pathways.

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

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Anthracycline antibiotics are inducers of an immunogenic form of apoptosis that has immunostimulatory properties because of the release of damage-associated molecular patterns. To study the mechanisms used by the innate immune system to sense this 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 rapid influx of neutrophils and increased levels of IL-6 and monocyte chemotactic protein-1. We demonstrate that acute inflammation induced by doxorubicin is associated with apoptosis of monocytes/macrophages and that it is specific for doxorubicin, an immunogenic chemotherapeutic. Further, the inflammatory response is significantly reduced in mice deficient in myeloid differentiation primary response gene 88 (MyD88), TLR-2 or TLR-9. Importantly, a TLR-9 antagonist reduces the recruitment of neutrophils induced by doxorubicin. By contrast, the acute inflammatory response is not affected in TRIF(Lps2) mutant mice and in TLR-3, TLR-4 and caspase-1 knockout mice, which shows that the inflammasome does not have a major role in doxorubicin-induced acute inflammation. Moreover, we have shown that in this model deficiency in TLR-2 or TLR-9 results in better protection against doxorubicin-induced intestinal damage. Our findings provide important new insights into how the innate immune system senses immunogenic apoptotic cells and clearly demonstrate that the TLR-2/TLR-9-MyD88 signaling pathways have a central role in initiating the acute inflammatory response to this immunogenic form of apoptosis.

Cell sensitivity to oxidative stress-induced apoptosis/necrosis is influenced by autophagy of iron-binding proteins

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Oxidative stress is often limited to the lysosomal compartment and mediated by iron-catalyzed Fenton-type reactions. Therefore, cellular iron is mainly stored safely in ferritin. Only during the continuously ongoing degradation of iron-containing proteins and organelles inside lysosomes, iron is liberated and temporarily redox-active before it is exported from the lysosomes and used in protein synthesis or being stored. Consequently, the lysosomal compartment is probably the only place where free redox-active iron is found. This makes the lysosomal membrane, that separates the lysosomal acidic milieu with many powerful hydrolytic enzymes from the rest of the cell, especially vulnerable to oxidative stress and subsequent rupture with resulting apoptotic or necrotic cell death. We demonstrated that oxidative stress-induced lysosomal membrane permeabilization can be prevented by small hydrophilic or lipophilic iron chelators, such as deferiprone and SIH. Furthermore, by allowing cells to endocytose an iron-phosphate complex or iron-rich ferritin, lysosomes could be made more sensitive to oxidative stress. On the other hand, endocytosis/autophagy of apo-ferritin, metallothionein or Hsp70 increased cellular resistance to oxidative stress. This suggests that intralysosomal iron binding may be a general mechanism by which stress-induced phase II proteins, to which the above proteins belong, protect cells against oxidative stress.

Role for endoplasmic reticulum stress in pancreatic beta-cell apoptosis in mitochondrial diabetes of Friedreich's ataxia

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Background and aims : Friedreich's ataxia (FA) is an autosomal recessive neurodegenerative disease caused by a GAA trinucleotide repeat expansion in the first intron of the frataxin (Fx) gene leading to a 60-90% reduction in the mitochondrial protein Fx. FA patients have a high prevalence of diabetes, which is caused by increased abdominal body fat accumulation and pancreatic beta-cell failure. Our aims were to study the pathogenic mechanisms involved in FA diabetes.
Materials and methods : Fx was knocked down by siRNA (siFx) in clonal INS-1E cells, primary rat beta-cells and dispersed human islets. Beta-cell apoptosis was examined by Hoechst 33342/propidium iodide staining and western blots for cleaved caspase-3, 24h after exposure to oleate (0.5 mmol/l, OL), palmitate (0.5 mmol/l, PAL) or the endoplasmic reticulum (ER) stressors cyclopiazonic acid (25 µmol/l, CPA), tunicamycin (5 µg/ml, TU) or brefeldin A (0.1 µg/ml, BR), alone or in combination with the adenylate cyclase stimulator forskolin (20 µmol/l, FK).
Results : Fx knockdown resulted in a 40-50% reduction in Fx protein. Fx deficiency increased apoptosis in rat beta-cells (10±1% apoptosis for siFx vs 8±1% for control siRNA (siCT), n=6, $p<0.05$) and dispersed human islets (27±1% for siFx vs 16±2% for siCT, n=4, $p<0.01$). Fx knockdown sensitized INS-1E cells to OL but not PAL-induced apoptosis (OL 20±2% for siFx vs 13±3% for siCT, n=6, $p<0.01$). These results were confirmed by caspase-3 activation. Fx deficiency increased caspase-3 cleavage by 56±16 in control and 39±10% in OL-treated INS-1E cells compared to siCT. Fx knockdown also sensitized INS-1E cells to ER stress-induced apoptosis (CPA 48±2%, TUN 25±3%, BR 37±2% apoptosis for siFX vs, respectively, 38±3%, 19±2%, and 22±4% for siCT, n=4, $p<0.05$). FK treatment partially prevented OL- and ER stress-induced apoptosis in Fx-deficient beta-cells (OL+FK 13±4% apoptosis vs OL 19±4%, n=8, $p<0.01$; TUN+FK 9±2% apoptosis vs TUN 27±2%, n=3, $p<0.01$).
Conclusion : Beta-cell failure plays a key role in the development of diabetes in FA patients. This may be due to metabolic stress-induced beta-cell apoptosis, a process that seems to be mediated by ER stress. Inducers of cAMP have a protective role and may have therapeutic potential.

Reconstitution of proapoptotic BAK´s function in liposomes reveals a dual role for mitochondrial lipids in the BAK-driven membrane permeabilization process.

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BAK is a key effector of mitochondrial outer membrane permeabilization (MOMP) whose molecular mechanism of action remains to be fully dissected in intact cells, mainly due to the inherent complexity of the intracellular apoptotic machinery. Here, we show that core features of the BAK-driven MOMP pathway can be reproduced in a highly simplified in vitro system consisting of recombinant human BAK lacking the carboxyl-terminal 21 residues (BAKDeltaC) and tBID in combination with liposomes bearing an appropriate lipid environment. Using this minimalist reconstituted system we established that tBID suffices to trigger BAKΔC membrane insertion, oligomerization and pore formation. Furthermore, we demonstrate that tBID-activated BAKΔC permeabilizes the membrane by forming structurally dynamic pores rather than a large proteinaceous channel of fixed size. We also identified two distinct roles played by mitochondrial lipids along the molecular pathway of BAKΔC-induced membrane permeabilization. First, using several independent approaches we showed that cardiolipin (CL) directly interacts with BAKΔC leading to a localized structural rearrangement in the protein which “primes” BAKDeltaC for interaction with tBID. Second, we provide evidence that selected curvature inducing lipids present in mitochondrial membranes specifically modulate the energetic expenditure required to create the BAKΔC pore. Collectively, our results support the notion that BAK functions as a direct effector of MOMP akin to BAX, and also add significantly to the growing number of evidence indicating that mitochondrial membrane lipids are actively implicated in BCL-2 protein family function

Comparative analysis of the mitochondrial proteome from primary B-cell malignancies and cultured cells

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Chronic lymphocytic leukemia (CLL) and Mantle Cell Lymphoma (MCL) are two of many forms of B-cell malignancy and are characterised by the relentless accumulation of small B lymphocytes in the blood, bone marrow, lymph nodes and other lymphoid tissue. CLL in particular is the most common form of adult leukemia in the Western world. In recent years, there has been renewed interest in the Warburg effect which describes fundamental differences in the metabolic pathways of cancer cells compared to normal cells. In transformed cells there is a shift from ATP-generation through oxidative phosphorylation to ATP-generation via aerobic glycolysis and converting most of the incoming glucose to lactate. Since aerobic glycolysis in cytosol is much less energy efficient than oxidative phosphorylation in mitochondria, cancer cells require increased amounts of glucose and glycolysis to meet their energy needs. However, metabolic analysis of CLL and Ramos cells shows that mitochondria of these cancer cells are functionally intact and do carry out coupled oxidative phosphorylation. We therefore wanted to characterise how the mitochondria of primary leukemic cells contribute to the overall metabolism of the cells and their sensitivity to apoptosis. As part of this study we are characterising the mitochondrial proteome and associated proteins of leukemia, in order to determine whether there are any differences in protein expression that would influence the balance between oxidative phosphorylation and glycolysis, and cell survival. We describe a method for isolating intact mitochondria from CLL and MCL patient samples as well as cultured cells (Ramos, Burkitt Lymphoma). The technique involves rupturing cells by homogenisation and immunoprecipitation of mitochondria using a Tom20 antibody cross-linked to magnetic Protein G Dynabeads. Mitochondrial purity was assayed by Western blotting for key proteins resident in different subcellular compartments. Purified mitochondria were then analysed by mass spectrometry using both shotgun proteomics approaches as well as mass spectrometry analysis of the total sample. The results showed extensive coverage of mitochondrial proteins and over 600 proteins were identified with high confidence and sequence coverage. We specifically analysed proteins involved in oxidative phosphorylation, the citrate cycle and the protein import machinery (TOM and TIM complexes) and observed significant differences in protein expression between CLL / MCL patient samples and the Ramos cell line. Selected mass spectrometry results were confirmed by Western blotting and densitometry of the resulting protein bands, using a Li-Cor Odyssey infrared imager. Data was normalised to VDAC1, an abundant protein resident in the outer mitochondrial membrane, which is expressed equally in CLL/MCL and Ramos cells. This innovative approach to mitochondrial proteomics allows us to characterise and analyse primary cells from cancer patients as well as cultured cells, and will provide the basis for the potential discovery of new marker proteins and therapeutic targets.

The Role of the new c-FLIP binding protein Ku70 in Death Receptor mediated apoptosis: The DUB Ku70 : Novel regulator of c-FLIP function

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FLIP proteins are known as major inhibitors of death receptor-mediated apoptosis by interfering with caspase-8 activation at the death-inducing signaling complex (DISC). The molecular mechanism allowing the high stability of FLIP proteins in tumour cells is totally unknown. We have identified Ku70 as a new binding protein of FLIP. We have investigated the role of Ku70 in FLIP proteins stability and in Death Receptor-mediated apoptosis. We have found that Ku70 silencing by SiRNA experiments induces ubiquitination and downregulation of FLIP and induces DR4/5 caspase-8-mediated apoptosis in tumour cell lines, not in “normal cells”. Ku70 can deubiquitinate Bax and seems deubiquitinate FLIP allowing an increase of FLIP stability. We have observed that Ku70 acetylation by a new HDAC inhibitor called Droxinostat identified as a specific FLIP inhibitor (CMPHB), or Vorinostat, induces Ku70-FLIP complex disruption and promotes FLIP ubiquitination allowing proteasomal degradation. We have found that Ku70 is recruited in DR4/5 DISC and plays a major role in Death Receptor mediated apoptosis. Our results show for the first time that the DUB Ku70 can be the link between Type I and Type II Death Receptor-mediated apoptosis and could be a promising new target for HDACi chemotherapy and TRAIL-mediated apoptosis.

The association of statins and taxanes exacerbates human gastric cancer cell apoptosis

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Gastric cancer has a dramatic prognosis, and several types of chemotherapy regimen have been used with only little improvement of the condition. Several clinical trials are being conducted or planned that associate novel combinations. Nevertheless, it is mandatory to analyze the possible benefit from additional experimental associations, and to investigate the mechanisms of tumour cell killing. Statins are major anti-hypercholesterolemia drugs, which have also been demonstrated to trigger apoptotic death of many cancer cell types. In this study, we looked at the combined effects of lovastatin and docetaxel, a potent microtubule-stabilizing agent, in the human gastric cancer cell line HGT-1. Microarray analysis showed that lovastatin induced profound changes in expression of a wide variety of genes, while docetaxel had only moderate effects. Both docetaxel and lovastatin triggered cell apoptosis, and the association of both drugs had a synergistic effect on apoptosis. The cell killing activity of the drug combination was linked to a drop of the anti-apoptotic Mcl-1 protein, the proteolytic cleavage of procaspase-3, PARP and Bax. A marked rise in p21, a powerful cell cycle inhibitor, together with reduced levels of aurora kinases A and B was brought about by docetaxel. Lovastatin, alone or combined with docetaxel, strongly suppressed expression of these proteins, together with that of cyclins B1 and D1. In addition, docetaxel strongly induced survivin expression, but this effect was counteracted by lovastatin, which repressed survivin levels, either alone or associated with docetaxel. Strikingly, isolated docetaxel resistant cells - which showed massive over-expression of the pGp protein - were more sensitive to lovastatin than docetaxel-sensitive cells. Taken together, these results suggest that the association of these drugs shows promise as a plausible strategy against gastric cancer, either as an initial therapy or to treat docetaxel resistant cancer cells.

Inhibitors of mitochondrial Kv1.3 channel induce tumor cell death

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Tumor resistance to treatment is a major clinical problem. Many tumors become resistant to treatment by downregulation of Bax and Bak. We have previously shown that mitochondrial Kv1.3 functions as a direct target of Bax (Szabo et al, 2008, PNAS, Szabo et al, 2011, CDD). Thus, we tested whether drugs inhibiting mitochondrial Kv1.3 are able to kill cells lacking Bax and Bak and whether these drugs are able to kill a panel of tumor cells by specific inhibition of Kv1.3. Three distinct membrane-permeant inhibitors of Kv1.3 induce apoptosis in different cancer cell lines expressing mitoKv1.3. Genetic deficiency or siRNA-mediated downregulation of Kv1.3 abrogated the effects of the drugs demonstrating Kv1.3 specificity of the inhibitors. The drugs were able to induce death in Jurkat lymphocytes and mouse embryonic fibroblasts (MEFs) lacking Bax and Bak. The efficacy of these drugs was strongly enhanced by inhibitors of the multidrug-resistance efflux pumps while the membrane-impermeant Kv1.3 inhibitors Margatoxin and Shk did not induce death, suggesting that apoptosis involves inhibition of mitochondrial and not of plasma membrane Kv1.3. In vivo studies demonstrate that one of these inhibitors prevented tumor growth of B16F10 melanoma. The studies indicate that inhibition of mitochondrial Kv1.3 might be a novel option to induce tumor cell death in vivo independent of Bax and Bak and may act as potential chemotherapeutic drugs.

The apoptotic response induced by the dependence receptor Met involves its relocalization to mitochondria

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The receptor tyrosine kinase Met and its ligand, the hepatocyte growth factor/scatter factor, are essential to embryonic development, whereas deregulation of Met signaling pathways is associated with tumorigenesis and metastasis. Ligand-activated Met induces multiple cellular responses including survival. Nonetheless, Met is cleaved in stress condition by caspases within its intracellular region, generating a 40 kDa fragment (p40 Met) which is able to induce apoptosis. By the survival and apoptosis responses that the receptor triggers in presence or absence of ligand, Met belongs to the family of the dependence receptor. While the signaling pathways triggers by membrane full length RTK to promote survival are well known, the molecular mechanisms induced by dependence receptor to promote apoptosis are poorly understood. Using an antibody directed specifically against p40 Met, we observed that the fragment is generated from 2h after induction of apoptosis. In addition, we demonstrate in Tet-on inducible epithelial cells expressing p40 Met that the fragment precipitates the cell death only when apoptosis is induced beforehand. Although p40 Met has the entire kinase domain, it does not display nor kinase activity or tyrosine phosphorylation demonstrating that the fragment is not an active kinase. However, p40 Met localizes both in mitochondria and nucleus, two essential compartments of the apoptotic machinery. Indeed, the first loop of the kinase region contains a nuclear localization domain. Deletion of this region induces an exclusive localization of the fragment in mitochondria, suggesting a shuttling between these two compartments. Forced relocalization of p40 Met at the plasma membrane, by grafting the myristoylation site of SRC, decreases its apoptotic properties, demonstrating that its subcellular localization is required for the apoptotic response. Taken together, while survival response triggered by membrane-anchored full length Met requires tyrosine kinase activity and induction of signaling pathways, precipitation of apoptosis by p40 Met is independent of kinase activity but involves localization of the fragment in apoptotic organelles.

Genetic control of Streptococcus pyogenes induced macrophage necrosis

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Group A streptococcus (GAS, Streptococcus pyogenes) is an important pathogen capable of causing a variety of diseases in humans, ranging from mild infections such as pharyngitis or impetigo to severe invasive diseases such as necrotizing fascitiis and Streptococcal Toxic Shock Syndrome (STSS). In the early stages of streptococcal infections macrophages play a key role in GAS host defence. They ingest and kill invading bacteria and recruit other immune effector cells to the site of infection through production and release of pro-inflammatory cytokines. By using a mouse model of STSS we found that S. pyogenes is able to evade the macrophage host defence through induction of necrotic cell death. Macrophages exposed to GAS exhibited an oncotic cellular phenotype with extensive cytoplasmic vacuolization, cellular and organelle swelling and rupture of the plasma membrane. This cell death was found to be mediated by the streptolysins S and O, involved loss of mitochondria transmembrane potential and was independent of caspase 1. By using a genetic linkage approach in crosses between GAS susceptible C3H/HeN mice and GAS resistant BALB/cByJ mice we have identified a quantitative trait loci (QTL) on proximal mouse chromosome 17 which contributes to the control of bacterial growth and survival. Most interestingly, this identified QTL was found to correlate with susceptibility to GAS induced macrophage necrosis. To identify the genes underlying this QTL we have generated new subcongenic mouse lines that carry different genomic intervals of the susceptible parental strain in the resistant BALB/cByJ genetic background. We will provide an update on our efforts to fine map the QTL interval through high-density SNP array mapping and report on the characterization of underlying cellular mechanisms that are associated with GAS induced macrophage necrosis.
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Accumulation of active caspase 3 in the nucleus of cells exposed to non-toxic dose of H2O2 is associated with cell growth arrest and DNA damage

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Caspases activation has been established as one of the hallmarks of apoptosis. Nevertheless, non-apoptotic roles of caspases have also been documented in recent years. The present study compares the effect of caspase 3 activation in cells exposed to a classical inducer of apoptotic cell death, 1µM of staurosporine (STS) or to a non-toxic dose (50µM) of the oxidative stress inducer, hydrogen peroxide (H2O2). Our results show that both treatments resulted in a significant activation of caspase 3. While STS activated caspase 3 through the well-established initiator caspase cascade pathway, activation of caspase 3 by H2O2 was independent of other caspases activation. Although STS-activated caspase 3 could transitorily be detected in the cells' nucleus, it ultimately accumulated in the cytosol while in H2O2-treated cells, activated caspase 3 accumulated mostly in the nucleus. Activation of caspase 3 by STS resulted in the cleavage of DNA damage repair proteins and structural proteins. In contrast, in H2O2 -treated cells only the cleavage of DNA damage repair proteins, such as PARP and RAD51, were detected. Intrigued by these findings, we next assessed the effect of caspase 3 activation on cells' fate. As expected, activation of caspase 3 following cells' exposure to STS correlated with cell death that was accompanied by the activation of the classical apoptotic pathway as revealed by a fragmented nucleus and an increase in the number of cells found in the sub-G1 population . On the contrary, activation of caspase 3 by H2O2 had no effect on the cells' nucleus morphology and no significant increase in numbers of cells in sub-G1 population. Inhibition of caspase 3 activation in H2O2-treated cells decreased the amount of gamma-H2AX detected and partially restored cell growth 72 to 96 hours following cells' exposure to H2O2. Taken together these results support that nuclear accumulation of activated caspase 3 participates in the induction of cells' growth arrest and DNA damage detected in cells exposed to mild-oxidative stress.

BARD1 is a target of miR-19a and miR-19b: a new selective epigenetic therapy for acute myeloid leukaemias?

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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. We found that BARD1 expression in leukaemia could be altered by epidrugs treatment, thanks to a mechanism mediated by miR-17-92 family. In the real it is not surprisingly that miRNAs activity appears to be related to stimulation by epigenetic compounds, in fact it is known that the promoters of miRNAs genes contain CpG islands and so are subjected to epigenetic control of gene expression by DNA-methyl transferases (DNMTs) and histone deacetylases (HDACs). The miR-17-92 family is composed of six mature miRNAs: miR-17, miR-18a, miR-19a, miR-19b-1 and miR-92-1; it is also known as oncomir-1 because there are in literature different papers that corroborate pro-oncogenic properties of miR-17-92 cluster, in fact it is over-expressed in lymphomas and several solid tumors compared to normal cells and appears to be able, togheter with c-myc, to induce B-cell lymphoma development in mouse model. On the other hand there are also evidences that miR-17-92 cluster deletion is linked to hepatocellular carcinoma, suggesting that these miRNAs could show onco-suppressor properties in different contexts. Maybe the diverse functionality of these miRNAs could be due to their pleiotropic expression and to the variability of their targets in different cell types or developmental and physio-pathological states. The aim of our work has been to investigate the effects of BARD1 and miR-19a/b epigenetic regulation in acute myeloid leukaemia models: we could show that miR-19a and miR-19b over-expression, and consequent BARD1 down-regulation, leads to a major sensitivity to epigenetic treatment in U937 cells, as demonstrated by cell cycle and differentiation analysis. These data will be further discussed.

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Targeting of distinct signalling cascades and cancer associated fibroblasts define the efficacy of Sorafenib against prostate cancer cells

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Sorafenib, a multi-tyrosine kinase inhibitor, kills more effectively the non-metastatic prostate cancer cell line 22Rv1 than the highly metastatic prostate cancer cell line PC3. In 22Rv1, constitutively active STAT3 and ERK are targeted by sorafenib, contrasting with PC3 cells, in which these kinases are not active. Notably, overexpression of a constitutively active MEK construct in 22Rv1 stimulates the sustained phosphorylation of Bad and protects from sorafenib-induced cell death. In PC3 cells, Src and AKT are constitutively activated and targeted by sorafenib, leading to an increase in Bim protein levels. Overexpression of constitutively active AKT or knockdown of Bim protects PC3 cells from sorafenib-induced killing. In both PC3 and 22Rv1, Mcl-1 depletion is required for the induction of cell death by sorafenib since transient overexpression of Mcl-1 is protective. Interestingly, co-culturing of primary cancer associated fibroblasts with 22Rv1 or PC3 cells protected the cancer cells from sorafenib-induced cell death, and this protection was largely overcome by co-administration with the Bcl-2 antagonist, ABT737. In summary, the differential tyrosine kinase profile of prostate cancer cells defines the cytotoxic efficacy of sorafenib and this profile is modulated by primary cancer associated fibroblasts to promote resistance. The combination of sorafenib with Bcl-2 antagonists, such as ABT737, may constitute a promising therapeutic strategy against prostate cancer.

Knockdown of PICK1 promotes low glucose-dependent cell death and mitochondrial changes

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PICK1 (Protein interacting with C-kinase 1) is a scaffold protein that predominantly locates to mitochondria in NIH 3T3 cells. Our previous study showed that PICK1 stabilized mitochondrial membrane potential by recruiting protein kinase C α (PKCα). Mitochondria are highly mobile organelles that continuously undergo fusion and fission. Disturbance in mitochondrial dynamic has been shown to be associated with several physiological and pathological conditions including programmed cell death. Our studies showed that down-regulation of PICK1 by shRNA in NIH3T3 cells significantly increased dependency on glucose for survival. Neither the activation of caspase-3 nor the apoptotic nuclei was observed after cells were cultured under low glucose conditions, suggesting apoptosis was not the predominant form of cell death under these conditions. Interestingly, we observed formation of autolysosomes as demonstrated by MDC (monodancyl-cadaverin) staining and increased LC3-II levels under low glucose conditions in NIH3T3 parental cells. These results suggested that glucose starvation induced autophagy instead of apoptosis in PICK1 deficient cells. Furthermore, knockdown of PICK1 promoted mitochondrial fragmentation under both normal and, with a significantly higher extent, low glucose conditions, suggesting a role of PICK1 in regulating mitochondria morphology. Glucose starvation also induced a rapid processing of L-OPA1, a mitochondrial protein known to regulate mitochondria fusion, to S-OPA1 in PICK1 knockdown cells. Taken together, this study suggests a novel function of PICK1 in maintaining mitochondria morphology and cell survival under low glucose conditions.

Ack1 is required for TRAIL induced apoptosis

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The Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is one of the most recent identified death inducing ligands of the TNF cytokine family. TRAIL induces apoptosis in many cancer cells, whereas most normal cells are resistant. TRAIL receptor agonists are therefore considered to be a promising anti-cancer therapeutic, however many cancer cells develop resistance to TRAIL, and TRAIL resistance mechanisms are therefore extensively studied. We have discovered that Activated Cdc42-associated kinase 1 (Ack1) is required for TRAIL induced apoptosis in human epithelial cells. Ack1 is a non-receptor tyrosine kinase with numerous protein-protein interaction domains, suggested to have a role in several cellular processes such as growth factor signalling, endocytosis and cell motility. Knockdown of Ack1 in various epithelial cell lines leads to impaired TRAIL induced apoptosis as evident by reduced cleavage of Caspase-8 and -3 and surface exposure of Annexin V. Ack1 is a known downstream effector of Cdc42; we therefore wanted to investigate if the role of Ack1 in TRAIL induced apoptosis is Cdc42 dependent. Indeed, knockdown of Cdc42 also leads to reduced levels of cleaved caspase-8 following TRAIL treatment, suggesting that Cdc42 is upstream of Ack1 in regulating TRAIL induced apoptosis. Exploring the underlying mechanism we found that the Ack1 knockdown leads to impaired TRAIL induced clustering of Death receptor 4 (DR4) and a reduction in the recruitment of Caspase-8 to the DISC complex. This is likely due to constitutive internalization of DR4 in the absence of Ack1, as shown by immunocytochemistry. These results show that Ack1 controls TRAIL induced apoptosis by regulating DR4 receptor dynamics and cell surface expression. We are currently investigating whether loss of Ack1 may underlie acquired TRAIL resistance in cancer treatment.

RIP1-mediated necroptosis essentially contributes to renal ischemia/reperfusion injury

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Loss of kidney function in renal ischemia/reperfusion injury (IRI) is caused by programmed cell death (PCD) but the contribution of necroptosis, a recently discovered form of programmed necrosis, has not been investigated. Initially, we identify the presence of death receptor-mediated caspase-independent cell death in murine tubular cells and characterize it as necroptosis by addition of necrostatin-1 (Nec-1), a highly specific receptor interacting protein kinase 1 (RIP1)-inhibitor. The detection of the necroptotic key players RIP1 and RIP3 in whole kidney lysates and freshly isolated murine proximal tubules led us to investigate the contribution of necroptosis in a mouse model of renal IRI. Herein, inhibition of RIP1 by Nec-1 reduces organ damage and renal failure, even if administrated after reperfusion, and resulted in a significant survival benefit in a model of lethal renal IRI. We functionally compared these results with the contribution of apoptosis to renal IRI by applying the pan-caspase inhibitor zVAD. Unexpectedly, the specific blockade of apoptosis by zVAD neither prevented organ damage in renal IRI nor the increase of retention parameters in vivo. Our results demonstrate the presence and functional relevance of necroptosis in the pathophysiologic course of ischemic kidney injury and a functional predominance of necroptosis over apoptosis in this setting. Above that, we identify the therapeutical potential of Nec-1 as a drug for the prevention and treatment of renal IRI.

The role of autophagy gene 5 in malignant melanoma

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Cutaneous melanoma is a common malignancy of melanocytes. In its early stage malignant melanoma can be cured by surgical resection, but it becomes extremely difficult to treat once it progresses to the metastatic stage due to its high resistance to current chemotherapeutic drugs. Autophagy is a highly conserved cellular self-eating process, in which proteins and organelles are sequestered and subsequently degraded in a double membrane structure called autophagosome. Autophagy related genes (ATGs) are the main players during this process. Autophagy has been shown to be involved in the pathogenesis of numerous diseases, including neural degenerative diseases, infections and cancer.
We are interested in the role of ATG5 in melanoma. We found that ATG5 expression is down-regulated in malignant melanoma comparing with benign melanocytic nevus patients. Downregulation of ATG5 in melanoma is at least partially due to ATG5 promoter methylation. Over-expression of ATG5 decreases colony formation of melanoma cells. With our data, we hypothesize that down-regulation of ATG5 may contribute to melanoma tumorigenesis.

Hijacking cellular survival pathways and redirecting them into induction of cell death - apoptin as a model

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The discovery of cancer stem cells and their preliminary characterization, in recent years, opens new ways for their selective targeting. Using apoptin, a viral protein that selectively kills cancer cells, we have investigated pathways changed in breast-, prostate-, and hematologic malignancies. We have employed a variety of molecular tools like shRNA-gene silencing, gene-knock-out model cell lines, dominant-negative and dominant active forms of studied proteins, adenoviral transduction, specific inhibitors, synthetic peptides mimicking active domains, and other tools. In our model, cancer cells were killed by apoptin through cooperation of various pathways: (i) interaction of apoptin with PI3-K and Akt led to nuclear translocation of Akt and phosphorylation of numerous nuclear substrates (Maddika et al., Cell Prolif., 2007; Maddika et al., Oncogene, 2008, Maddika et al., J. Cell Sci., 2008), (ii) activation of the Akt->Nur77 led to cytoplasmic translocation of the later one, with subsequent activation of mitochondrial death pathway (Maddika et al., Oncogene, 2005), (iii) apoptin triggered activation of CDK2 via Akt-mediated phosphorylation and Akt mediated inhibition of its inhibitor p27Kip (Maddika et al., Mol. Cell. Biol., 2008). These cellular changes, together lead to deregulation of mitosis, and activation of mitochondrial apoptotic pathway, selectively in cancer cells. Thus, in our experimental model, the pro-survival PI3-K/Akt signaling pathway that is upregulated in majority of cancers, could efficiently be “hijacked” and redirected towards selective induction of cell death in cancer cells. Various, potential experimental treatment strategies will be discussed.

Autophagy inhibition affects melanoma cell survival, redox and metabolic homeostasis by altering mitochondrial function

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Background: Cutaneous Malignant Melanoma (CMM), the most lethal type of skin cancer, is largely refractory to existing therapies and has a very poor prognosis. This urges better understanding of melanomagenesis to allow the development of 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 and/or malfunctioning blood supply) and has emerged as a synergistic factor in melanocyte transformation and CMM therapy resistance. Decreased availability of oxygen is a cellular stress known to stimulate autophagy, an evolutionarily conserved lysosomal pathway for the recycling of cytoplasmic materials, including proteins and damaged organelles, like mitochondria. The role of this catabolic process in melanomagenesis remains largely unclear. Beside the well known prosurvival role of autophagy, several lines of evidence suggest a tumor suppressive function for autophagy. Certain common events in CMM, such as hyper-activation of the PI3K-Akt pathway, known to negatively regulate autophagy, suggest that autophagy mitigation might support oncogene-driven cancer progression. Objectives: In this study we want to unravel the effect of an (oncogene-driven) reduction in autophagic flux on melanomagenesis. Results: Here, we show that constitutive activation of the oncogene Akt decreases autophagic flux in CMM cell lines. To further investigate the effect of this decreased autophagic flux on melanomagenesis we used the pharmacological inhibitor chloroquine and siRNA targeting the key autophagy gene product ATG5 and BNIP3, an important mediator of (mito)autophagy induction under hypoxia. Attenuation of autophagic flux led, especially under hypoxia, to the accumulation of damaged and superoxide-producing mitochondria, which was functionally linked to increased oxidative stress and a metabolic shift towards glycolysis. The increased mitochondrial superoxide levels promoted the stabilization/activation of HIF-1 and upregulation of its downstream targets (e.g. Glut-1 and VEGF), thus suggesting that autophagy may be essential to maintain metabolic homeostasis and prevent the tumor-promoting accumulation of HIF-1 under hypoxia. A severe blockage of autophagic flux, induced massive mitochondrial superoxide production, mitochondrial membrane depolarization, caspase activation and apoptosis. Intriguingly, in a xenograft model of advanced melanoma, administration of chloroquine (50mg/kg) not only reduced tumor size but also decreased tumor necrosis and hypoxia, suggesting that blockage of autophagy in vivo may profoundly affect the tumor micro-environment. We are currently exploring these effects in more detail. Conclusion: Our result suggest that oncogene-driven reduction in autophagic flux in CMM increases cellular oxidative stress, stabilize/activate HIF-1 and shifts the metabolism towards glycolysis, supporting melanomagenesis. On the other hand, a severe blockage of autophagy induces cell death in vitro and in in vivo conditions autophagy inhibition appears to have an impact not only on tumor cell fitness and survival but also on the tumor micro-environment.

RxDL motifs of the c-FLIP DED domains are critical for its anti-apoptotic function

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Introduction: cFLIP is a major anti-apoptotic protein that blocks the apoptotic pathway mediated by death receptors. cFLIP is overexpressed in many cancers resulting in chemoresistance and limiting the effectiveness of commonly used anticancer therapies. Both the long (FLIPL) and the short (FLIPS) splice forms compete with procaspase 8 for binding with the adaptor protein FADD at the death inducing signaling complex (DISC), which is formed after stimulation of death receptors by their ligands. Developing a strategy to prevent recruitment of cFLIP to the DISC would be of potential therapeutic value. The aim of this project was to identify the critical regions in cFLIP which are required for binding to FADD and to examine whether disruption of this binding can promote death receptor mediated apoptosis.
Results: As a member of the death effector domain (DED) protein family, cFLIP contains tandem DED domains, each with a characteristic RxDL conserved binding motif, which were shown previously to be important for interaction and function of viral FLIP. The crystallographic structure of cFLIPS remains unresolved, so computer modelling using an homology model of cFLIPS based on viral FLIP was carried out. This suggested that the two conserved RxDL motifs in each DED were important sites of interaction with FADD. To validate the model's predictions, site-directed mutagenesis was performed to generate mutations in the RxDL motif in each individual DED or in both. We have demonstrated that the ability of RxDL cFLIPS mutants to interact with FADD and be recruited to the DISC is significantly impaired compared to wild-type cFLIPS,. Moreover overexpression of cFLIP RxDL mutants no longer protects cells from death ligand-induced apoptosis.
Conclusion: This study demonstrates that the RxDL motifs in the DEDs of FLIP are important for cFLIPS function.

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Differences in apoptosis-related gene expression in CD77-positive and CD77-deficient Burkitt lymphoma cells

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We have shown that Daudi cells, CD77-positive Burkitt lymphoma cells, are more susceptible to apoptosis inducers including camptothecin and serum deprivation than VT500 cells, Daudi-derived CD77-deficient mutants, as determined using Annexin V staining, mitochondrial membrane depolarization and FDA-based assays in combination with flow cytometry. Further investigations have been performed in order to identify possible components of signaling pathways involved in the observed differential sensitivity to apoptosis inducers. For microarray analysis, total RNA was isolated, labeled with Cy3, and hybridized to Agilent Human Whole Genome microarrays. Bioinformatic analysis for significant fold changes and relevant pathways was performed using Genespring GX10 and Ingenuity Pathways Analysis (IPA) software. Western blot analysis was performed to determine expression of proteins related to known apoptosis pathways. Microarray analysis indicates that a number of genes are differentially expressed in CD77-positive versus CD77-deficient Burkitt's lymphoma cells including the apoptosis-related genes JNK1, GAS-2, PARP and ICAD. In addition, pro-caspase 3, pro-caspase 4 and pro-caspase 5 genes were all up-regulated in Daudi versus VT500 cells. Western blot analysis has confirmed differences in PARP, ICAD and caspase expression. In addition, differentially expressed genes related to other pathways known or suspected to involve CD77 in these cell lines including interferon signaling, cell adhesion and MHC class II-mediated antigen presentation were identified by microarray analysis. Initial results of RT-PCR assays are consistent with the microarray results for differential expression of genes for IRF-7, HLA-DM and GAS-2.

Analysis of FOXO and Sirtuin proteins family in canine coronavirus type II-induced apoptosis

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Sirtuins proteins are highly conserved nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases and constitute a family of regulatory molecules modulating the organism life span in many species. Until now, in mammalian, seven members (SIRT1 to SIRT7) are known. They have been implicated in the control of critical cellular processes such as differentiation, proliferation, metabolism, senescence and apoptosis. Moreover, sirtuins regulate viral transcription interacting with the activity of Forkhead box O (FOXO) gene family proteins. Canine coronavirus type II (CCoV-II), a member of antigenic group 1 of the family Coronaviridae, is a single positive-stranded RNA virus responsible for enteric disease in young puppies. It has been shown that infection of a canine fibrosarcoma cell line (A-72 cells) by CCoV-II resulted in an apoptotic process, depending on the activation of both intrinsic and extrinsic pathways of the caspases cascade. Herein we investigated the role of Sirtuin and FOXO families during CCoV-II infection in A-72 cells, using Nortern Blot and Western Blot analysis. Our results demonstrated that mitochondrial SIRT3 and SIRT4 protein expression increased from 24 h post infection (p.i.) on, whereas the nuclear SIRT1 expression increased during the first 12 h p.i. followed by a decrease after 36 h p.i.. Moreover, we observed that FOXO3A and FOXO1 expression increased significantly and stably from 12 h p.i. on. Furthermore, the expression of pro-apoptotic member bax significantly decreased in the cytosolic fraction from 12 h p.i. on; whereas, in the mitochondrial fraction, significantly increased at the same times, indicating the translocation of this protein from the cytosol into the mitochondrial compartment following infection. By contrast, the expression of the anti-apoptotic protein bcl-2, was detected only in the cytosolic fraction where decreased significantly in a time-dependent manner during infection. These data suggest that FOXO transcription factors mediate pro-apoptotic effects of CCoV-II, in part due to activation of extrinsic apoptosis pathway, while Sirtuin family may be involved in intrinsic apoptotic pathway. These findings represent a further evidence that sirtuins are key regulators of cell survival and apoptosis through their interaction with nuclear and mitochondrial proteins.

Apoptosis regulation and chemoresistance in head and neck squamous cell carcinoma

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Dysregulation of apoptosis interrupts the balance between cell growth and cell death and is associated with cancer including HNSCC (head and neck squamous cell carcinoma). A conventional cancer therapy is a cyclic application of cytostatic drugs to induce apoptosis in cancer cells. An impaired ability of cancer cells to undergo apoptosis causes to cancer development and resistance towards chemotherapy. Usually a tumor recurs afterwards, whereas the molecular processes of the involved anti-apoptotic signal transduction pathways that prevent cell death are mostly unknown. To induce apoptosis in cancer cells permanent HNSCC cell lines were treated with the conventional cytotoxic drug Paclitaxel. In order to identify the changes in the anti-apoptotic signal transduction pathways microarray gene expression analysis using Affymetrix human gene chip was performed by an comparison of untreated and Paclitaxel treated HNSCC cell lines. According to this microarray analysis some markers of the programmed cell death were significant up- and down-regulated. These changes are probably responsible for the resistance of the tumor to chemotherapy. We will show the progress of our investigation.

The antiapoptotic oncoprotein Aven- a key modulator of tumor development and progression ?

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Aven, which has recently been assigned an additional function in DNA damage repair, has originally been described as an antiapoptotic interactor of Apaf-1 and Bcl-XL. 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 the lysosomal aspartic protease Cathepsin D to inhibit Apaf-1/Casp-9-mediated apoptosis in mammalian cells. N-terminal processing of Aven by CathD is particularly pronounced in the human breast adenocarcinoma cell line MCF-7. We stably transduced these cells with hAven shRNA and investigated their tumorigenic properties using a mouse xenograft model. Compared to control shRNA cells, Aven-deficient MCF-7 cells failed to initiate tumor development until more than 10 weeks after injection and display severe proliferation and apoptosis defects in vitro. Interestingly, in preliminary experiments we could demonstrate that the downregulation of Aven seems to increase invasiveness of MCF7 cells. We are currently trying to decipher Aven´s molecular function in breast carcinogenesis and in normal mammary gland development. In childhood acute lymphoblastic leukemia, Aven overexpression has been correlated with bad prognosis. Using different mouse models established in our lab, we are investigating the role of Aven in initiating and maintaining leukemia. Here, we show that in a p53-deficient background Aven overexpression is able to accelerate T-ALL development. At the same time, Aven (over-) expression is required for tumor growth in subcutaneous xenografts of ALL and AML cell lines. Taken together, our findings place this protein in focus as a novel potential therapy target in several malignancies.

Role of bim and p53 in transcription - independent apoptosis induced by combretastatin a-4 treatment in human non-small lung cancer H460 cells

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The BH3-only Bcl-2 subfamily member Bim is a well known apoptosis promoting protein. However, the mechanisms upstream of mitochondrion membrane permeability by which Bim is involved in apoptosis has been poorly investigated, particularly in response to agents capable of interfering with the cytoskeleton architecture and arresting cells in mitosis. Based on the observation that Bim is sequestered on the microtubule-array by interaction with the light chain of dynein, we have investigated upon depolymerisation, whether and possibly how Bim could be involved in the commitment of apoptosis. With this purpose H460 Non Small Lung Cancer Cells (NSLC) were treated with the microtubule damaging agent combretastatin-A4 (CA-4) (7.5nM; 8-48 h), and various parameters were investigated. Upon treatment, cells arrested in mitosis and died through a caspase-3-dependent mitotic catastrophe. Transient knock down of Bim drastically reduced apoptosis, indicating that this protein was involved in cell death as induced by microtubules disorganisation. In response to increasing conditions of microtubules depolymerisation, we found that the protein level of Bim was strongly upregulated in a time-dependent manner at transcriptional level. Furthermore, Bim was released from microtubule-associated components and translocated to mitochondria, even in a condition of protein synthesis inhibition, where it showed a markedly increased interaction with Bcl-2. In turn, the fraction of Bax bound to Bcl-2 decreases in response to treatment, thereby indicating that Bim possibly promotes Bax release from the pro-survival protein Bcl-2. Overall, we demonstrated that Bim is required for the CA-4-induced cell death in the H460 lung cancer cell line via activation of the mitochondrial signalling pathway. Defining the contribution of Bim to the mechanism of apoptosis may offer some different clues in view of developing new strategies for chemotherapy with CA-4, underlining the relevance of the cytoskeleton integrity in the apoptotic response.

Transmission-through-dye microscopy as a tool for cell death research

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Shrinkage vs. swelling has been long used as a distinguishing feature between apoptosis and necrosis, but more recently, cell volume has additionally emerged as an important signaling factor that can profoundly influence the development of apoptosis. Quite naturally, in order to study cell volume one would need to measure cell volume – and this step often presents a problem when working with adherent cells. Indeed, there have been very few direct measurements of the apoptotic volume decrease (AVD) in cells attached to a substrate, and the majority of studies of AVD have been limited to cells that either naturally grow in suspension or were trypsinized to enable the measurement by electronic sizing or by light scatter. The technique of transmission-through-dye (TTD) microscopy is based on the simple principle of attenuation of transmitted light as it passes through an absorbing medium. Cells are placed in a shallow compartment, such as a flow chamber with a thin spacer or simply a gap between a coverslip and a slide, and a strongly absorbing, cell-impermeable dye is added to the medium. Transmission images are collected at the wavelength of maximum dye absorption; since the depth of the absorbing dye layer is complementary to cell thickness, thicker cells or parts of cells appear brighter. For quantitative analysis, cell thickness is calculated from the logarithm of transmitted intensity, and its sum over the cell area gives volume. The method is fast (the entire volume information is contained in a single image), precise (with vertical resolution exceeding the diffraction limit), and insensitive to possible fluctuations in the light source. The food colorants acid blue 9 or patent blue V have absorption peaks around 630 nm and are nontoxic for living cells at concentrations necessary to produce sufficient contrast. They can be used to continuously observe cellular 3D shape and volume in a perfusion chamber; at the same time, the assay is compatible with confocal or wide-field fluorescence (for example, caspase activation using NucView 488 and the mitochondrial membrane potential using TMRE can be monitored simultaneously with the volume). In addition to that, intracellular fluorophores with emission above 600 nm can be used to test the membrane integrity, as they are efficiently quenched by direct contact with the dye. For longer incubations, the fixed-time variant of TTD, where the average cell volumes are compared among different samples, may be more practical than the real-time assay. We believe that TTD microscopy opens new possibilities for studying cell volume regulation in dying cells. We are currently applying it to the actinomycin D-induced model of apoptosis in adherent HeLa and T24 cultures. In particular, we are interested in the relationship between AVD, the loss of attachment and biochemical manifestations of apoptosis.

Quantitative measurement of activated and non-activated Caspase 14 in skin

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Activation of caspase-14 occurs during terminal differentiation of keratinocytes and may play a role in filaggrin degradation. Therefore, down-regulation of caspase-14 may lead to impaired barrier function. To compare the levels of active and total caspase-14 in healthy subjects in various age groups and in patients with atopic dermatitis (AD), using two enzyme-linked immunoassay (ELISA) systems. We established four clones of monoclonal antibodies to caspase-14 and used clone 3 as the immobilizing antibody. A cleavage site-directed antibody, h14D146 [4] was used for specific quantification of active caspase-14 in extracts of tape-stripped corneocytes. Total caspase-14 was measured with a commercial antibody, H-99. The amount of caspase-14 remained constant (ca. 0.1% of extractable proteins) in healthy males from their twenties to their fifties. Caspase-14 was mostly in active form (71–94%) in these extracts. In contrast, caspase-14 level and active caspase-14 ratio were significantly decreased in females in their fifties and sixties. Contents of free amino acids were decreased in females in their sixties, and transepidermal water loss was increased in females in their forties and sixties. In patients with AD, active caspase-14 was markedly down-regulated compared to age-matched controls in both lesional (7.5%) and non-lesional skin (10.6%). Staining of active caspase-14 was considerably weaker in non-lesional skin and was hardly detectable in lesional skin with parakeratosis. Conclusion: Our new ELISA systems are effective tools to quantify activation of caspase-14. Our results indicate a role of caspase-14 in epidermal barrier function.

siRNA- mediated knock-down of nucleostemin in leukemia cell lines: differentiation or apoptosis

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Leukemia is a disease arised from CD34+ blood stem cells. Despite, depth of knowledge concerning pathogenesis of leukemia, long-term survival still remains a challenge in medical treatments. Obviously, attempts for identification and targeting of signaling pathways involved in proliferation, differentiation and apoptosis of leukemic stem cells may improve treatment strategies in leukemia patients. Nucleostemin (NS), a novel nucleolar GTP-binding protein, plays a critical role in cell cycle, self-renewal and proliferation of both stem cells and cancerous cells. Expression of NS gene in leukemic stem cells has been demonstrated, but there is no comprehensive research concerning NS mode of action. The aim of the present study was to introduce NS-siRNA into human leukemic stem cell models (K562 and NB4) and to investigate probable effects of NS gene silencing in growth, differentiation and apoptosis of leukemic stem cells. To do this, NS specific double-stranded small interfering RNA (NS-siRNA) was transfected into leukemic stem cell models (K562 and NB4). The results showed that NS gene was highly expressed in K562 and NB4 cells. After 72 h transfection of 200 nM siRNA, a significant decrease in growth (up to 30-40%) accompanied with G1 cell cycle arrest were observed in both cell lines. In this condition, however, viability was reduced only in K562 cells but not in NB4 cells. In addition, differentiation characteristics such as decrease in nuclear cytoplasm ratio and ingestion of latex particles were observed after longer transfection time in NB4 cells. Although, these results suggest that NS inhibition in leukemia cell lines induced differentiation and/ or apoptosis in a cell-dependent manner. Attain to apoptotic and differentiating effects of NS-siRNA in human leukemic cells, the inhibition of this gene can be proposed as a new marker for stem cell therapy of leukemia patients.

The autophagy protein Atg7 is essential for hematopoietic stem cell maintenance and leukemia prevention

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Autophagy is the cell's major regulated lysosomal degradation pathway. It has been described to play roles in cell death and survival and has been shown to prevent cellular damage. As such, its role in the maintenance of adult hematopoietic stem cells (HSCs) remains unknown. While normal HSCs sustain life-long hematopoiesis, malignant transformation of HSCs leads to leukemia. Mechanisms protecting HSCs from cellular damage are therefore essential to prevent hematopoietic malignancies. Here we removed autophagy in HSCs by conditionally deleting the essential autophagy gene Atg7 in the hematopoietic system. This resulted in a dramatic loss of normal HSC functions, development of acute myeloid leukemia (AML) and death of the mice within weeks. The Atg7-deficient hematopoeitic stem and progenitor cell compartment displayed an accumulation of mitochondria and reactive oxygen species, which in turn resulted in increased proliferation and DNA damage. While the rare HSCs within the Lin-Sca-1+c-Kit+ (LSK) compartment were significantly reduced, the overall LSK compartment was expanded and contained leukemic stem cells. The Atg7-/- AML was transplantable and the malignant myeloid cells expressed significantly higher levels of the myeloid leukemia marker CD47. Taken together, these data show that Atg7 is an essential regulator of adult HSC maintenance and prevents leukemogenesis. These results highlight the pathway of autophagy as a potential target for the prevention and/or treatment of AML.

To prevent embryonic lethality, IAPs must limit activation of RIP kinases by TNF Receptor 1

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Inhibitor of apoptosis proteins cIAP1, cIAP2, and XIAP regulate apoptosis and cytokine receptor signaling, but their overlapping functions make it difficult to distinguish their individual roles. To do so, we deleted the genes for IAPs separately and in combination. While lack of any one of the IAPs produced no overt phenotype in mice, deletion of cIap1 with cIap2 or Xiap resulted in mid embryonic lethality. In contrast, Xiap-/-cIap2-/- mice were viable. The death of cIap2-/-cIap1-/- double mutants was rescued to birth by deletion of TNF receptor 1 genes. Remarkably, hemizygosity for receptor-interacting protein kinase-1 (RIPK1) allowed Xiap-/-cIap1-/- double mutants to survive past birth, and prolonged cIap2-/-cIap1-/- embryonic survival. Similarly, deletion of Ripk3 was also able to rescue the mid gestation defect of cIap2-/-cIap1-/- embryos, as these embryos survived to E15.5. cIAPs are therefore required during development to limit activity of RIP kinases in the TNF receptor signaling pathway.

Analysis of the proteasome impairment and cell death pathways caused by the neurodegenerative-diseases associated proteins UBB+1 and UCH-L1

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The ubiquitin-proteasome system (UPS) is the major proteolytic pathway that degrades intracellular proteins in a regulated manner. Protein degradation by the UPS involves a group of enzymes that ultimately attach ubiquitin, a small well-conserved protein, to an internal lysine residue in the target protein. Multiple ubiquitin proteins are connected to form a polyubiquitin chain which serves as a degradation signal recognized by the proteasome (Johnson ES, 1992). Deficiencies in the ubiquitin or the proteasome can cause the accumulation of misfolded proteins, which can aggregate, leading to proteotoxic stress and, unless timely corrected, cellular dysfunction or death. Protein aggregation is a hallmark of many neurodegenerative diseases including Alzheimer disease (AD) and Parkinson disease (PD). Aggregates associated with disease show ubiquitin deposition including a mutant form of ubiquitin, named UBB+1, which it is itself a substrate for degradation by the UPS but it can act as a natural proteasome inhibitor when accumulates. Although UBB+1 exemplifies that ubiquitin itself can be a cause for UPS impairment, the molecular mechanisms is unclear and it is therefore not clear if UBB+1 causes ubiquitin stress or affects other events critical for proteasomal degradation (Dantuma N, 2009). The levels of ubiquitin are strictly controlled by the balance of ubiquitinating (E1, E2 and E3) and deubiquitinating enzymes (DUBs). Mutation in ubiquitin processing enzymes such as the ubiquitin carboxyl-terminal esterase L1 (UCH-L1), a DUBs enzyme, may contribute to the pathogenesis of PD and AD where is oxidatively damaged and loses about 40-80% of its activity. However, the ways in which the function and localization of UCH-L1 are regulated are largely unknown. Interestingly, we found that UBB+1 and UCH-L1 interact in yeast two-hybrid screen. We use the yeast Saccharomyces cerevisiae to investigate the cellular effects of UBB+1 and UCH-L1 (alone and combined) and their role on UPS function and cell death pathways. Previous work in the group has linked UBB+1 to the proteasome system showing a beneficial effect on survival when expressing UBB+1 at low levels, but inhibition, at low and high expression levels, of all three types of proteolytic subunits of the proteasome.

Bortezomib primes neuroblastoma cells for TRAIL-induced apoptosis by promoting mitochondrial outer membrane permeabilization

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Despite recent advances of therapeutic strategies for treatment of childhood malignancies, the outcome of children with high-risk neuroblastoma (NB) remains poor. So, there is still a need of novel therapeutic approaches to treat aggressive NB. In this study we investigated the potential of the proteasome inhibitor Bortezomib to sensitize NB cells for TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis. Combination therapy of Bortezomib and TRAIL led to significant enhancement of cell death in comparison to single agent treatment in a synergistic manner and also inhibited longterm survival. This was demonstrated in various NB cell lines as well as in primary cultured NB cells, underscoring the clinical relevance of this finding. Importantly, Bortezomib and TRAIL also acted in concert to suppress tumor growth in an in vivo model of neuroblastoma. Exploring the underlying molecular mechanism of sensitization, we found enhanced cleavage of caspase-8, -3 and Bid by combination treatment compared to single-agent treatment. Furthermore, Bortezomib profoundly enhanced TRAIL-induced cleavage of Bid into tBid, accumulation of tBid in the cytosol and its insertion into mitochondrial membranes, pointing to a concerted effect on Bid cleavage (TRAIL) and stabilization of tBid (Bortezomib), which links the death receptor to the mitochondrial pathway. Also, combination treatment with Bortezomib and TRAIL compared to single agent treatment led to enhanced conformational change of Bax and Bak, loss of the mitochondrial membrane potential and cytochrome c release. By overexpressing the anti-apoptotic protein Bcl-2, which inhibits the activation of the mitochondrial apoptotic pathway, apoptosis induction after combination treatment was partially blocked. Our data demonstrate that the proteasome inhibitor Bortezomib sensitizes NB cells for TRAIL-induced apoptosis by increasing TRAIL-triggered Bid activation, thereby enhancing mitochondrial outer membrane permeabilization. Thus, the combination of Bortezomib and TRAIL represents a promising therapeutic approach for NB treatment.

Mechanisms of ER stress and apoptosis induction by saturated and unsaturated fatty acids in human pancreatic beta-cell line NES2Y

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Chronically elevated concentrations of fatty acids in blood contribute to apoptosis of pancreatic beta-cells of patients with type 2 diabetes. Induction of endoplasmic reticulum (ER) stress is considered as an important factor involved in fatty acid-induced apoptosis. In our previous experiments, we found that saturated fatty acids (i.e. palmitic and stearic acid), in contrast to unsaturated fatty acids (i.e. palmitoleic and oleic acid), induce cell death in human pancreatic beta-cell line NES2Y. In this study, we tested the effect of saturated and unsaturated fatty acids on the activation of apoptotic and ER stress signaling pathways (ATF6, IRE1alfa and PERK pathway) in NES2Y cells. Experiments were performed in chemically defined, serum-free media containing individual fatty acids bound to bovine serum albumine (BSA). Cell death was induced by palmitic and stearic acid (1 mM/2% BSA, 24 h). 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. Cell death induced by stearic acid was associated with significant activation of caspase-6, -7, -9, -2 and -8, but surprisingly not with significant activation of caspase-3. The activation of caspases was significantly decreased by coincubation with 0.2 mM unsaturated fatty acids. We did not detect the activation of mitochondrial pathway of apoptosis (no cytochrome c release, no change in ROS level and mitochondrial membrane potential) nor p53 activation after stearic acid treatment. We also did not find any change in Fas receptor and Fas ligand expression. However, the expression of ER stress markers BiP and CHOP was strongly induced by stearic acid. Stearic acid treatment lead to XBP1 mRNA cleavage, JNK activation and eIF2alfa phosphorylation, but not ATF6 cleavage. These findings indicate the involvement of IRE1alfa and PERK pathway of ER stress signaling, but not ATF6 pathway of ER stress signaling. Induction of the expression of ER stress markers BiP and CHOP as well as the activation of ER stress signaling pathways were inhibited by coincubation with oleic acid. Taken together, cell death induced by stearic acid in human pancreatic beta-cell line NES2Y is a p53- and mitochondrial apoptotic pathway-independent process related to the activation of caspase-6, -7, -9, -2 and -8, but not caspase-3. Stearic acid activates IRE1alfa and PERK pathway of ER stress signaling. All detrimental effects of stearic acid tested were blocked very effectively by oleic acid coincubation.

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Mitochondrial “priming” is a meaureable event which governs clinical response to chemotherapy

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Cytotoxic chemotherapy is the mainstay of current cancer treatment it targets ubiquitous elements such as DNA and microtubules. Despite decades of clinical use of chemotherapy, determinants of response to such treatments are poorly understood. Here, we showed that clinical response to cytotoxic agents is largely governed by the initial proximity of tumors mitochondria to the apoptotic threshold, prior to treatment. Cells that are close to the apoptotic threshold are referred to as “primed” for death, this can be measured by a functional assay that measures mitochondrial response to standardized death signals in the form of BH3 peptides. Patients with highly “primed” mitochondria demonstrated a better clinical response to therapy across a variety of treatment regimens in both hematological and solid malignancies. We also showed that increasing “priming” in cancer cells improved chemosensitivity to cytotoxic agents. The greatest discord in response to chemotherapy is between normal tissues and cancer cells. Significantly, we found that most normal tissues tested exhibited a low level of “priming” and this may explain the relative resistance to chemotherapy and the existence of a therapeutic window for cytotoxic agents. Hopefully, with this new understanding of a determinant of response to chemotherapy we can utilize it to logically improve efficacy of cytotoxic agents or to devise better combination of drugs for maximal killing efficiency.

FOXO3-induced reactive oxygen species are regulated by BCL2L11/Bim and SESN3

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FOXO transcription factors induce apoptosis and regulate cellular reactive oxygen species (ROS) production. To identify the sequence of molecular events underlying FOXO3/FKHRL1-induced apoptosis, we studied FOXO3 regulation and function by expressing an ECFP-tagged FOXO3 or a 4OH-tamoxifen (4OHT)-inducible FOXO3-ERtm fusion protein in the neuronal cells SH-EP and STA-NB15. By FOXO3-knockdown or expression of a dominant-negative FOXO3 mutant we observed that etoposide- and doxorubicin-induced elevation of cellular ROS depends on FOXO3-activation and induction of its transcriptional target BCL2L11/Bim. Activation of FOXO3 by its own induced two sequential ROS waves as measured by reduced MitoTrackerRed via live cell microscopy. Induction of Bim by FOXO3 is essential for this phenomenon since Bim-knockdown or ectopic expression of BCL2L1/Bcl-xL prevented FOXO3-mediated ROS overproduction and apoptosis. Tetracycline-controlled expression of Bim per se impaired mitochondrial respiration and caused ROS production, suggesting that FOXO3 induces uncoupling of mitochondrial respiration via Bim. FOXO3 also activated a ROS rescue pathway by inducing the peroxiredoxin SESN3/Sestrin3, which was responsible for the biphasic ROS accumulation. SESN3-knockdown caused an increase of ROS and accelerated apoptosis by FOXO3. The combined data clearly demonstrate that FOXO3 activates ROS overproduction as a consequence of Bim-dependent impairment of mitochondrial respiration in neuronal cells leading to apoptosis.

Targeting of histone deacetylase 10 causes neuroblastoma cell death via lysosomal dysfunctions and ROS release

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Pan-HDAC (histone deacetylase) inhibitors are known to induce cell death in tumor cells and are currently being evaluated in clinical trials. However, due to their unselective nature, these compounds exhibit dose limiting side effects restricting their full anti-cancer potential. Therefore, characterization of the molecular function of single HDAC isozymes is of major importance. Here, we show that only those HDAC inhibitors covering class IIb HDAC10 in their inhibitory profile induced accumulation of lysosomes in several neuroblastoma cell lines associated with p53-independent cell death. Enforced expression of HDAC10 enhanced tumor cell survival and reduced endogenous as well as induced lysosome formation, whereas a catalytically inactive HDAC10 did not. Co-immunoprecipitation studies revealed the binding of the lysosomal chaperone HSC70 to HDAC10. RNAi-mediated knockdown of HSC70 or HDAC10 expression induced the accumulation of dysfunctional lysosomes in BE(2)-C neuroblastoma cells. Consequently, autophagic flux was disturbed and resulted in an accumulation of autophagosomes upon targeting of HDAC10. Additionally, the accumulation of dysfunctional lysosomes was accompanied by ROS release, leading to programmed cell death. Simultaneous inhibition of apoptosis and necroptosis with zVAD-fmk and necrostatin efficiently rescued cell death mediated by HDAC10 knockdown. These results demonstrate for the first time that class IIb family member HDAC10 is involved in the regulation of lysosomal functions, such as chaperone-mediated autophagy in neuroblastoma cells. Selective targeting of this HDAC family member induces an alternative lysosomal-mediated tumor cell death pathway and may thus be a novel strategy for neuroblastoma therapy.

Distinct roles of Bcl-2 and Bcl-xL in apoptosis and autophagy of bone marrow mesenchymal stem cells during differentiation.

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Adult mesenchymal stem cells (MSCs) can be maintained over extended periods of time before activation and differentiation. Little is known about the programs that sustain the survival of these cells. Undifferentiated adult human MSCs (hMSCs) did not undergo apoptosis in response to different cell death inducers. Conversely, the same inducers can readily induce apoptosis when hMSCs are engaged in the early stages of differentiation. The survival of undifferentiated cells is linked to the expression of Bcl-xL and Bcl-2 in complete opposite ways. Bcl-xL is expressed at similar levels in undifferentiated and differentiated hMSCs while Bcl-2 is expressed only in differentiated cells. In undifferentiated hMSCs, the down-regulation of Bcl-xL is associated with an increased sensitivity to apoptosis while the ectopic expression of Bcl-2 induced apoptosis. This apoptosis is linked to the presence of cytoplasmic Nur 77 in undifferentiated hMSCs. In hMSCs, the expression of Bcl-2 depends on cellular differentiation and can be either pro- or anti-apoptotic. Bcl-xL, on the other hand, exhibits an anti-apoptotic activity under all conditions.

The chemotherapeutic agent 5-Fluorouracil induces a calcium-calmodulin dependent pathway required for p53 activity and apoptotic cell death in colon carcinoma cells

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The chemotherapy agent 5-FU (5-Fluorouracil) is an antimetabolite which has been in use against cancer for over 40 years. It acts in several ways but principally as a thymidylate synthase inhibitor, thereby blocking synthesis of the pyrimidine thymidine required for DNA replication and repair, with sequential impairment of DNA, resulting in cell death. In addition, early works described that loss of carcinogenic properties in colon cancer cells may be caused by incorporation of the 5-FU metabolite fluorouridine triphosphate into RNA. It is generally accepted that cell death, mediated by the hierarchically-ordered ATM/ATR–Chk1/Chk2–p53 signaling pathway, can occur in response to DNA damage. In comparison, significantly less has been reported regarding death signaling pathways originating from RNA damage, although it has been suggested that transcriptional stress can lead to p53 activation. Current knowledge also states that 5-FU-induced stress is triggering a p53-dependent induction of death inducing complex (DISC) formation and subsequent caspase-8 activation. Accordingly, death receptors DR5 and CD95, members of the TNF-receptor family and p53 target genes, have been reported essential for the process. In our recent work we define DR5 and not CD95 as the main receptor required for activation of the caspase cascade. Moreover, by using a library of potential upstream inhibitors of the caspase cascade, we discovered that 5-FU-mediated cell death in colon carcinoma cells depends on calcium-signaling. Thus, the calcium-chelating agent BAPTA inhibited cell death, as determined by a lack of effector caspase activity and FACS analysis of subG1 cells. Interestingly, although core p53 levels remained, we observed a reduction in three out of eight phospho-p53 events analyzed. Similar results were obtained using two separate calmodulin (CaM) inhibitors and when verapamil, an inhibitor of L-type plasma membrane calcium-channels, was added to the experimental system. Consequently, it is likely that 5-FU exerts its effect in some tumor cell lines by a calcium-CaM dependent activation of p53. Results from experiments using verapamil, calcium-free cell culture medium and thapsigargin also indicated that calcium originating from extracellular sources but not the ER is required for the process. To confirm these data, we analyzed three factors which has been described to be important for posttranslational modifications of p53, checkpoint kinase 2 (Chk2), calcium-dependent protein kinase C (PKC) and ataxia telangiectasia mutated (ATM). However, by using HCT116 Chk2-/- cells and specific inhibitors of PKC and ATM, we could conclude that these proteins did not contribute significantly to any of the 5-FU induced phospho-p53 events analyzed. Current work is focusing on functional assays to reveal the implications of specific p53 modifications for 5-FU-induced cell death. The fact that a widely used therapeutic drug, such as 5-FU, is using calcium as a messenger could provide new therapeutic intervention points, or specify new combinatorial treatment regimes.

ROS generated in specific compartment mediate either cell death or senescence in lung cancer cells

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ROS are continuously generated in vivo acting as important modulators of several biological processes such as signaling, proliferation and differentiation, but increases in their steady states are regarded to be responsible for a variety of pathologies, including cancer and aging. Recent research demonstrates that redox dysregulation originating from metabolic alterations and disruption of mitogenic and survival signaling through ROS modulation represents a specific vulnerability of malignant cells that can be selectively targeted by pro-oxidant chemotherapeutics or stress inducers leading to senescence. It is well established that the majority of the cell death inducers lead to formation of ROS. However, it is still unclear whether ROS production is an early event during apoptotic/necrotic signaling or rather a consequence of cell death. In order to assess whether ROS generation and accumulation in different subcellular compartments can lead to cell death or senescence U1810 NSCLC, a therapy resistant cell line, was used as model. Treatment with 12.5 mU of Xanthine Oxidase and 25 uM Xanthine (XH) generated high levels of H2O2 readily accumulating in cytosol, nucleus and mitochondria. ROS production was sustained in all subcellular compartments but declined differentially within 1 h in cytosol and nucleus and within 35 min in mitochondria. Under the same conditions PI-Annex V staining revealed that this treatment initiates cell death within 3 h (16% dead cells vs CTR) and progressively increases its cytotoxicity overtime peaking at 18 h (93% dead cells). Most of dead cells displayed PI+ AnnexV+ staining (70% necrotic cells) while less amount displayed single AnnexV+ staining (30% apoptotic cells). Pretreatment with 20 uM zVAD failed to prevent cell death to a significant extent while pretreatment with 40 uM necrostatin-1 was confering 50% protection from necrotic cell death up to 6h treatment without affecting apoptotic cell death. Absence of caspases-2,-3, -8, -9 activities confirmed that under these conditions cells are dying in a caspase-independent way. Western blot analysis revealed absence of PARP-1 cleavage, strong decrease in Akt phosphorylation and progressive increase of gammaH2AX expression peaking at 9 h of XH treatment. Comet Assay confirmed that DNA damage occurs after 1 h and becomes prominent after 6 h in these experimental settings (Tail moment 3.39 and 36.08 respectively). On the other hand, treatment with 1.25 mU of Xanthine Oxidase and 25 uM Xanthine (XL) generated low levels of H2O2, accumulating only in cytosolic compartment, whose production declined to basal level after 45 min. FACS analysis revealed that under these conditions cell viability is unaffected up to 24 h and decreases considerably after 48 h (30% dead cells vs CTR). Western Blot revealed increase in Akt phosphorylation, absence of PARP-1 cleavage and undetectable levels of gammaH2AX. Activity of all measured caspases was negligible. Similar results were obtained upon treatment with 5 uM Antymicin A (AA) except for total absence of ROS accumulation in any cellular compartments and unaffected cell viability up to 48h of treatment. AA was shown to induce increased expression of senescence-associated marker p21 and more importantly both AA and XL treatments were able to significantly increase SA-β-Gal staining at 24 h, 10% vs CTR for XL, and 35% vs CTR for AA, respectively. Thus, in U1810 cells sustained production of high levels of ROS induces caspase-independent necrosis-like cell death, while sustained production of ROS at low levels induces an adaptive response leading either to survival or senescence only marginally affecting cell viability.

Apaf-1 regulates cytochrome c release from mitochondria and provides control for apoptosis-injured cell recovery and long term survival

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Apoptosis is a cell death program that allows controlled elimination of unwanted cells. Defects in the regulation of apoptosis are at the root of a variety of diseases. When cells acquire resistance to induction and execution of apoptosis it frequently correlates with cancer or autoimmune diseases. In contrast, excessive apoptosis induces unwanted cell death and promotes several pathological conditions related to tissue infarction, stroke, ischemia-reperfusion damage, degenerative diseases and AIDS. The intrinsic apoptotic pathway that is activated upon intracellular insults depends on mitochondrial signalling and further formation of the apoptotic platform called the apoptosome. Apaf-1 is the core protein of the apoptosome and is activated by cytochrome c (Cyt c) release which occurs upon mitochondrial membrane permeabilization (MMP). Small molecules that inhibit Apaf-1 are a promising approach for developing unwanted apoptosis inhibitors. We have reported on a family of small molecules that inhibits apoptosis by interfering with Apaf-11-3. In this study we show that the apoptosis inhibition provided by the Apaf-1 inhibitor SVT016426 at the level of apoptosome and Cyt c release contributes to maintain functional cells and allows long term cell survival, thus raising hope for the development of future treatments of unwanted pathological apoptosis.

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Role of autophagy in paracetamol-induced liver damage

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Paracetamol overdose causes acute liver injuries as a result of extensive hepatic necrosis with high morbidity and mortality. The complete cellular mechanisms leading to paracetamol-induced liver failure and necrosis are still illusive. Paracetamol is metabolized to NAPQI which reduces cellular glutathione levels, binds to cellular structures and proteins and therefore causes cellular damage. Free cytosolic components released by injured and necrotic hepatocytes induce the release of pro-inflammatory cytokines in the liver leading to inflammation and cirrhosis. Damaged cellular organelles are usually removed by autophagy, a fundamental cellular rescue mechanism. Although many regulators of apoptotic cell death, such as Jun kinase and the BH3-only protein Bim, are involved paracetamol-induced cell death, tissue and cells show a more necrotic phenotype. We have previously shown that paracetamol strongly induces Bim expression in a Jun kinase- and Foxo3a-dependent manner. Furthermore, the TNF-related apoptosis-inducing ligand TRAIL amplifies paracetamol-induced cell death in a Bim dependent manner. As paracetamol-treated hepatocytes and liver tissue show increased vacuolized structures we are currently investigating the role of autophagy in paracetamol-induced hepatocyte death and liver injury. The anti-apoptotic Bcl2 homologs have been shown to inhibit the autophagy-related protein beclin-1. As Bim binds and neutralizes these Bcl-2 homologs, paracetamol may cause the release and activation of beclin-1 and induce autophagy. Current data indicate that treatment of hepatocytes with paracetamol leads to a lipidation of LC3 and a conversion of LC3-I to LC3-II. Furthermore, using immunofluorescence staining we have seen that treatment of cells with paracetamol causes an aggregation of LC3 protein, indicative of the formation of autophagosomes. Therefore, we propose the possibility that autophagic, necrotic and apoptotic pathways may synergize in paracetamol-induced liver injury.

Midostaurin (PKC412) inhibits tumor growth of non-small lung carcinoma in vivo

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Midostaurin (PKC412), one of the staurosporine cognates, is a selective inhibitor of protein kinase C as well as other kinases. PKC412 showed a broad antiproliferative activity against various tumors and normal cell lines in vitro and in vivo. Recently we have shown that PKC412 sensitizes non-small cell lung carcinomas (NSCLC) resistant to conventional radio- and chemiotherapy to apoptosis. The goal of our work was to investigate the therapeutic efficacy of PKC412 in human NSCLC xenografts established from the U1810 cell line in nude mice. BALB/c female mice at the age of 1,5-2 months were subcutaneously transplanted with U1810 cells (5 x 106). When tumors could be detected, mice were randomly divided into 2 peer groups (on 16th day after inoculation). After randomization, the mice from the first group (control) were treated with vehicle Gelucire 44/14 (Gattefossé) with PBS and mice of the second one (experimental group) were administrated with PKC412 dissolved in vehicle by gavage for 7 days. The dose of PKC412 (100mg/kg/day) used in our study was shown earlier to inhibit tumor growth in other xenograft models. The tumor volume was measured every day during treatment (7 days) and each second day after finishing treatment for 39 days. The inhibition of tumor growth was observed during injection of PKC412 from 12% on day 2 to 65% on day 7 compare with control. In the experimental group significant decrease of the size of tumors was observed after the end of drug administration (from day 24 to day 36, 65% to 78%, $p<0,001$) as compared with control. It is worth to notice that one mouse in experimental group had complete remission at day 42 after inoculation. There was no significant difference in the mean body weight of mice exposed and non-exposed to the drug. The mechanism of PKC412 action in NSCLC cells is discussing.

Mutual regulation of Bcl-2 proteins independent of the BH3 domain as shown by the BH3-lacking protein Bcl-xAK

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The BH3 domain of Bcl-2 proteins was regarded as indispensable for mutual regulation of pro- and antiapoptotic family members as well as for apoptosis induction. We have recently described Bcl-xAK, a proapoptotic splice product of the bcl-x gene, which lacks BH3 but encloses BH2, BH4 and a transmembrane domain. It remained however unclear, how Bcl-xAK may trigger apoptosis. For its efficient overexpression, Bcl-xAK was subcloned in an adenoviral vector under Tet-OFF control. Strong induction of apoptosis was seen in melanoma and nonmelanoma cell lines in a time-dependent manner, reaching up to 50% of apoptotic cells at 72 h. Interestingly, Bcl-xAK shared typical characteristics with other proapoptotic Bcl-2 proteins, namely mitochondrial translocation, disruption of mitochondrial membrane potential and cytochrome c release, clearly indicating its regulation of the mitochondrial apoptosis pathway. Importantly, Bcl-xAK activity was critically dependent on the expression of either Bax or Bak, as shown in genetic models, and apoptosis was abrogated in Bax/Bak double knockout cells as well by overexpression of antiapoptotic Bcl-2 proteins as Bcl-2 or Bcl-xL. A direct interaction with Bcl-2 or Bax was however ruled out by immunoprecipitation. Bcl-xAK proves the existence of an additional level of mutual regulation of Bcl-2 proteins that is independent of the described BH3-mediated interaction between family members. Therein, mitochondrial translocation appears as a critical step, and this type of regulation may also play a role for other proapoptotic family members. New pathways may be used for overcoming therapy resistance frequently determined by Bcl-2 protein of cancer cells.

Healing of skin wounds with mitochondria-targeted antioxidants: cellular mechanisms

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We have shown previously that mitochondria-targeted antioxidant SkQ1 stimulated healing of full-thickness dermal wounds in mice and rats (Demianenko et al., 2010). This effect was strongly expressed in old animals and in mice of db/db-line with obesity and type II diabetes. It was shown that SkQ1 affected the critical phases of wound healing: it accelerated the progress of inflammatory phase, stimulated formation of granulation tissue and epithelialization. It was shown that SkQ1 stimulated differentiation of fibroblasts into myofibroblasts, the important components of granulation tissue, as well as migration of epitheliocytes into the wound. We have identified some signaling pathways modulated by SkQ1 in the model of fibroblast differentiation in vitro (Popova et al., 2010). Histological analysis of the wounds demonstrated that SkQ1 significantly reduced accumulation of neutrophils in the wound during the phase of inflammation. It was earlier shown that antioxidants could inhibit inflammatory reaction in endothelium and suppress penetration of neutrophils through the vessel wall. We have studied the effect of SkQ1 on activation of endothelial cells (EaHy926 line) by the inflammatory cytokine TNFa. It was shown that SkQ1 (0.2nM) inhibited expression of the adhesion molecule ICAM1 and adhesion of lymphocytes at the monolayer of endothelial cells induced by subtoxic concentrations of TNFa. SkQ1 also prevented reorganization of actin cytoskeleton and disassembly of intercellular contacts (which determined the barrier function of endothelium) and suppressed expression of cytokines Il-6 and Il-8 which are important for development of inflammation. Apoptosis of endothelial cells induced by high doses of TNFa(5-50ng/ml) was also prevented by SkQ1. We suggest that the anti-inflammatory effects of SkQ1 could be mediated by modulation of signaling pathways which included activation of MAPK cascade and transcription factors NFκB and AP1. The drugs like SkQ1 could become an important component of the complex anti-inflammatory therapy.

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Mechanism of Betulinic acid induced tumor killing

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Betulinic acid (BetA) is a plant-derived compound that shows selective cytotoxicity for tumor cells while healthy cells are unaffected. We have previously shown that BetA induces tumor cell killing in a wide variety of tumor cell lines in a death receptor and Bcl-2 family-independent manner. Moreover, we found that induction of cell death can be inhibited by cyclosporine A, an inhibitor of the permeability transition pore (PT-pore) suggesting an important role for the PT-pore in BetA-induced cell death. Using electron microscopy, we observed that upon BetA treatment mitochondria, change structure significantly and display an adapted structure with concentric cristae. Such concentric cristea are also observed in Barth syndrome patients, an X-linked disorder, caused by a defect in the Taffazin gene leading to an altered cardiolipin (CL) homeostasis. CL is an important structural phospholipid in the inner membrane of the mitochondria where it has a role in energy metabolism, mainly by providing stability for the individual enzymes and enzyme complexes involved in energy production. Moreover, CL is involved in different stages of the mitochondrial apoptotic process and in mitochondrial membrane dynamics and also associates with the PT-pore. We found that CL levels in BetA-treated cells contain more saturated fatty acid chains compared to untreated cells. We hypothesize that the changes in fatty-acid backbone modify the rigidity of CL and thereby affect the function and eventually result in PTpore opening. In agreement, we observed an increase in cell death when we treated cells with BetA and the saturated fatty acid Stearic acid, which aggravates CL saturation, while unsaturated fatty acids prevent this saturation and BetA-induced cell death. Currently we are determining how BetA can affect the saturation of CL and how this differs in healthy cells that are not sensitive to BetA by looking into the role of Cardiolipin synthase (CLS) in this process.

Platinum sensitize ovarian carcinoma cells to ABT-737 in vitro and ex vivo through inhibition of Mcl-1

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In ovarian carcinoma, chemoresistance is the main responsible for the poor 5-year overall survival, remaining under 30%. Protection against apoptotic cell death is particularly involved in this chemoresistance, and among the observed alterations, overexpression of anti-apoptotic Bcl-2 proteins is of first importance. Among them, Bcl-xL and Mcl-1 appeared to cooperate to protect ovarian cancer cells against apoptosis, thus constituting together pertinent targets. In this context, the use of BH3 mimetic molecule ABT-737, that targets Bcl-xL, could constitute an alternative strategy to reverse the platinum chemoresistance in ovarian carcinoma. However, ABT-737 remaining unable to efficiently inhibit Mcl-1 activity, its clinical use in ovarian carcinoma thus requires defining of another tool able to inhibit Mcl-1. In this study, we investigated in vitro and ex vivo the capacity of platinum derivatives (cisplatin and carboplatin) to inhibit Mcl-1 expression or activity and therefore sensitize ovarian carcinoma cells to ABT-737. SKOV3 and IGROV1-R10 platinum-resistant ovarian cancer cell lines were exposed to these drugs, as single agents or associated, using various exposure protocols in vitro or ex vivo (SKOV3 and IGROV1-R10 xenografted tumor nodes developed in nude mice and subsequently used for slicing and ex vivo treatment). We thus studied apoptosis induction as well as Mcl-1 and pro apoptotic BH3-only proteins expression. In vitro study: whereas neither cisplatin nor ABT-737 alone presented any toxicity, the association of ABT-737 and platinum was highly cytotoxic in both cell lines. We tested several protocols and showed that ABT-737 must be present during the 24h following carboplatin exposure to allow cell death. These observations are in agreement with the needed down-regulation or inactivation of Mcl-1 by platinum prior or concomitant with ABT-737 exposure to induce cell death. As expected, cisplatin as well as carboplatin were able either to inhibit Mcl-1 expression or to induce BH3-only expression (particularly Noxa and Bim). Moreover, we observed a synergetic effect of the association ABT-737/platinum on BH3-only Bim and Noxa expression, these events being correlated to the ability of platinum to sensitize to ABT-737. Ex vivo study: we confirmed these observations in SKOV3 and IGROV1-R10 ex vivo tumor slices models. Whereas ABT-737 and platinum derivatives remained poorly cytotoxic or completely ineffective as single agents, their association was highly cytotoxic. In conclusion, this strategy associating ABT-737 to platinum appears as an attractive way to reverse resistance to platinum derivatives that remain the most active drugs in ovarian cancer. Moreover, this study presents platinum derivatives as pertinent sensitizers to ABT-737, through its direct or indirect Mcl-1 inhibition, opening new perspectives for the clinical use of this promising BH3-mimetic molecule.

Caspase-dependent cleavage of Scythe is a common feature of apoptosis

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Scythe (also known as BAT3 or BAG6) has been implicated in apoptosis in various model systems. First, Scythe modulates the Drosophila melanogaster apoptotic regulator, Reaper. Second, mice lacking Scythe show pronounced defects in organogenesis and in the regulation of apoptosis and proliferation during development. Furthermore, studies in human cells have shown that Scythe co-localizes with apoptosis-inducing factor (AIF) in the cytosol after thapsigargin treatment. Additional evidence for the role of Scythe in apoptosis comes from the observation of the formation of a caspase-3-generated C-terminal Scythe fragment with pro-apoptotic activity after ricin treatment. We have observed that cleavage of Scythe by caspase-3 is a general phenomenon during apoptosis and occurs both after the activation of the extrinsic, Fas-mediated pathway or intrinsic pathway (i.e. staurosporine-triggered apoptosis). Moreover, the size of the fragments generated from this cleavage seems to depend on the nature of the apoptotic stimulus. The cleavage of Scythe is required for the cytosolic translocation of the AIF-binding fragment of Scythe since experiments with T cell leukemic Jurkat cells pre-treated with the pan-caspase inhibitor Z-VAD-FMK showed that: a) Scythe is retained in the nucleus, b) no cleavage product is generated, and c) no cytosolic interaction with AIF occurs. Interestingly in the B lymphoma cell line Raji, in which caspase-3 is activated upon Fas ligation but not following other agents targeting the intrinsic pathway of apoptosis, Scythe is retained in the cell nucleus despite the cleavage of this protein by caspase-3. We conclude that Scythe cleavage occurs in response to numerous pro-apoptotic stimuli and we provide evidence that cleavage of Scythe may serve as a biomarker of apoptosis resistance since no cleavage of Scythe is observed in cells which lack caspase activation.

Protein phosphatase 2A and CIP2A enhances mTORC1 signaling and inhibits autophagy.

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Macroautophagy (autophagy) is an evolutionarily conserved catabolic process that enhances the survival of cells exposed to various stresses thereby enhancing tumor growth. Autophagic mechanism plays an essential function in survival of multicellular organisms from cellular damage, development of cancer, neuronal damage and aging. Mammalian target of rapamycin complex 1 (mTORC1) and Akt – mTORC1 signaling pathways have been identified as major positive and negative regulators of autophagy, respectively. However, little is known about mechanisms of phosphatases regulating autophagy in cancer. Thus, we screened human phosphatome siRNA library targeting 317 phosphatases and their regulators for siRNAs that either increase the number of autophagosomes in normally growing MCF-7 breast carcinoma cells or reduce their number upon inhibition of autophagosome maturation by lysosomotropic anti-cancer agent siramesine. We demonstrate that under normal nutrient conditions autophagy is regulated by numerous of serine/threonine, - tyrosine and lipid phosphatases and related regulatory genes of those. Bioinformatics analysis of the candidates revealed strong links to known autophagy regulatory pathways especially, such as Akt - mTORC and MAP kinase pathways, via regulators of protein phosphatase 2A (PP2A). PP2A might have an essential function in regulation of Akt – mTORC1 signaling pathway for autophagy activation and inhibition. We focused to enlighten the role of identified PP2A holoenzyme and regulatory subunits in regulation of autophagy trough Akt – mTORC1 pathway. Further analysis of these candidates validated cancerous inhibitor of PP2A (CIP2A) as a potent endogenous autophagy inhibitor in breast cancer. We therefore describe a novel role for PP2A and its associates and provide insight into the regulation of autophagy. Mechanistic knowledge of this process is crucial for understanding and targeting therapies for several human diseases, including cancer and neurological diseases, in which abnormal autophagy might be pathological.

Sensitization of melanoma cells for death ligand-induced apoptosis by the potassium channel inhibitor TRAM-34 correlates with the intrinsic pathway and SMAC release

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Melanoma only poorly responds to chemotherapy, and the death ligand TRAIL, which may trigger apoptosis in melanoma cells via TRAIL-R1/DR4 and TRAIL-R2/DR5, appears as a promising therapeutic strategy. However, prevalent and inducible TRAIL resistance may limit its clinical use, as previously shown in melanoma cells. Potassium channels as IK1 may play significant roles in tumor progression and may serve as therapeutic targets. Functional expression of IK1 in melanoma cells is demonstrated by quantitative RT-PCR analysis and patch clamp recordings. We prove that TRAM-34, a selective IK1 inhibitor, strongly enhanced TRAIL sensitivity of melanoma cells and overcomes prevalent and inducible TRAIL resistance. Unraveling the signaling pathways revealed that TRAM-34 was able to overrule the lack of caspase-3 processing in selected TRAIL-resistant cells. Disruption of the mitochondrial membrane potential and release of proapoptotic mitochondrial factors, such as cyctochrome c, AIF and SMAC, clearly indicated the involvement of mitochondrial apoptosis pathways. Importantly, TRAM-34 mediated enhancement of the TRAIL-induced apoptosis was critically dependent on the expression of either Bax or Bak, and apoptosis was abrogated in Bax/Bak double knockout cells as well as by overexpression of antiapoptotic Bcl-2. Taking into account the physiological role of death ligands in immune surveillance, sensitization of melanoma cells for death ligands may be supportive for an anti-tumor immune response. This data prove the critical role of mitochondria in TRAIL resistance of melanoma cells and present a new strategy for TRAIL sensitization based on the targeting of ion homeostasis. Furthermore, combinations with the potassium channel inhibitor TRAM-34 may help for a breakthrough of TRAIL-mediated strategies in melanoma.

A System For Quantitative Production Of Murine Basophils Ex Vivo

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Basophils are the least common granulocytes in the circulation. They have recently been recognized as important immunomodulatory cells and play a major role as effector cells in allergic inflammation. However, functional and biochemical assays on basophils have long been hampered due to their extremely low number in the blood (<1% of peripheral blood leukocytes in the mouse). Consequently, very little is known about the regulation of apoptotic signalling pathway in basophils. In addition, basophils are short-lived cells with an expected half-life of approximately a few days. For these reasons, we established a system for quantitative production of murine basophils in vitro. We modified the method published by Wang et al. (Nat Meth 2006), where they described a method for the quantitative production of macrophages or neutrophils using conditional Hoxb8. Besides different cytokine cocktails, we use a 4-hydroxytamoxifen-inducible lentiviral system for conditional Hoxb8 expression (Vince et al. Cell 2008). In order to generate Hoxb8-immortalised, basophil-committed myeloid progenitor cell lines, we infect an enriched fraction of bone marrow-derived multipotent cells with a conditional Hoxb8 expression construct. This progenitor cell line is genetically stable (immortalised but not transformed), can be maintained in culture for a long time and progenitors can be differentiated into mature basophils in near unrestricted number upon shutdown of Hoxb8 expression Importantly, after differentiation, these cells exhibit many characteristics of mature murine basophils. They stop cycling, have multilobed nuclei, exhibit mouse basophil specific surface markers (c-kit-/FcεRIhi), express high-levels of basophil specific mast cell proteases 8 (Mcpt8) and MCP-11, degranulate in response to IgE crosslinking and start to produce IL4. To our knowledge, this is the first report presenting an in vitro model to produce large numbers of mouse basophils. This system is important not only to study the molecular mechanisms of basophil differentiation, but also because of the possibility of using genetically modified mice to further investigate basophil biology. In terms of apoptosis signalling, we have started to look into the role of BH3-only proteins (Bid, Bim) in death receptor-induced and mitochondrial apoptosis.

Protein expression profiles of apoptosis regulators in malignant melanoma are highly heterogeneous, but can be exploited in systems analyses to case-specifically predict drug responsiveness with high accuracy

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Key to the personalisation of anti-cancer therapies are tools that allow to determine a priori whether treatments are likely to be effective. However, owing to high inter-individual heterogeneity of many cancers, reliable markers for drug responsiveness are often non-existent or only apply to sub-populations of patient cohorts. Using malignant melanoma cell lines as pre-clinical model systems, we here report a novel approach that is able to classify responsiveness to apoptosis-inducing chemotherapeutics with high accuracy. In a panel of 11 cell lines we determined the absolute expression (nM) of 17 key regulatory proteins of apoptosis. Expression profiles were highly heterogeneous between cell lines and did not correlate with disease progression. We therefore conducted a systems-level analysis building on functional protein clustering, multi-variate statistics and mathematical discriminant analysis to investigate whether the complex information contained in baseline protein expression profiles can be exploited to predict drug responsiveness. When comparing the results from the analysis of protein profiles with cell death readings in response to TRAIL, cisplatin or dacarbazine, cell death susceptibility (classified as non-responsive, low, medium or high) was correctly assigned for most cell lines by the systems approach (100%, 91% and 82% correct classifications in response to TRAIL, cisplatin or dacarbazine, respectively). In addition, our approach can be exploited to generate predictions on which proteins should be targeted to most efficiently enhance drug responsiveness of individual cell lines. Our results and novel methodology therefore serve as a first proof-of-principle for the successful exploitation of systems biological approaches to generate accurate, case-specific predictions of drug responsiveness and provides means for the rational selection of additional targets to enhance cell death in poorly responding cells. Our strategy can easily be expanded to investigate other drugs or drug combinations, not only in malignant melanoma but also in other heterogeneous cancers.

Switching from aerobic glycolysis to oxidative phosphorylation modulates tumor cell sensitivity to TRAIL

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Aerobic glycolysis, as described by the Warburg Effect, is believed to be the main energy source for cancer cells and is potentially involved in the resistance of some cancers to apoptotic agents. TRAIL is one such potential anti-cancer agent that induces apoptosis through DISC-mediated activation of caspase 3 and/or cleavage of the pro-apoptotic Bcl-2 family member BID. Therefore, we investigated the effect of prolonged glucose deprivation on the metabolism of tumor cells and their sensitivity to TRAIL. Tumor cells cultured with pyruvate under glucose-free conditions undergo a switch in metabolism with increased oxidative phosphorylation capacity and decreased glycolysis compared to cells cultured with glucose and pyruvate. In line with this switch, cells adapted to glucose-free media further exhibit a concentration- and time-dependent loss in sensitivity to TRAIL-induced apoptosis. In contrast, inhibition of glycolysis with the anti-glycolytic 2-deoxyglucose, did not bring about a metabolic switch and cells were sensitised to TRAIL-induced apoptosis. We therefore propose that inhibition of glycolysis through glucose-deprivation and 2-deoxyglucose occurs through different modes. Further investigation into the loss of TRAIL-sensitivity in glucose-free cells found that caspase 8 activity at the DISC was decreased and the Bax: Bcl-2 ratio was altered to a more anti-apoptotic phenotype.

Non-apoptotic role of caspases in microglia activation

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Microglia cells are resident immune cells of the central nervous system. Activation of microglia can lead to release of pro-inflammatory factors, which can result in neuronal damage. We have recently showed the orderly activation of caspase-8 and caspase-3 in microglia cells, without triggering of cell death. Inhibition of this caspase cascade hindered the microglia activation and lead to a reduction in neurotoxicity.
Stroke is caused by disturbance of blood supply to a part of the brain and can lead to permanent neurological damage or death. We are now investigating the non-apoptotic role of caspase-8 and caspase-3 in activated microglia cells adjacent to the infarct site.

Investigating rat retinal neural cell death induced by glutamate: intracellular mechanisms and the neuroprotective role of Neuropeptide Y (NPY).

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Glutamate is the main excitatory neurotransmitter in the Central Nervous System (CNS), including in the retina. Excitotoxity is responsible for neuronal cell death giving rise to several acute and chronic neurodegenerative diseases (Ozawa, Kamiya et al. 1998) also in the retina such as glaucoma and diabetic retinopathy (Kowluru and Abbas 2003; Santiago, Hughes et al. 2008). Neuropeptide Y (NPY) and NPY receptors are widely distributed in the CNS, including the retina. We previously showed that cultured rat retinal neural cells express NPY and NPY Y1, Y2, Y4 and Y5 receptors (Alvaro, Rosmaninho-Salgado et al. 2007). NPY has a neuroprotective role against excitotoxicity in rat hippocampus (Silva, Pinheiro et al. 2003; Xapelli, Silva et al. 2007). Moreover, we have also demonstrated that NPY has a protective role against cell death induced by MDMA (3,4-methylenedioxymethamphetamine) (Alvaro, Martins et al. 2008). The aim of this study is to investigate the type of cell death induced by glutamate and the neuroprotective role of NPY and NPY receptors against this insult. Additionally the intracellular mechanisms that mediate the glutamate excitotoxicity and the NPY protective role in this neuronal cell death will be investigated. Rat retina neural cell cultures were prepared from newborn Wistar rats and were exposed to glutamate (500 μM) for 24 h. Necrotic cell death was evaluated by propidium iodide (PI) assay and apoptotic cell death by TUNEL assay. The NPY receptors involved in the neuroprotective role of NPY were studied by using receptor agonists and antagonists and the intracellular mechanisms involved were evaluated by PI assay using several intracellular pathway inhibitors. Results: The exposure of retinal cells to glutamate increased the number of necrotic cells (PI-positive cells) by 517.3±71.7% and apoptotic cells (TUNEL-positive cells) by 294.1±41.7% compared to control. Glutamate induced cell death of neurons (TUJ1 positive-cells), astrocytes/Müller cells (GFAP positive-cells) and microglia cells (cd11b positive-cells). NPY (100 nM) pre-treatment inhibited glutamate-induced necrotic cell death by 208.0±41.8% and apoptotic cell death by 87.9±32.6%, compared to glutamate and also protected all different cell types in culture. NPY Y2, Y4 and Y5 receptors activation inhibited necrotic glutamate toxicity although glutamate induced apoptotic cell death was prevented by the activation of Y5 receptor. Glutamate excitotoxicity was inhibited by U0126, an inhibitor of extracellular signal-regulated kinase 1/2 (ERK 1/2) and the neuroprotective effect of NPY against glutamate cell death was decreased by H89, an inhibitor of Protein Kinase A. Thus glutamate induces neuronal cell death mediated through ERK 1/2.

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Stress induced death of T cells cultivated with deficit of autocrine factors is connected with decrease of intracellular ATP level

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A lot of data has shown that survival of mammalian cells is under control of growth factors and autocrine survival factors (AF). We studied the influence of AF deficit on survival, intracellular ATP content, lactate production and transmembrane mitochondrial potential of cytotoxic IL-2-dependent CTLL-2 cells under oxidative stress and alkalosis (pH 8.3). Autocrine factor deficit in cell culture was formed by cultivation of CTLL-2 cells at high density (2x106 cells/ml) for 14–16 h and following transfer of the cells in fresh medium into low density culture (1x105 cells/ml). CTLL-2 cells cultivated in AF deficit conditions have been shown to be more susceptible to oxidative injury in comparison with the cells cultivated without deficit of AF (control); they died at smaller concentrations of H2O2 than control cells did. Alkalosis (pH 8.3) combined with AF deficiency caused a substantial decrease of cell survival too. ATP content in CTLL-2 cells was transiently decreased under AF deficit conditions even without any stress. But treatment of the cells by hydrogen peroxide or cell cultivation under pH 8.3 resulted in additional large decrease of intracellular ATP level. This ATP reduction was accompanied by drop of mitochondrial potential and suppression of lactate production. Cell death under oxidative stress or alkalosis in the presence of AF deficit developed predominantly via necrosis rather than apoptosis pathway. Thus oxidative stress and alkalosis in AF-deficient culture of T cells provoked irreversible changes in energy metabolism resulting eventually in cell death.

Cell death induced by photodamage to different cellular compartments

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Cell death induced by photodynamic treatment (PDT) based on photosensitisers localised to different cellular compartments has been studied. Photodynamic treatment induces ROS, mainly singlet oxygen, so the cellular localisation of the photosensitiser determines the site of ROS production and that of the primary damage. Response of human epidermoid carcinoma cell line A-431 to PDT mediated by photosensitisers localised to mitochondrial interior (lipophylic cation 3,7-diamino-2,8-dimethyl-5-phenylphenazinium chloride, Safr), lysosomes (aluminium phthalocyanine tetrasulphonate, AlPcS4) or multiple intracellular membranes (meso-tetrahydroxyphenyl chlorin, mTHPC) was examined. At moderate cytotoxic doses, PDT targeted to mitochondrial interior (Safr-PDT) did not induce cell death, and the observed cell viability decrease for 50% can be ascribed to cell cycle arrest, reducing amount of cells in S phase. At high extent of the damage to mitochondrial interior by Safr-PDT, reducing cell viability for 80%, apoptosis was induced. mTHPC-mediated PDT targetted to membranes of multiple cellular compartments (ER, Golgi, mitochondria) as well as AlPcS4–mediated PDT targetted to lysosomes induced the pronounced apoptosis. An accumulation of the autophagy marker LC3-II was observed after PDT mediated by any of the photosensitisers. However, at moderate or high doses of PDT targeted to lysosomes, autophagic flux (studied using lysosomal protease inhibitors) was not registered, implying photodamage to autophagic machinery.

Mechanism of Sestrin2 Induction During ER Stress-Mediated Autophagy

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This work was conducted in order to elucidate the mechanisms that connect ER stress and autophagy. Microarray data performed on colon cancer cells treated with ER stress inducing agents has shown that a number of genes, responsible for autophagy initiation were upregulated during unfolded protein response (UPR). This work is focused on one of them, Sestrin2, a p53 responsive gene. This protein is known to be responsible to induce autophagy through activation of AMPK and subsequent inactivation of mTOR. Here we show that during ER stress, but not genotoxic stress, Sestrin2 is induced independently of p53. Moreover, the activation of Sestrin2 correlates with p70 S6 kinase inhibition and autophagy activation. The inhibition of UPR main branches suggest that they have a role in Sestrin2 activation in response to ER stress. This important connection could show relation between autophagy and ER stress, two mechanisms that are important in cellular survival and death.

IAP inhibitors induce apoptosis via NF-κB-activation and TNFα-secretion in a RIP1-dependnet manner in acute leukemia cells

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Patients with high risk acute lymphoblastic leukemia (ALL) or relapse of ALL do not respond well to current treatments and still have a poor prognosis. Because this failure is, in part, due to defects in apoptosis programs, new strategies are required that counter apoptosis resistance in order to improve the poor prognosis. Since "Inhibitor of Apoptosis" (IAP) proteins are expressed at high levels in acute leukemia and block apoptosis at a central point of the apoptotic machinery, they present a suitable molecular target for therapeutic intervention. We already showed that neutralizing IAP proteins by small molecule IAP inhibitors is an effective approach to sensitize childhood acute leukemia cells for death receptor- or chemotherapy-induced apoptosis. Here, we report that in some acute leukemia cell lines small molecule IAP inhibitors alone induce apoptosis - also at nanomolar concentrations. Cell lines which are sensitive for apoptosis induction by IAP inhibitors alone show rapid degradation of cIAPs, activation of NF-κB and secretion of TNFα, leading to an autocrine, apoptosis inducing TNFα-loop. Further analysis of signaling pathways reveals that IAP inhibition causes TNFα-dependent loss of mitochondrial membrane potential, caspase activation and apoptosis. In addition, this signaling pathway is dependent on the availability of RIP1. Leukemia cells with RIP1-knockdown show significant reduction in IAP inhibitor induced loss of mitochondrial membrane potential, caspase activation and apoptosis. Also primary leukemia cells are in part sensitive for IAP inhibitor induced cell death. Since not all acute leukemia cell lines and primary leukemic cells are sensitive for IAP inhibitor induced apoptosis, it is very important to find markers, which indicate IAP inhibitor-sensitivity to provide a new successful treatment approach for acute leukemia.

Disruption of Oxygen & Metabolic Sensing in Cancer

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Oxygen-sensing mechanisms are often dysfunctional in tumors. Oxygen sensing is mediated partly via prolyl hydroxylases, a family of enzymes that require molecular oxygen for enzymatic activity. Our work focuses on how prolyl hydroxylases execute apoptosis in neural precursors during development and how disruption of this process can lead to certain forms of nervous system tumors. Hydroxylase activity is not only dependent upon oxygen, but also equally dependent upon α;-ketoglutarate, a metabolite that takes part in the Krebs cycle. To identify novel oxygen sensing mechanisms, we are trying to identify novel EglN3 hydroxylation substrates. We recently have shown that EglN3 mediated apoptosis requires hydroxylation activity and is independent of HIFα; regulation. Therefore the identification of novel substrates will reveal mechanisms of how the prolyl hydroxylase EglN3 executes neuronal apoptosis. We have now completed a genome wide loss of function screen to identify EglN3 downstream targets that are required for apoptosis function. In an initial pilot screen we provided a proof of concept of this study and identified a novel tumor suppressor called KIF1Bβ, a kinesin motor protein located on chromosome 1p36.2, a region of the genome that is frequently deleted in neural crest derived tumors including neuroblastoma. Our current screen includes a new and more complex short hairpin library provided by the Broad Institute. Using this library we identified additional hits that are required for EglN3/KIF1Bβ mediated apoptosis. We therefore investigated further how this kinesin Kif1Bβ induces apoptosis. Interestingly, its kinesin domain is indispensible for apoptosis function. Large-scale affinity purification coupled with mass spectrometry identified KIF1Bβ binding partners, which specifically interact to the minimal region that is necessary and sufficient for apoptosis function. One such binding partner called DHX9 was identified in acting as a critical mediator of KIF1Bβ-induced apoptosis. Interestingly, localization studies revealed that cytoplasmic DHX9 locates towards the nucleus upon KIF1Bβ induction and that nuclear localization of DHX9 is neccesary for KIF1Bβ mediated apoptosis. Further, a loss of function screen for KIF1Bb apoptosis revealed several chromatin remodeling enzymes and transcription factors. We are now trying to understand what gene expression signature is mediatiated by DHX9 in the context of KIF1Bβ induction.

Involvement of Casein Kinase 2 in the resistance of glioma cells towards TNF-α mediated apoptosis

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Casein kinase-2 (CK2), a broad range kinase associated with cell survival and resistance to apoptosis, is over-expressed in several malignancies. CK2 induces aberrant NFkB activation and its inhibition has anti proliferative and anti-inflammatory effects. We observed an elevated CK2 level in Glioblastoma multiforme (GBM) tumor sample as compared to the surrounding normal tissue. GBM are resistant to TNFa induced apoptosis and blockade of TNF&α; induced NFkB activation sensitizes glioma cells to apoptosis. Since CK2 plays an important role in sustained NF&κ;B activation in several tumors, we investigated the potential of CK2 inhibitors (CK2-Is)- DRB (5,6-dichloro-1-beta-D-ribofuranosyl-benzimidazole) and Apigenin in sensitizing glioma cells to TNF&α;-induced apoptosis. Treatment with CK2-Is reduced glioma cell viability and sensitized cells to TNFa induced apoptosis in a Caspase 8 independent manner. TNFa induced NFkB activation was abrogated upon CK2 inhibition. CK2-Is decreased STAT3 phosphorylation and induced suppressor of cytokine signaling (SOCS-1) expression which is frequently silenced in GBM by its promoter methylation. SOCS-1 sensitized cells to TNFa and CK2-I induced death and down-regulated TNFa mediated release of pro-inflammatory cytokines. Importantly, we found activation of wild type p53 function in cells treated with CK2-Is in the presence and absence of TNFa. Activation of p53 function involved its increased transcriptional activation, DNA binding ability, increased expression of p53 target genes associated with cell cycle progression (p21) and apoptosis (GADD45b, NOXA), and decreased telomerase activity in CK2-Is treated cells. Since p53 and NF&κ;B drive opposing network circuitries associated with apoptosis and survival respectively, this ability of CK2-Is to sensitize glioma to TNFa induced cell death via multiple mechanisms involving abrogation of NFkB activation, SOCS-1 reintroduction and reactivation of wild type p53 function warrants investigation.

Voltage-Dependent Anion Channel-1-based peptides interact with hexokinase to prevent its anti-apoptotic activity

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Malignant cancer cells typically display high rates of glycolysis, even when fully oxygenated. Glycolysis offers several advantages to cancer cells, such as producing ATP faster than oxidative phosphorylation and providing precursors for anabolic biosynthesis. The high level of glycolysis in cancer cells is associated with overexpression of the glycolytic enzyme, hexokinase (HK), able to bind to the voltage-dependent anion channel (VDAC). This association appears to protect tumor cells from cell death. The HK–VDAC interaction offers an intriguing target for the selective triggering of cancer cell death. Our strategy aimed at disrupting the interaction between HK and VDAC involves VDAC1-based peptides. VDAC1 domains interacting with HK-I and HK-II were defined using site-directed mutagenesis. VDAC1-based peptides were subsequently designed and found to preferentially kill tumor cells. These synthetic peptides specifically bind to purified HK-I. The same VDAC1-based peptides also detached HK bound to brain or tumor-derived mitochondria. Moreover, expression of the VDAC1-based peptides in cells over-expressing HK-I or HK-II prevented HK-mediated protection against staurosporine-induced release of cytochrome c and subsequent cell death. A cell-penetrating VDAC1-based peptide entered the cell and prevented the anti-apoptotic effects of HK-I and HK-II. Both cell-expressed and exogenously added cell-penetrating peptide detached mitochondrial-bound HK-I-GFP. Furthermore, this cell-penetrating peptide induced apoptosis in several cancer cell lines but not in healthy lymphocytes. When tested on lymphocytes derived from B-cell chronic lymphocytic leukemia (CLL) patients, the peptide induced dramatic and selective apoptotic cell death of the cancer cells. These results suggest that HK over-expression in cancer cells promotes tumor cell survival through its direct interaction with VDAC1, inhibiting cytochrome c release and thereby, apoptotic cell death. Together, these findings suggest that interference with the anti-apoptotic effect of HK-I by VDAC1-based peptides may offer a novel strategy to potentiate the efficacy of conventional chemotherapeutic agents.

Varicose veins: relations between apoptosis, Bcl-2- associated protein Bax and NF-κ B

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Introduction: Endothelial cells (EC) regulate the permeability of blood vessels to leukocytes and inflammatory mediators. EC dysfunction promotes infiltration of inflammatory cells into the intima and increases the migration and proliferation of smooth muscle cells (SMC).With a heightened level of apoptosis (Ao) the expression of anti-coagulants by EC is decreased. Thus functional peculiarities caused by apoptosis as well as a patients age may contribute to an increased risk of varicosites. The intrinsic pathway of apoptosis is regulated by Bcl-2- associated protein x (Bax). Expression levels of apoptotic proteins have been reported to be upregulated by nuclear transcription factor κ B (NF-κ B). Vascular endothelial growth factor (VEGF) plays a centrale role in maintaining vascular integrity, and cyclooxygenase 2 (COX-2) may serve as an inducer to VEGFs in endothelial cells. We aimed to investigate the apoptosis of EC and SMC together with the Bax, NF-κ B, VEGF-R2, COX-2 and COX-1 expressions in the wall of varicose veins in women of different ages.
Methods: Women (n=34) undergoing the excision of varicose veins were divided into 3 groups: younger than 35 years (I), 35-50 years (II), older than 50 years (III). Apoptosis was determined in situ using the TUNEL method and the apoptotic cell percentage was counted in ECs and in SMCs. The Bax, NF-κ B, VEGF R2, COX-1 and COX-2 expressions were investigated immunohistochemically.
Results: The percentage of apoptotic EC and SMC of varicose veins increased in Group II as well as in Group III. Interestingly, the most intensive Bax staining was seen in the media and the endothelium in Group II, while in Groups I and III Bax staining intensity was equally lower. In contrary to Bax staining, the expression of NF-κ B and COX-2 was very low in the media in Group II. The VEGF R2 expression also decreased in middle-aged women (Group II) in the media. When the expression of Bax in Group III decreased in the media and the endothelium, the NF-κ B ,VEGF R2 and COX-2 expressions rose in the same group. There were no significant changes in the COX-1 level in the media but an increase was noticed in the endothelium in Group III.
Conclusion: Apoptosis of EC and SMC rose in older age group (Group III) and in the same group similar changes were seen in the expressions of NF-κ B, VEGF R2 and COX-2 in the endothelium and the media. Changes of the expression of Bax were different and its level rose in middle-aged women (Group II) but decreased in the older age group (Group III).

Docosahexaenoic acid is a promising agent sensitizing colon cancer cells to TRAIL-induced apoptosis

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Polynsaturated fatty acids (PUFAs) have been shown to influence the development of colorectal cancer. Docosahexaenoic acid (DHA) is a PUFA of n-3 series, with unique abilities that alow it to affect diverse physiological processes including cell membrane function and different cell signaling pathways, thus providing protection against various human diseases. It is also documented that DHA can modulate events that are associated with induction of apoptosis in cancer cells. We hypothesized that DHA exerts beneficial effects in sensitizing colon cancer cells to apoptosis induced by TRAIL (tumor necrosis factor-related apoptosis inducing ligand). TRAIL is a tumor necrosis factor family cytokine which selectively induces apoptosis in cancer cells while sparing normal cells. Because of these unique selective effects, it is considered as a promising anticancer agent. However, some cancer cells including colon are resistant to TRAIL-induced apoptosis. Here we showed that DHA can stimulate TRAIL-induced apoptosis in colon cancer cells, which was accompanied by enhanced activation of caspases-8 and -3, and cleavage of caspase substrates. At the same time, caspase-2 and -10 remained unaffected We also demonstrated an important role of mitochondrial apoptotic pathway in the effects observed, and bring evidence of potent stimulation of proapoptotic Bcl-2 family proteins such as Bax and Bak following combined treatments. A significant downregulation of selected inhibitor of apoptosis proteins (IAPs) was also observed in the cells treated with DHA and TRAIL. While pretreatment with physiologically relevant concentrations of DHA followed by subsequent treatment with TRAIL induced apoptosis in several different colon cancer cell lines, it was not harmful to normal colon epithelial cells. This supports the selective cytotoxic effects of the interesting drug combination on colon cancer cells. Our research highlights an important role of DHA in modulation of colon cancer sensitivity to TRAIL-induced apoptosis.

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Celecoxib counteracts curcumin-induced apoptosis via cell cycle perturbation in hematologic malignancies

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Chronic inflammation is an important tumor promoter by favoring cell proliferation and by inhibiting cell death. Many pro-inflammatory factors may be responsible for this effect, such as cyclooxygenase-2 (COX-2) or NF-κB. Molecules targeting these pathways have demonstrated beneficial effects in cancer prevention and treatment (1). Recently, the administration of anti-inflammatory drugs has appeared to be an effective strategy to inhibit tumor progression (2). However, these data concern mostly solid tumors and this strategy has not been fully investigated in hematologic malignancies. In this study, we studied the effects of celecoxib, a specific COX-2 inhibitor and curcumin, an anti-inflammatory natural product on different hematologic cancer cell lines (Hel, Jurkat, K562, Raji and U937). We found that both drugs separately induce apoptosis in all cell models. Further investigations on the most sensitive one, U937 cells, indicated that these effects were tightly associated with an accumulation of the cells in G0/G1 phase of cell cycle for celecoxib and in S and G2/M for curcumin, respectively. In the case of combination experiments, the pretreatment of U937 cells with celecoxib at non-apoptogenic concentrations, made the cells more resistant to curcumin-induced apoptosis. We found that this effect correlated with the prevention of the accumulation in S and G2/M phase of cell cycle induced by curcumin. Similar results have been obtained when celecoxib and curcumin were co-administrated at the same time. In literature, the induction of autophagy has been associated with an arrest of cell cycle in G0/G1 (3) and with chemoresistance (4). Our preliminary findings show that celecoxib treatment is associated with the appearance of autophagic features such as an accumulation of vesicles in the cytoplasm, an increase of lysosomal mass and the conversion of LC3-I to LC3-II, a well-known marker of autophagosomes. Our findings show that celecoxib counteracts the apoptotic effect of curcumin in U937 cells by accumulating the cells in the G0/G1 phase of cell cycle, possibly through an induction of autophagy. Moreover, our data underline a possible link between autophagy, cell cycle and apoptosis resistance.

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Conserved features of cancer cells define their sensitivity to HAMLET-induced death; c-Myc and glycolysis

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HAMLET is the first member of a new family of tumoricidal protein–lipid complexes that kill cancer cells broadly, while sparing healthy, differentiated cells. Many and diverse tumor cell types are sensitive to the lethal effect, suggesting that HAMLET identifies and activates conserved death pathways in cancer cells. Here, we investigated the molecular basis for the difference in sensitivity between cancer cells and healthy cells. Using a combination of small-hairpin RNA (shRNA) inhibition, proteomic and metabolomic technology, we identified the c-Myc oncogene as one essential determinant of HAMLET sensitivity. Increased c-Myc expression levels promoted sensitivity to HAMLET and shRNA knockdown of c-Myc suppressed the lethal response, suggesting that oncogenic transformation with c-Myc creates a HAMLET-sensitive phenotype. Furthermore, HAMLET sensitivity was modified by the glycolytic state of tumor cells. Glucose deprivation sensitized tumor cells to HAMLET-induced cell death and in the shRNA screen, hexokinase 1 (HK1), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 and hypoxia-inducible factor 1-α modified HAMLET sensitivity. HK1 was shown to bind HAMLET in a protein array containing ~8000 targets, and HK activity decreased within 15 min of HAMLET treatment, before morphological signs of tumor cell death. In parallel, HAMLET triggered rapid metabolic paralysis in carcinoma cells. Tumor cells were also shown to contain large amounts of oleic acid and its derivatives already after 15 min. The results identify HAMLET as a novel anti-cancer agent that kills tumor cells by exploiting unifying features of cancer cells such as oncogene addiction or the Warburg effect.

Role of microRNA machinery in mechanisms of lung cancer cells sensitivity/resistance to treatment

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Histopathologicaly lung cancer (LC) is divided into small cell lung carcinoma (SCLC), and non-small cell lung carcinoma (NSCLC). Despite high sensitivity to treatment, LC is characterized by development of severe resistance to therapy during the course of disease. Therefore, understanding of the mechanisms of resistance of LC to the current treatment modalities is of great interest. The microRNAs (miRNAs), a well-conserved and abundant class of regulatory RNA, were implicated as oncogenes or tumor suppressors in human cancers, including LC. Meanwhile, the associations of dysregulation of miRNAs with chemoresistance of human cancers are attracting more attention. Altered balance of proteins involved in production and function of miRNAs can contribute to the development of malignances in a miRNA-guided fashion and independently of the RNA interference pathway. Dramatic change in expression of most genes encoding proteins of the miRNA machinery was shown using the screening tissue microarray set and NSCLC cell lines. However, little is known about the role of proteins regulating miRNA synthesis and miRNAs in lung tumor proliferation and radiotherapy/drug resistance. The main goal of this study was to elucidate the role of microRNA synthesis machinery and expression of miRNAs in molecular shifts responsible for the malfunction of cell death of certain LCs and mechanisms of their resistance to treatment. The expression of core microRNA machinery proteins (Drosha, Dicer1, Exportin-5, Argonaute-2, Tudor-SN, PACT and FXR1) was assessed in a panel of NSCLC and SCLC with different sensitivity to radiation treatment (SF2 value). In parallel the profiling of miRNA expression was performed in the same panel using microarray approach. Analysis of protein expression revealed that Drosha, Dicer1 and Exportin-5 are expressed at higher level in radioresistant cells comparing to the sensitive ones within NSCLC panel. The number of microRNAs expressed in each cell line was also positively correlating with radioresistance of NSCLC. Based on these observations the knockdown of Dicer1, Drosha and Ago-2 proteins was accomplished using transfection of U1810 cells (representative resistant NSCLC cell line) with target siRNAs. Cells were subjected to radiation treatment (8 Gy) using Co60 source and cell death (PAPR cleavage, caspases processing and activity, sub-G1) was assessed 48h after treatment. We showed that down-regulation of Dicer 1, or Drosha or AGO2 do not sensitize U1810 cells to irradiation. Further, the effect of knockdown of studied proteins on sensitivity to treatment with DNA-damaging drug cisplatin was assessed in U1810 cells. Obtained data indicate that elimination of Dicer1 and Drosha but not Ago-2 results in decrease of apoptotic response to treatment with cisplatin. Overall, our results suggest the connection between altered expression of main proteins involved in microRNA biosynthesis and resistance of LC cells to DNA-damaging agents.

Primary enamel knot - apoptotic cell death, executive caspases and debris clearance

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Apoptotic cell death accompanies many stages of tooth development (odontogenesis), including odontoblast and ameloblast organisation in the course of tooth mineralization and final shape formation. However, the most evident apoptosis occurs during elimination of the signalling centres, enamel knots, where clear clusters of dying cells can be identified. In the first mouse molar, PEK appears at the embryonic day (E) 13 at the tip of the epithelial bud and becomes gradually eliminated by apoptosis around E 15.5. Additionally, we studied the third mouse molar, which develops postnatally and create a welcome comparative model to the first molar. In the third molar, PEK was visible at the postnatal day 3 and was confirmed by in situ hybridisation for Fgf-4 and Shh. Apoptotic bodies were evident in the next days, accompanying the gradual elimination of the PEK. As at PEK stages, vascularisation of the tooth germs just starts, neighbouring cells are considered to perform the clearance of apoptotic bodies. In the second part of this research, vascularisation, macrophage differentiation and their distribution related to position of forming vessels and apoptotic cells was investigated. Immunolabelling of VEGF and macrophage markers showed presence of macrophages around the tooth germ already at the stage ED 13. Previously, we have shown the primary enamel knot (PEK) apoptosis to proceed via mitochondrial mediated cell death as caspase-9 and caspase-3 mutants have no apoptosis in the PEK (Matalova et al. 2006, Setkova e al. 2007). Here we investigated, whether caspase-7 is engaged in the PEK apoptosis. Immunohistochemistry and mutant analysis were performed to achieve the goal. Activated caspase-7 was not detected in the PEK and the mutants displayed normal PEK apoptosis. However, activated caspase-7 was found in the forming jaw bone and our results in the mutants suggest its non-apoptotic role in osteoclast/osteoblast differentiation. Later stages are recently under study to reveal contribution of scavenger cells to the PEK clearance.

Matalová et al. (2006) Int J Dev Biol 50: 491-497
Setkova et al. (2007) Arch Oral Biol 52: 15-19
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Platelets induce proliferation of human bronchial smooth muscle cells through a mechanism dependent on 12-lipoxygenase and reactive oxygen species

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Increased proliferation has been shown to play an important role in asthma, a chronic inflammatory disease in which repeated cycles of damage and repair change the structure of the airways. These structural alteration, referred to airway remodelling, is a process that includes increased smooth muscle mass. Several cell types and inflammatory mediators have been suggested to contribute to airway remodelling and a growing amount of evidence support at role of platelets as important players in inflammatory airway diseases. 12-lipoxygenase (12-LOX) is an enzyme involved in the metabolism of arachidonic acid resulting in the metabolite 12-hydroxyeicosatetraenoic acid. 12-LOX is thought to be expressed in platelets but not in airway smooth muscle cells and inhibition of 12-LOX have been shown to induce apoptosis in e.g. human gastric cancer cells. The aim of the present study was to investigate the importance of 12-LOX in the interaction between platelets and human bronchial smooth muscle cells (HBSMC) and what impact it might have on reactive oxygen species (ROS)-production and platelet-induced proliferation. The interaction between platelets, and HBSMC was analysed morphologically by phalloidin staining of the F-actin and DAPI staining of the nucleus followed by fluorescence microscopy. Proliferation was measured by using the MTS assay and the fluorescent dye DCFDA was used to quantify ROS. Our results show that co-incubation of platelets and HBSMC results in a significant increase in proliferation (75%) and in intracellular ROS-production (35%). We also found that platelets bind to HBSMC and to the area surrounding the HBSMC and that platelets become activated and undergo shape change due to this interaction. Furthermore, we found that two substances known to inhibit 12-LOX, CDC and baicalein, both inhibited platelets ability to undergo shape change although had no effect on platelets ability to interact with HBSMC or the area surrounding the HBSMC. Interestingly, platelet-induced proliferation and ROS production was also reduced by CDC and baicalein. In conclusion, our results show that platelet activation initiated through interaction with HBSMC or the area surrounding the HBSMC is 12-LOX dependent and important for the mitogenic effect on HBSMC and on ROS-production generated after coincubation of HBSMC and platelets. This action of platelets represents a potential important and novel mechanism that may contribute to airway remodelling. The results may also have an impact in the development of new pharmacological strategies in the treatment of patients with inflammatory airway diseases.

Macrophages engulfing apoptotic cells produce retinoids to promote apoptosis and clearance of the neglected thymocytes

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T lymphocytes that mature to the double-positive (CD4+CD8+) stage and begin to express a productivelyrearranged TCRαbeta on their cell surface become susceptible to repertoire selection. Of these thymocytes 90% bear TCRs that cannot interact with self-MHC/peptide complexes and die by neglection. It is generally believed that apoptosis of these neglected cells is driven by the glucocorticoid hormone produced by thymic epithelial cells. Here we demonstrate that in vivo apoptosis induction of thymocytes triggers an enhanced retinoid production in the thymus. The retinoids are produced by macrophages engulfing apoptotic cells. Apoptotic cells uptake triggers retinaldehyde dehydrogenase expression via the three lipid sensing receptors LXR, PPAR gamma and PPAR delta. Retinoids than promote phagocytosis of apoptotic cells in a feed back way by enhancing the signaling via these receptors. In addition to enhancing phagocytosis, retinoids contribute to the appearance of transglutaminase 2 in thymocytes entering apoptosis, induce apoptosis in neglected thymocytes by activating a Nur77-dependent cell death program and promote the glucocorticoid-mediated apoptosis of thymocytes by stimulating the transcriptional activity of the glucocorticoid receptor. Our data show that besides glucocorticoids, retinoids contribute to the apoptosis regulation of immature thymocytes and reveal a complex interplay between apoptotic cells and engulfing macrophages in the in vivo apopto-phagocytosis program of the mouse thymus which ensures the fast death and removal of the high number of improperly produced thymocytes.

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Androgen depletion induces autophagy of LNCaP cells, a process which increases their resistance to apoptosis

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Standard treatments for high-grade/high-stage prostate cancer (PCa) are based on pharmacological or surgical androgen deprivation therapy. However, androgen deprivation is only temporarily efficient. After a few months or years without androgenic stimulation, the tumor inevitably relapses as hormone-refractory cancer (HRPCa) and progresses to metastatic disease. Although autophagy causes chemoresistance in other cancers, little is known about its role in the HRPCa transition. In this study on LNCaP cells, a model of HRPCa, we show that upon androgen deprivation or treatment with the anti-androgen bicalutamide (Bic), the conversion of LC3-I to LC3-II, a key early step in autophagosome formation and relocalization of LC3-GFP into autophagosomes were significantly increased. LC3-II accumulation was enhanced by the combination of lysosomal proteases inhibitors, E64D and pepstatin A, suggesting that androgen deprivation or Bic stimulated the rate of autophagy rather than inhibited LC3-II degradation. In contrast, LC3-I/II conversion and LC3-GFP relocalization were dramatically reduced after siRNA-mediated depletion of Atg5 and Beclin-1, two key genes involved in the early autophagic process. Both androgen deprivation and Bic decreased the phosphorylation level of Akt-S473 and p70S6k-T389, suggesting that autophagy induction by androgen receptor inhibition involved Akt/mTOR pathway. To further investigate the biochemical pathways involved in the androgen deprivation-induced autophagy, mRNAs from LNCaP cultured in the absence or presence of Bic were compared by a PCR array exploring the expression of 84 autophagy-related genes. Among these, levels of DAPK1 and ULK1 mRNAs, two kinases involved in autophagy and/or apoptosis were most dramatically increased in response to Bic. To assess if autophagy induced by androgen depletion or Bic treatment was a prosurvival mechanism or rather a cell death process, we analyzed cell death induced by Bic or androgen deprivation after Atg5 and Beclin-1 depletion. Depletion of both genes increased cell death, as determined by sub-G1 FACS analysis and dissipation of mitochondrial potential. From a therapeutical perspective, the anti-malarial drug, chloroquine, a well-known inhibitor of autophagy, dramatically potentiated cell death after androgen deprivation and Bic treatment. Taken together, our data suggest that autophagy is a protective mechanism against androgen deprivation in HRPCa cells and that restoration of hormone sensitivity in LNCaP cells could be achieved by concomitant treatment with the safe anti-malarial drug, chloroquine. We suggest these findings could pave the way to clinical trials using chloroquine in combination with anti-androgen therapy in HRPCa.

Discovery of an apoptosis-inducible compound, TLSC702, targeting human glyoxalase I

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The human glyoxalase I (hGLO I), which is a rate-limiting enzyme in the pathway for detoxification of apoptosis-inducible methylglyoxal (MG), has been expected as an attractive target for the development of new anti-cancer drugs. We have previously identified a natural compound myricetin as a substrate transition-state (Zn2+-bound MG-glutathione (GSH) hemithioacetal) mimetic inhibitor of hGLO I. Here, we constructed a hGLO I/inhibitor 4-point pharmacophore based on the binding mode of myricetin to hGLO I. Using this pharmacophore, in silico screening of chemical library was performed by docking study. Consequently, a new type of compound, which has a unique benzothiazole ring with a carboxyl group, named TLSC702, was found to inhibit hGLO I more effectively than S-p-bromobenzylglutathione (BBG), a well-known GSH analog inhibitor. Furthermore, TLSC702 inhibited cell proliferation and induced apoptosis in HL-60 cells. The computational simulation of the binding mode indicates the contribution of Zn2+-chelating carboxyl group of TLSC702 to the hGLO I inhibitory activity. This implies an important scaffold-hopping of myricetin to TLSC702. Thus, TLSC702 may be a valuable seed compound for the generation of a new lead of anti-cancer pharmaceuticals targeting hGLO I.

Dimer formation at the DISC: Interaction partners of Caspase-8

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Caspase-8, -10 and c-Flip are recruited to the CD95 DISC upon stimulation of the receptor. Experiments with substitution of DEDs by artificial dimerization domains or kosmotropic salts showed dimer formation and activation of these molecules. Nevertheless the exact dimer composition in cells has never been shown before. In order to visualize dimer formation of initiator caspases at the DISC in living cells, we applied an adapted bimolecular fluorescence complementation (BiFC) approach. The unique feature of this method is its ability to allow visualization of homodimers in a cellular context. The two proteins of interest, e.g. caspase-8 and -10, are coupled with two different halves of the fluorescent protein Venus. If the two nonfunctional fragments are brought into close proximity they are reunited and form the functional fluorescent protein. Fluorescence can be quantified via FACS or localization can be analyzed using confocal microscopy. We present data showing that caspase-8 forms homodimers upon anti-CD95 stimulation and heterodimers with c-Flip and caspase-10. We quantified interactions using flow cytometry and localization was analyzed via confocal microscopy. In the overexpression state we show homodimers of caspase-8 and heterodimers with c-Flip and caspase-10 in HeLa cells and HT1080 cells. Induction of apoptosis cannot be shown in these cells due to cytotoxicity of caspase-overexpression and possible impact of high endogenous caspase-8 expression in these cell lines. To exclude these effects we analyzed homodimer formation of caspase-8 in caspase-8-deficient Jurkat cells using active centre mutants of caspase-8. Stimulation of caspase-8-reconstituted cells with an anti-CD95 antibody leads to formation of homodimers. Untreated cells show a weak background fluorescence signal, which increases over the next 24 hours after anti-CD95 stimulation. Further analyses will show whether heterodimers are formed in caspase-8-deficient cells, as well, and how dimer composition regulates the outcome: apoptosis, proliferation or survival. We show for the first time in living cells that upon anti-CD95 stimulation caspase-8 homodimers are formed. These dimers are very likely to play a role in CD95-induced signaling leading to apoptosis. Caspase-8 interacts as well with caspase-10 and c-Flip. Therefore heterodimers of these molecules in CD95 signaling seem very probable.

Membrane Remodeling Induced by the Dynamin Related Protein Drp1 Stimulates Bax Oligomerization

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Apoptosis is an essential physiological process required for normal development and maintenance of tissue homeostasis. When misregulated, apoptosis can contribute to various diseases including cancer, autoimmune and neurodegenerative diseases. Although many of the components of the apoptotic machinery have been identified, our understanding of the mechanism of action of some of them is still incomplete. This is particularly true for several apoptosis-regulatory factors acting at the mitochondrial outer membrane (MOM) level, including Bcl-2 family proteins and components of the mitochondrial fission-fusion machinery. Bcl-2 family proteins can be divided into antiapoptotic members (e.g. Bcl-2 and Bcl-XL) and proapototic members, the latter including Bax-type proteins (e.g. Bax and Bak) and BH3-only proteins (e.g. tBid and Bim) [1]. The main function of Bcl-2 family protein is to regulate MOM permeabilization (MOMP). Bax-type proteins are thought to be direct effectors of MOMP. Although the molecular pathway by which Bax becomes activated remains ill defined, it is clear that (i) Bax is amphitropic protein that translocates from the cytosol to the mitochondria during apoptosis, (ii) shifting Bax from monomeric to an oligomeric state is a critical step in Bax activation. During the past decade, tBID and a mitochondrion specific-lipid cardiolipin (CL) were identified as two critical factors implicated in Bax activation at the MOM-level. Importantly, although tBid and CL can be sufficient for triggering Bax oligomerization under certain in vitro experimental conditions [2], it was also recognized that additional proteins are required to induce efficient Bax oligomerization in vivo [3]. However, the nature of these "missing" Bax-activating factors has remained elusive. Recently, we obtained different lines of evidence supporting a role for the Dynamin-Related Protein 1 (Drp1) as a Bax-activating factor [4]. Previously it was shown that upon apoptotic stimulation Drp1 is recruited to the MOM where it co-localizes with Bax at mitochondrial constriction sites [5] apparently implicated in mitochondrial division, but the connection between these phenomena and Bax-driven MOMP remained obscure. We now show that Drp1 stimulates tBid-induced Bax oligomerization and cytochrome c release by promoting tethering and hemifusion of membranes in vitro. This function of Drp1 is independent of its GTPase activity and relies on arginine 247 and the presence of CL in the membrane. In cells, overexpression of Drp1 R247A/E delays Bax oligomerization and cell death. In summary, our findings reveal a novel function of Drp1 and provide important insight into the mechanism of Bax activation and MOMP induction during apoptosis.

Retinoic acid induces upregulation of autophagy in acute promyelocytic leukemia cells: a pro-survival mechanism that occurs during the terminal differentiation

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Acute promyelocytic leukemia (APL) is characterized by an arrest of the terminal differentiation of myeloid cells into neutrophils/granulocytes. Administration of pharmacological doses of All-trans retinoic acid (ATRA) to APL patients produces a clinical remission of the disease by inducing the maturation of promyelocytes and the degradation of the PML-RARα; protein, leading to the eradication of the leukemic stem cells. Here, we demonstrate that both autophagy and Beclin 1, an autophagic protein, are upregulated during the course of ATRA-induced differentiation of an APL-derived cell line named NB4. Treatment of NB4 cells with ATRA results in Bcl-2 downregulation and mTOR activity inhibition, two responses known to be involved in the regulation of autophagy. Small interfering RNA (siRNA)-mediated knockdown of Beclin 1 expression does not affect ATRA-mediated differentiation of NB4 cells but increases the apoptotic death of the differentiated cells. Similar results were found when autophagy was inhibited by using a specific siRNA against ULK1. These results demonstrate the anti-apoptotic function of Beclin 1 and ULK1 during the course of ATRA-induced differentiation of APL-derived cell line. The clinical significance of this pro-survival role of Beclin 1 and ULK1 in the outcome of APL patients treated with ATRA, in particular those who develop ATRA syndrome caused by an accumulation of mature cells, deserves to be investigated.

Synergistic induction of melanoma cell death through modulation of autophagy by pharmacological inhibitors of p38 MAPK and PI3K signaling

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Metastatic melanoma is often refractory to available systemic treatments and is associated with poor prognosis for the majority of patients. Much of current melanoma research therefore focuses on the identification of cellular pathways required for tumor growth and survival that could be therapeutically targeted in patients. In line with this effort, we studied the responses of model melanoma cell lines to SB202190, a small molecule inhibitor of the p38 mitogen-activated protein kinase (MAPK) pathway. While the inhibitor had only a minor effect on cell proliferation, we observed a strong increase in cellular vacuolization and upregulation of autophagy in SB202190-treated cells. Autophagy inhibitors Bafilomycin A1 and 3-methyladenine significantly reduced the viability of SB202190-treated cells by triggering p53-independent apoptosis, suggesting a protective role for SB202190-induced autophagy in melanoma cells. Importantly, class III phosphatidylinositol 3-kinase (PI3K) enzymes have been reported to be involved in the early stages of autophagy and this led us to hypothesize that pan-specific inhibitors targeting multiple classes of PI3Ks might be able to synergize with p38 inhibitors in melanoma cells. Experimental tests showed that the combination of SB202190 with a pan-specific PI3K inhibitor LY294002 can synergistically activate melanoma cell death. Taken together, our results show that pharmacological inhibition of the p38 MAPK pathway and simultaneous modulation of autophagy can influence melanoma cell viability and provide a rationale for developing novel anti-melanoma therapeutic strategies combining the cellular activities of small molecule drugs targeting the p38 MAPK and PI3K signaling pathways.

Inhibition of photoinduced apoptosis of crayfish glial cells mediated by glutamate and N-acetylaspartylglutamate

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Objectives. Intercellular interactions play an important role in responses of a complex tissue to external impacts such as oxidative stress induced by photodynamic treatment. In the crayfish nervous system, stimulated axons are known to release N-acetylaspartylglutamate (NAAG), which is split by N-acetylaspartyl and L-glutamate by glutamate carboxypeptidase II. We studied the role of neuroglial interactions based on glutamate-mediated signaling in photodynamic injury of crayfish mechanoreceptor neurons and surrounding glial cells (GC).

Methods. Possible involvement of NAAG, glutamate and its receptors in photoinduced necrosis and apoptosis of crayfish neurons and GC was evaluated using inhibitors, ion channel agonists and blockers. Alumophthalocyanine Photosens was used as a photosensitizer, diode laser (670 nm) as a light source.

Results. Glutamate and NAAG significantly decreased photoinduced GC apoptosis but not necrosis of glial cells and neurons. PBDA, an inhibitor of glutamate carboxypeptidase II, significantly increased photoinduced apoptosis of GC but did not influence necrosis of neurons and glia. This confirmed the involvement of NAAG in the anti-apoptotic action on GC. NMDA, an agonist of ionotropic glutamate receptors or their blocker MK-801 did not influence photoinduced necrosis and apoptosis of GC. Therefore, NMDA receptors did not participate in photoinduced death of glial cells.

Conclusion. The present data indicated the protective, antiapoptotic effect of glutamate and its natural precursor NAAG in the crayfish nervous system on photoinduced apoptosis of crayfish glial cells. These compounds played presumably a mediator role in the protective effect of crayfish neurons on surrounding glial cells. Their action was possibly associated with metabotropic but not ionotropic glutamate receptors.

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DNA methylation machineries are responsible for the total or partial methylation of the bax gene in human gliomas.

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Aberrant DNA methylation occurs frequently during tumorigenesis and is considered as a major oncogenic event in many cancers. We have observed that the expression of Bax was under the control of its promoter methylation and was correlated with patient survival in gliobastoma multiforme (GBM) (Cartron et al., Hum. Mol. Genet.2002; 11: 675-82). Here we confirm that the total methylation of the Bax promoter induced its silencing in GBM and is associated with a poor prognosis while its partial methylation led to the expression of a N-terminal truncated form of Bax, Bax psi, which is associated with a longer survival in GBM. Using primary cultures of human gliomas, we found that both common and distinct DNA methylation machineries are responsible for the total or partial methylation of bax gene through specific interactions of transcription factors (TF) with demethyltransferases (Dnmts). We also found that these TF/Dnmts interactions are differentially implicated in the methylation of genes such as mgmt, blu or socs3. Taken together our results suggest that DNA methylation of an essential pro-apoptotic gene is correlated with that of other genes also involved in survival / response to treatments in human gliomas. Thus, the development of therapeutic approaches using DNA demethylating drugs acting as activators of the intrinsic apoptotic pathway and inhibitors of survival mechanisms might lead to a major therapeutic improvement in the treatment of human gliomas with poor prognosis.

Caspase-6 cleaves RIPK1 and prevents cell-death induced sterile inflammation

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Two main forms of programmed cell death have been described: Apoptosis and necroptosis. The first is caspase-dependent and is a clean form of cell death, whereby the immune system is not activated, whereas the latter depends on the sequential activation of the receptor-interacting protein kinases (RIPKs) 1 and 3 and results in the potent induction of inflammation. During apoptosis, the RIPKs are cleaved in a caspase-dependent manner, preventing the late-phase RIPK1-dependent production of pro-inflammatory cytokines. It is commonly assumed that caspase-8 cleaves the RIPKs. However, we show here that RIPK1 cleavage occurs during intrinsically triggered apoptosis in the absence of caspase-8 activation. Instead, we demonstrate that caspase-6 potently cleaves RIPK1, downstream of caspase-3 activation, thus preventing the late-phase production of tumor necrosis factor-α (TNF-α) and other pro-inflammatory cytokines. Caspase-8 is activated either through homodimerization or heterodimerization with the inactive caspase-8 homologue FLIPL. The homodimer of caspase-8 has a pro-apoptotic function, whereas the heterodimer, which is preferentially formed, has a proliferative function in the absence of caspase-8 cleavage. We furthermore show that caspase-6 cleaves caspase-8 in the intersubunit linker, thus turning the proliferative heterodimer in a pro-apoptotic species of caspase-8. Finally, we demonstrate that full activation of caspase-6 crucially depends on cleavage by caspase-3, whereas auto-processed caspase-6 has limited potential. This finding suggests that the function of caspase-6 is restricted to apoptosis. However, the implications of these findings are still under investigation.

A comprehensive LPS-induced shock study unveils its complexity involving inflammation, apoptosis and necrosis

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The primary contribution of inflammatory and cell death pathways in the pathology of septic shock is only fragmentarily reported. To clarify this issue, we analyzed the susceptibility of CASP1-, CASP3-, CASP7-, RIPK3- and IL1β/IL18-deficient mice for LPS-induced shock in one comprehensive study. We found that either CASP1- or IL1β/IL18-deficiency, but not CASP7-deficiency, fully protected against LPS-induced mortality, demonstrating that caspase-7 as an apoptotic executioner caspase downstream of caspase-1, is not implicated. Interestingly, CASP3- and RIPK3-deficient mice were partially protected, indicating an additional apoptotic and necrotic component, respectively. Protection against LPS-induced shock correlated with reduced systemic inflammatory cytokine levels and sustained Goblet cell numbers, but not with splenocyte cell death. Corroborating the importance of CASP1 and CASP3, their joint inhibition with qVDoph strongly protected, indicating that specific caspase inhibition could still be of therapeutic value.

Endonuclease G interacts with histone H2B, AIF, and DNA topoisomerase II α during apoptosis as revealed by FRET analysis of living cells

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Apoptosis is a natural form of cell death involved in many physiological changes in the cell. During some forms of cell death, proteins endonuclease G (EndoG) and apoptosis-inducing factor (AIF) are released from mitochondria and then they translocate into the cell nuclei, where they participate in chromatin degradation in a caspase-independent way. The C. elegans homolog of AIF was shown to induce apoptosis and to interact with a homolog of EndoG and together they mediated chromatin DNA degradation. Interaction of human AIF and EndoG has not yet been shown, although one result from in vitro protein analysis suggests its possibility. Also interactions of EndoG and AIF with other proteins inside cell nucleus are studied in this work. We have conducted living-cell confocal fluorescence microscopy followed by analysis of fluorescence resonance energy transfer (FRET) to observe the protein interaction of EndoG with AIF and their interactions with other proteins in human cell nuclei after induction of apoptosis. Our results show that EndoG interacts with histone H2B, AIF, and DNA topoisomerase II α (TOPO2a). Also AIF was found to interact with TOPO2a. Therefore we can conclude that EndoG, AIF, and TOPO2a may form a protein complex allowing chromatin degradation in apoptotic nucleus. These results offer an important insight into the mechanism of apoptotic cell death, which plays a major role in development and progression of degenerative diseases, cancer, and inflammation.

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Iminophosphorane-organogold (III) complexes induce cell death through mitochondrial ROS production.

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During the last few years there has been a remarkable increase in the interest of gold (I) and gold (III) derivative compounds to be applied in cancer chemotherapy. The high toxicity exhibited by gold (III) complexes against solid tumour cancers in combination with a minor systemic toxic effect is probably the main reason for that renewed interest. We have studied the cellular effects exerted by three different iminophosphorane-organo gold (III) compounds in leukemia cells (a neutral compound with two chloride ligands, 1¸and two cationic compounds with either a dithiocarbamate ligans 2¸or a water-soluble phosphine with a chloride ligand 3). Our work has focused mainly on the role that Bcl-2 family of proteins, caspases and ROS production could play in the cellular events activated by these compounds. We observed a remarkable toxic effect of the three compounds against T-cell leukemia Jurkat cells and B-CLL leukemia cells. Nevertheless, normal lymphocytes exhibited a considerably higher resistance. Experiments concerning typical apoptotic morphologic features led us to conclude that cell death mechanisms triggered by these compounds implicate both apoptosis and necrosis. Further analysis of the cell death pathways activated after the treatment with the compounds pointed to a direct implication of mitochondrial ROS generation.

PERK is an essential component of the ER-to-mitochondria contact sites required to convey mitochondrial apoptosis following ROS-mediated ER stress

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Endoplasmic reticulum (ER)-stress and subsequent activation of the unfolded protein response (UPR) can either promote cellular survival but also play an important role in various physiopathological conditions as well as in cancer cell death in response to certain anticancer treatments. The molecular effectors that mediate the switch from a pro-survival response to pro- death signaling are being thoroughly scrutinized. ER stress induced cell death is often caused or associated with an increase in reactive oxygen species (ROS), but how these molecules modulate the molecular events leading to ER stress-mediated mitochondrial apoptosis is currently unknown. To investigate this link in more detail, we used a new approach to generate ROS at the ER. This entails the irradiation of a photosensitizer (hypericin) that localizes preferentially to the ER membranes. Subsequently, high and local concentrations of ROS are generated, which then cause rapid depletion of ER Ca2+, disturbing ER homeostasis and ultimately leading to apoptotic cell death. This rapid and selective burst in ROS generation at the ER, which we dubbed as 'photo-oxidative ER stress (phox-ER stress)', is furthermore associated with the induction of a cluster of UPR genes as was shown in previous work [1], indicating the functional propagation of an ER stress response. Here we show that MEFs deficient for PKR-like ER kinase (PERK), one of the three mammalian sensors of ER stress, in contrast to canonical ER stressors like thapsigargin, are protected from phox-ER stress and H2O2 induced cell death compared to their WT counterparts. This was illustrated by a delay in cytochrome c release, caspase activation and PARP cleavage as well as a strong reduction in mitochondrial membrane depolarization. Moreover, phox-ER stressed PERK-deficient cells display increased clonogenic survival as compared to the treated WT MEFs. Further analysis of these cells revealed that PERK-/- cells not only display an aberrant ER morphology resulting in disturbed Ca2+ signaling but also a significant reduction in the amount of contact sites with mitochondria. Furthermore, while in WT MEFs phox-ER stress resulted in a substantial increase/stabilization of these contact sites, no change in ER-to mitochondria interaction was observed for PERK-/- MEFs. Interestingly, weakening the ER-mitochondria juxtapositions confers protection specifically against ROS mediated ER stress-induced cell death, by hampering the propagation of oxidative damage between these organelles, as shown by a significant reduction of oxidized cardiolipin following phox ER-stress in PERK-/- cells. Finally, subcellular fractionation using mouse liver and a human cancer cell line indicated that PERK is enriched at the mitochondria associated membranes (MAMs). Thus, our study reveals an unprecedented role of PERK as a MAMs component crucially involved in maintaining ER-mitochondria juxtapositions, which are required to propel the pro-apoptotic branch of the UPR following ROS-mediated ER stress.

Cell death induced by the novel V-ATPase inhibitor archazolid B depends on autophagy and the mitochondrium.

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Archazolid B, a macrolide produced by the myxobacterium archangium gephyra inhibits the function of the V-ATPase, a proton pump responsible for pH regulation, by binding to the subunit c. It is reported that the V-ATpase is overexpressed in many tumor cells which correlates with a resistance towards chemotherapeutics in these tumor cells. Not much is known about the apoptosis inducing properties of archazolid B and V-ATPase inhibitors in general. This is why we investigated the effects of archazold B on the highly resistant breast cancer cell line SKBR3. In first results we could show that treatment with 10 nM archazolid B for 48 hours leads to apoptosis induction in SKBR3 cells which could be inhibited by a pan-caspase inhibitor. Furthermore, incubation for short time points lead to the induction of autophagy. Autophagy was detected by the conversion of the autophagy marker LC3I to LC3II and by the formation of autophagosomes. A decrease of mitochondrial membrane potential, a downregulation of anti-apoptotic Bcl-2 members and a strong activation of the pro-apoptotic Bcl-2 member BNIP3 were indicators of archazolid B induced apoptosis. BNIP3 is a BH-3 only protein which integrates to the mitochondrial membrane and its activation promotes either apoptosis or non-apoptotic cell death such as autophagy. We propose that cell death induced by archazolid does not just involve normal apoptosis but also autophagy. In further investigations we want to analyze the molecular mechanisms of apoptosis induction and elucidate the role of autophagy induced by archazolid B.

Smac mimetic sensitizes glioblastoma cells to temozolomide-induced apoptosis via NF-κB activation and RIP1

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Since Inhibitor of Apoptosis (IAP) proteins are expressed at high levels in many cancers, they represent attractive targets for therapeutic intervention. Here, we report for the first time that the Smac mimetic BV6 sensitizes glioblastoma cells towards temozolomide (TMZ), the first line chemotherapeutic agent in the treatment of glioblastoma, in a NF-κB- and RIP1-dependent manner independently of an autocrine/paracrine TNFα; loop. BV6 and TMZ synergistically reduce cell viability and trigger apoptosis in glioblastoma cells (combination index <0.4-0.8), which is accompanied by increased loss of mitochondrial membrane potential, cytochrome c release, caspase activation and caspase-dependent apoptosis. Analysis of the molecular mechanisms reveals that BV6 causes rapid degradation of cIAP1 leading to stabilization of NIK and NF-κB activation. Interestingly, BV6-stimulated NF-κB activation is critically required for sensitization towards TMZ, since inhibition of NF-κB by overexpression of the mutant IκBα; superrepressor profoundly reduces loss of mitochondrial membrane potential, cytochrome c release, caspase activation and apoptosis. By comparison, BV6-mediated sensitization to TMZ does not involve increased TNFα; mRNA expression. Also, the TNFα; blocking antibody Enbrel does not prevent the combination treatment-induced activation of caspases or apoptosis, pointing to a TNFα;-independent mechanism. Interestingly, BV6 and TMZ cooperate to trigger the formation of a caspase-8/RIP1/FADD complex independently of TNFα;. Transient knockdown of RIP1 by siRNA significantly reduces BV6- and TMZ-induced caspase-8 activation and apoptosis, showing that RIP1 is critical for apoptosis induction. These findings build the rationale for further (pre)clinical development of Smac mimetics in combination with TMZ as a novel treatment approach in glioblastoma.

Methylselenol formed by spontaneous methylation of selenide is a superior selenium substrate to the thioredoxin and glutaredoxin systems

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Selenium compounds can undergo reduction and form selenide. This highly reactive form of selenium can be methylated and form monomethylated and multimethylated compounds. Selenite and selenodiglutathione can efficiently be reduced by the mammalian thioredoxin and glutaredoxin systems. In the presence of SAM the kinetics of the well-established non-stoichiometric reactions of selenite/GS-Se-SG and the thioredoxin and glutaredoxin systems changed with a threefold increased velocity aerobically, indicating the formation of a novel metabolite, which is a superior substrate to both the thioredoxin and glutaredoxin systems. By LDI-MS, we identified the novel compound as methylselenol. In vitro cell experiments demonstrated that the presence of SAM increased the cytotoxicity of selenite and selenodiglutathione, which could neither be explained by altered selenium uptake nor impaired extra cellular redox environment. Our data suggest that selenide and SAM reacts spontaneously forming methylselenol, a highly nucleophilic and cytotoxic agent.

Differential responsiveness of SENP3 to ROS levels and the consequent interaction with p300 are responsible for the biphasic redox regulation of HIF-1 transactivation

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Oxidative stress, a common challenge to cellular homeostasis, is caused predominantly through the excessive production of reactive oxygen species (ROS). The extent of the increase of ROS production usually determines the consequences of cellular response to oxidative stress, during which global alterations of gene expression pattern and protein post-translational modifications are differentially regulated. Previous studies showed that a mild oxidative stress stabilizes HIF-1α and enhance HIF-1 transactivation, while a severe oxidative stress suppresses HIF-1 transactivation. However the mechanism of the biphasic redox regulation of HIF-1 transactivity has not been elucidated. We took HeLa cells exposed to a series of concentrations of hydrogen peroxide as a simple model of mild and severe oxidative stress, to investigate the effects of ROS to HIF-1 activation and the SUMOylation status of p300, a transcriptional coactivator of HIF-1, and the protein level and enzymal activity of SENP3, a SUMO protease that deSUMOylate p300. We found that SENP3 was stabilized by a mild oxidative stress, but its enzymal activity was inactivated by a severe oxidative stress, thus rendering opposite SUMOylation status to its substrate p300 as we as HIF-1 transactivation. These results suggest that the biphasic regulation of HIF-1 transactivation by ROS is correlated with and dependent on the differential SENP3/p300 interaction and consequent SUMOylation status of p300. This study reports for the first time that different cysteine sites of the same protein molecule can sense different levels of oxidative stress so as to modulate the protein function and generate diverse cellular responses. This investigation reveals the mechanism underlying that the function of proteins is regulated by ROS, and provides new insight for understanding how ROS influence the fate of tumor cells.

Lysosomal Chymotrypsin B Potentiates Apoptosis via Cleavage of Bid

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Apoptosis is a fundamental physiological process in mammals in which cells die by activating a suicide mechanism. Mitochondria serve as sensors and amplifiers of cellular damage are a major checkpoint of apoptotic regulation. Recently accumulating evidence has indicated that, in addition to mitochondria, lysosomes are tightly linked with apoptotic signaling. During our study of Bid-mediated mitochondrial outer membrane permeabilization (MOMP), an unknown caspase-8-like activity capable of converting Bid into truncated Bid (tBid) at neutral pH was detected in highly purified rat liver lysosomal extracts, which was eventually identified as chymotrypsin B. We further provide evidence indicating that chymotrypsin B is widely expressed in different rat tissues including cerebrum, cerebellum, heart, lung, liver, kidney, and spleen in the form of both activated enzyme and zymogen (chymotrypsinogen B). Upon exposure to different apoptotic stimuli such as oxidative stress (H2O2) or free fatty acids (palmitate), lysosomal chymotrypsin B is released into the cytosol of RH-35 hepatoma cells as a result of lysosomal membrane permeabilization (LMP) via a caspase-8- and Bid-dependent mechanism. Transient overexpression of recombinant chymotrypsin B significantly increased H2O2-induced apoptotic cells, while silencing the endogenous chymotrypsin B expression showed a statistically significant reduction on H2O2-induced apoptosis, suggesting that endogenous chymotrypsin B is involved in apoptosis. LMP was found to be an upstream event of MOMP. A low concentration of tBid is sufficient to initiate LMP. Chymotrypsin B, but not cathepsin B or cathepsin D, significantly cleaves Bid at cytosolic conditions (pH 7.4), thereby presumably initiating a positive feedback leading to the accumulation of cellular tBid, which eventually results in MOMP and apoptosis. Thus, our findings further demonstrate that lysosomal chymotrypsin B is an important molecule that mediates apoptosis via a Bid-dependent mechanism.

Epithelial FADD Regulates Intestinal Homeostasis

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The maintenance of immune homeostasis in the gut depends on a tightly regulated cross talk between mucosal immune cells, intestinal epithelial cells and commensal bacteria. The intestinal epithelium consists of a single cell layer of tightly connected cells separating the intestinal lumen from the mucosa. Intestinal epithelial cells (IECs) provide a structural barrier preventing translocation of luminal bacteria into the mucosa but they also actively participate in the intestinal antimicrobial defense by secreting antimicrobial factors. In addition, IECs perform immunoregulatory functions that are believed to prevent the mucosal immune system from overreacting to harmless luminal antigens. The intestinal epithelium therefore has a very important role in the maintenance of intestinal homeostasis and disruption of the intestinal barrier is being discussed as one mechanism leading to Inflammatory Bowel Diseases (IBD). Among many regulators of inflammation, TNF plays a pivotal role in the pathogenesis of IBD, exemplified by the efficacy of anti-TNF treatment in a subset of IBD patients. To study the role of death receptor signalling in the intestinal epithelium, we generated mice with IEC-specific knockout of FADD (FADD-IEC-KO mice), a central adaptor in death receptor induced apoptosis. FADD-IEC-KO mice spontaneously developed severe erosive colitis, loss of Paneth cells and enteritis. Increased numbers of dying IECs showing morphological features of necrosis were present in the intestinal epithelium of FADD-IEC-KO mice. Genetic ablation of RIP3, a central regulator of programmed necrosis, prevented IEC death and inflammation in FADD-IEC-KO mice, demonstrating that RIP3-mediated necrosis of FADD-deficient epithelial cells causes intestinal inflammation. Genetic experiments revealed important roles for TNF, the intestinal microbiota and MyD88-dependent signalling in the pathogenesis of intestinal inflammation in FADD-IEC-KO mice. These findings suggest that inhibition of RIP3 dependent cell death by FADD is essential for intestinal homeostasis and that programmed necrosis might have a role in the pathogenesis IBD, where defects in Paneth cells and impairment of the epithelial barrier have been suggested to contribute to intestinal inflammation.

Stabilization of c-Myc directs transcription of NOXA and BIM

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The c-Myc oncogene directs a varity of tumor promoting programs in tumor cells. Besides proliferation c-myc can also trigger apoptosis under distinct conditions. The molecular mechanisms of c-Myc induced apoptosis is still incomplete understood. In this study we analysed c-Myc-dependent apoptosis in pancreatic cancer cells, melanoma cells and genetically defined mouse embryonic fibroblasts (MEFs). We used the proteasome inhibitor bortezomib for c-Myc protein stabilisation. Bortezomibinduced apoptosis was blocked with c-myc specific siRNAs and an c-myc inhibitor (10058-F4). Consistent, deletion of both c-myc alleles by Tamoxifen treatment of 3T9-Mycfl/flCreER MEFs leads to inhibition of bortezomib-induced apoptosis, We further analysed the expression of the pro-apoptotic BCL2 family members and observed mRNA and protein induction of the BH3-only NOXA after 4 hours of bortezomib treatment. The induction of NOXA could be reverted after c-Myc inhibition using RNAi and small molecule inhibition, suggesting a direct transcriptional role for c-Myc. Consistent with a function of NOXA in the bortezomib response, NOXA knockout MEFs were partially protected from bortezomib-induced cell death. Interestingly, bortezomib-induced apoptosis of NOXA knockout MEFs was completely blocked by c-Myc inhibtion. We detected induction of the BH3-only protein BIM in NOXA knockout MEFs. BIM was also induced in tumor cells by the bortezomib treatment in an c-myc-dependent fashion. Furthermore, the contribution of BIM towards Bortezomib induced apoptosis was demonstrated with RNAi. To show direct binding of c-myc to the NOXA and BIM promoters we used quantitative promoter scanning chromatin immunoprezipitations. We observed bortezomib-induced direct binding of c-Myc, accompanied by RNA polymerase II recruitment, to non-canonical binding sites at the proximal promoter of the NOXA and BIM gene. All together, our data demonstrate that c-myc controls transcription of NOXA and BIM genes to efficiently induce apoptosis in response to the proteasome inhibitor bortezomib.

The role of the anti-apoptotic enzyme paraoxonase-2 in hematopoetic stem cell development

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Cancer therapies are often hampered as tumor cells acquire apoptosis resistance. Because many malignant cells are under pro-oxidant stress, pharmacological interference of redox-balance in cancer cells gained increasing attention. We recently found that the anti-oxidative human enzyme paraoxonase-2 (PON2) plays a significant role in apoptosis resistance of tumor cells and their sensitivity to chemotherapeutics. PON2 localizes to both the endoplasmic reticulum (ER) and mitochondria. Moreover, PON2 was demonstrated to suppress stress-induced JNK activation, CHOP expression and pro-apoptotic generation of oxidative stress (i.e. superoxide) which together lowered mitochondrial cytochrome C release. Concordantly, PON2 expression levels negatively correlated with cellular rates of apoptosis: cell death was strongly reduced upon PON2 overexpression, whereas PON2 knock-down increased cell death and even caused spontaneous apoptosis in selected tumor lines, among them K562, a human chronic myeloid leukemia blast crisis line. In addition, imatinib-induced K562 cell death was enhanced upon PON2 deficiency, arguing for additive effects. This is in line with the reported association of PON2 levels with pediatric ALLs and imatinib resistance in CML patients. We therefore performed pathway finder arrays to reveal which of the main cellular pathways are deregulated in response to PON2 deficiency causing spontaneous death of blast crisis cells. In addition, because redox signaling is regulated by PON2 and since redox-triggered pathways controls senescence, apoptosis, differentiation and self-renewal of hematopoetic stem cells, we next analyzed the hematopoetic system of PON2-/- mice. The studies revealed significant abnormalities, i.e. an altered number of long-term and short-term hematopoetic stem cells, together with an imbalance in subpopulations as granulocyte / monocytes progenitors, or structural aspects of erythrocytes and thrombocytes. In order to reveal underlying mechanisms and the role of PON2 in stem cell differentiation, we addressed oxidative stress and apoptosis rates in such cells. Our studies thus suggest that the anti-oxidative enzyme PON2 plays a yet undiscovered role in hematopoetic stem cells which emphasizes the importance of physiologic redox signaling.

PARP-1 and PARP-2 mobilize Ca2+ from intra- and extracellular sources in oxidant-induced cell death.

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Aim: An increase of cytosolic Ca2+ is a harmful event in cell physiology that can lead to a variety of complex responses ranging from survival to cell death. Therefore cytosolic changes of Ca2+ concentrations can be used as a marker for cell stress. In this study we investigated the involvement of the DNA damage sensor enzymes poly(ADP-ribose) polymerases 1 and 2 (PARP-1, PARP-2) as modulating factors for cytosolic Ca2+ shifts in cells challenged with a killing dose of H2O2.
Methods: High levels of cell death were induced by H2O2 in wildtype and parp-1-/- mouse embryonic fibroblasts (MEF). Cytosolic Ca2+ alterations as an immediate response to the cytotoxic challenge were measured in a fluospectrometric assay using the high affinity Ca2+ binding dye Fluo-4 up to 30 min. The distinct roles for PARP enzymes were analyzed by either chemical inhibition or RNAi approaches in both wildtype and parp-1-/- MEFs. Moreover the involvement of the transmembrane and lysosomal Ca2+ gating channel TRPM2, known to function in PARP dependent cell death, was analyzed using a chemical inhibitor and RNAi, respectively.
Results: - Parp-1-/- cells are more resistant to H2O2 compared to their wildtype counterparts.
- Under killing conditions both cell types show an increase of cytosolic Ca2+ levels, but with different kinetics.
- The shifts of cytosolic Ca2+ after H2O2 result from mobilization of extra- and intracellular Ca2+ sources in both cell types.
- Whereas PARP-1 is the major upstream regulator of the influx of extracellular Ca2+ in wildtype cells, a role for PARP-2 is negligible.
- The stress-induced activity of PARP-1 leads to IP3 mediated and TRPM2 gated Ca2+ via monomeric ADP-ribose.
- PARP-2 can mobilize Ca2+ from internal TRPM2 mediated Ca2+ sources in the absence of PARP-1.
Conclusion: PARP-1 and PARP-2 regulate alterations of cytosolic Ca2+ in H2O2-induced cell death. This Ca2+ originates from the combined activity of transmembane and lysosomal TRPM2 channels and through IP3 mediated mechanisms.

Dual Roles of Caspase-2 in response to DNA Damage:DNA Repair and Mitotic Catastrophe

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Caspase-2 is one of the most evolutionary conserved caspases and is unique caspase only constitutively present in cell nucleus. Pro-caspase-2 has been proposed to form several kinds of complex with other protein(s). Caspase-2 has been implicated in oocyte death, in DNA damage- and heat shock-induced death and in mitotic catastrophe, yet neither how caspase-2 responses to DNA damage nor to which caspase-2 associates in mitotic catastrophe is clear. cIAP-1,the most studied member of inhibitor of apoptosis proteins(IAPs) as ubiquitin E3 ligase, can induce ubiquitination of related proteins such as TRAF2, RIP1 or caspase-3 and therefore play import roles in NF-κB signaling and apoptosis. In addition, cIAP-1 BIR2 domain had been suggested to interact with Caspase-2 and inhibit caspase-2 activity in cell free system. We reported here that procaspase-2 as well as activated caspase-2 interacted with cIAP-1 in head neck squamous carcinoma cells (HNSCCs) after radiation or etoposide treatments. cIAP-1 was localized in nucleoplasm.. cIAP-1 knockdown caused sustaining DNA damage during 24h. Smac mimic compound SM-164 mediated cIAP-1 degradation and thus led to impairment of DNA repair. Meanwhile, caspase-2 knockdown regained clonogenic formational capacity of radio-resistant HNSCC after radiation, and specific inhibitor of caspase-2 Z-VDVAD abolished mitotic catastrophe 38h after radiation. Taken together, these data demonstrated that caspase-2 might play dual roles in different phases in response to DNA damage: DNA repair in early time and mitotic catastrophe in later time, through interaction with cIAP-1. Thus, both of Caspase-2 and cIAP-1 hold promises for future clinical development as novel radiosensitizer targets for the treatment of a subset of head and neck cancer patients.

Expression changes of autophagy-related genes as monitoring tools for programmed cell death

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Autophagy has been recognized as self-eating process of the cells during starvation, and thought as programmed cell death as like apoptosis. Recent extensive works have revealed that the autophagy is conserved from yeast to mammalian cells. So far ATG7 and BECN1 have been shown to be required for autophagic cell death. BECN1 is also known as a haploinsufficient tumor suppressor gene indicating that transcriptional regulation of autophagy-related gene is significant for its biological function. DRAM, a p53-induced modulator of autophagy, has been shown to play an important role for apoptosis as well as autophagy, suggesting that autophagy-related gene might play a bifurcation control between death and survive. More than a few dozens of autophagy-related genes have been identified in mammalian cells; however, their transcriptional regulation during programmed cell death is still elusive. In the present work, we intended to reveal unique autophagy-related genes whose transcriptional regulation could be applied as unique biomarkers for detecting the autophagy and apoptosis. First of all, we treated HeLa cells with etoposide for 48 hours, and then detected apoptosis based on the caspase-3/7 activity. We performed RT-PCR analysis to detect mRNA expression changes of autophagy-related genes; ULK1, ULK2, ATG2A, ATG2B, ATG3, ATG4A, ATG4B, ATG4C, ATG4D, ATG5, ATG6, ATG7, GABARAP, GABARAPL1, GABARAPL2, MAP1LC3B, ATG9A, ATG9B, ATG10, ATG12, ATG16L1, WIPI1, WIPI2, WIPI3, WIPI4 and DRAM. Among these genes, ATG2A was solely upregulated by etoposide. In addition, doxorubicin was added to the cells for 48 hours to induce apoptosis. The mRNA level for ATG2A was upregulated by doxorubicin. Doxorubicin induced ATG2A mRNA level in MCF7 cells in a time-dependent manner, and this upregulation was unchanged when the p53-dominant negative vector was overexpressed. Next HeLa and A549 cells were treated with C2-ceramide for 24 hours and A549 cells were treated with thapsigargin for 24 hours to induce autophagy. As commonly upregulated genes, we identified MAP1LC3B and WIPI1 by RT-PCR. The mRNAs induction of MAP1LC3B and WIPI1 was in advance of MAP1LC3B form II protein accumulation, suggesting mRNA detection could be rapid indicator of autophagy. Taken together, our present results suggest that expression changes of autophagy-related genes could be monitoring tools for programmed cell death especially for discriminating the autophagy and apoptosis.

Identification for a novel target of p53 that controls microRNA metabolism in the apoptotic response to DNA damage

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Tumor suppressor p53 functions as a transcription factor to induce its target genes involved in cell cycle arrest, DNA repair, and apoptosis in response to DNA damage. Selective transactivation of p53 target genes is determined by post-translational modification of p53. In particular, phosphorylation of p53 at Ser46 is indispensable for commitment to induce apoptotic cell death. However, little is known about target genes that are induced in a Ser46 phosphorylation-specific manner. By using microarray analysis, we have identified one of the epidermal growth factor family (in this abstract, this molecule is abbreviated as EGF-p53). EGF-p53 was up-regulated by ectopic expression of wild type p53, but not the S46A mutant in p53-null cells. Results from ChIP and luciferase reporter assay demonstrated that EGF-p53 is a direct target of p53 and induced to trigger apoptotic cell death upon DNA damage. To further investigate the mechanism of EGF-p53-mediated apoptosis induction, we analyzed EGF-p53-interacting proteins by mass spectrometry. One of the candidates, DEAD box protein 5 (DDX5), was found to co-localize with EGF-p53 and regulated microRNA processing in response to DNA damage. These findings indicate that EGF-p53, which is specifically induced by Ser46-phosphorylated p53, interacts with DDX5 and regulates microRNA processing in the apoptotic response to DNA damage.

The vertebrate-specific outer membrane protein MIEF1 recruits Drp1 to mitochondria but promotes mitochondrial fusion

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BACKGROUND Mitochondrial morphology is controlled by two opposing processes: fusion and fission. Drp1 and hFis1 are two key players of mitochondrial fission, but how Drp1 is recruited to mitochondria and how Drp1-mediated mitochondrial fission is regulated in mammals is poorly understood.
METHODS: This work used a number of cell biological techniques, eg cell culture, generation expression constructs, mutational analysis, subcellular fractionation, immunoblotting, double and triple immunofluorescence stainings, confocal microscopy, transmission electron microscopy, RNAi silencing, in vivo protein-protein cross-linking, co-immunoprecipitation and cell fusion assays.
RESULTS Here, we identify a novel vertebrate-specific protein MIEF1 (mitochondrial elongation factor 1), which is anchored to the outer mitochondrial membrane. Elevated MIEF1 levels induce extensive mitochondrial fusion, whereas depletion of MIEF1 causes mitochondrial fragmentation. MIEF1 interacts with Drp1 and recruits Drp1 to mitochondria in a manner independent of hFis1, Mff and Mfn2, but inhibits Drp1 activity, thus executing a negative effect on mitochondrial fission. MIEF1 also interacts with hFis1 and elevated hFis1 levels partially reverse the MIEF1-induced fusion phenotype. In addition to inhibiting Drp1, MIEF1 actively promotes fusion, in a manner distinct from mitofusins.
CONCLUSION Our findings uncover a novel mechanism, which controls the mitochondrial fusion-fission machinery in vertebrates. As MIEF1 is vertebrate-specific, these data also reveal important differences between yeast and vertebrates in the regulation of mitochondrial dynamics.

Simvastatin induces apoptosis in HCT116 colorectal cancer cells via an enhanced oxidative stress and activation of JNK.

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Statins are widely used as cholesterol-lowering drugs that selectively inhibit the enzyme 3-hydroxy-3-methylglutaryl CoA reductase, leading to decreased cholesterol biosynthesis. Recent studies show that statins induce apoptotic cell death in several types of cancer cells; however, the underlying molecular mechanisms are still not understood. Here, we showed that colorectal cancer cells HCT116 showed higher sensitivity towards simvastatin treatment in terms of its anti-proliferative and colony forming inhibitory effects. Exogenously added mevalonate or geranylgeranylpyrophosphate (GGPP) but not farnesylpyrophosphate(FPP) in combination with simvastatin prevented the growth inhibitory effect of simvastatin, suggesting that simvastatin exerts effects via blocking the mevalonate biosynthetic pathway and its downstream geranylgeranylated protein synthesis instead of farnesylated proteins. In addition, simvastatin induced a significant increase in intracellular ROS production in HCT116 cells. Notably preincubation with ROS scanverger completed prevented simvastatin-mediated cell death, thereby suggesting that enhanced oxidative stress may be an important signal. Finally, more studies on HCT116 cells have shown that simvastatin treatment activates stress response via c-Jun NH2-terminal kinase (JNK) and triggers mitochondrial cell death pathway. In conclusion, simvastatin-induced growth arrest and apoptosis of colorectal cancer cells is mediated by a cross talk between intracellular ROS production and JNK signaling.

Analysis Of The Novel Acyl-Coa Thioesterase Them5 Reveals A Role In Mitochondrial Morphology And Fatty Liver Disease Development

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Acyl-CoA thioesterases hydrolyze thioester bonds in acyl-CoA metabolites. The majority of mammalian thioesterases are α/beta-hydrolases and have been well studied, but Hotdog-fold enzymes have been less well described. Here, we present a structural and functional analysis of a new mammalian mitochondrial thioesterase, Them5. Them5 and its paralog Them4 adopt the classical Hotdog-fold structure and form homodimers in crystals, representing a new group of these enzymes. In vitro Them5 shows strong thioesterase activity with long-chain acyl-CoAs. Them5KO mice have highly interconnected mitochondria; this effect depends on the enzymatic activity of Them5. Loss of Them5 specifically alters the remodeling process of the mitochondrial phospholipid cardiolipin. Them5KO mice show deregulation of lipid metabolism and development of fatty livers. Consecutively, mitochondrial respiration and beta-oxidation are impaired. Thus, we present novel mitochondrial thioesterase Them5 and its critical and specific role in the cardiolipin remodeling process, connecting it to the development of fatty liver and related conditions.

Anti-cancer activity of synthetic analogues of poly-APS

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Natural and synthetic monomeric, oligomeric and polymeric alkylpyridinium compounds (APs) are known to exert a wide range of biological effects. Among the most studied naturally occurring polymeric APs are 3-octylpyridinium salts (poly-APS). These polymers were isolated from the marine sponge Reniera sarai, and show a plethora of biological activities, including inhibition of acetylcholinesterase, antimicrobial and non-toxic antifouling activity, hemolytic and cytotoxic properties and stable transfection of mammalian cells. Furthermore, the most prominent activity of poly-APS is their effects on certain types of cancer cells. They showed selective toxicity toward NSCLC cells whilst having no apparent toxicity towards normal lung fibroblast cells and tissue in vitro and in vivo. In view of the potential use of poly-APS like compounds as new promising anti-cancer agents, synthetic analogues have been made. Synthetic analogues of poly-APS (3APSA) mimic natural ones and make them suitable for commercial production and application. NSCLC cells are characteristic for the most common form of lung cancer, and have been shown to express molecules that are part of the cholinergic system, such as α 7 nicotinic acetylcholine receptors (nAChR), cholino-acetyltransferase, the vesicular ACh transporter and acetylcholinesterase. Nicotine, the principal active component of cigarettes, has been found to act through by nAChRs. It was found that nicotine and structurally related carcinogens could induce the proliferation of a variety of small cell lung carcinoma cell lines. This led to the hypothesis that nicotine is directly involved in the induction and progression of human lung cancers. Nicotine induces an increase in proliferation of lung cancer cells via α 7-nAChRs-mediated signal transduction pathways. The therapeutical approach against cancer is to use drugs enhancing cell death and blocking cell proliferation of cancer cells. Alpha 7 nAChR antagonists (α-bungarotoxin or methyllycaconitine) can attenuate the proliferative effects of agonists. We have shown that synthetic analogues of poly-APS have high affinity for nAChRs. They inhibit α 7 nAChRs with concentration of 0.1 ng/ml and are therefore strong antagonists of α 7 nAChR. 3APSA APS8 compounds show a significant toxicity towards NSCLC (A549). They inhibited tumor cell growth in a concentration dependent manner. We tested influence of APS8 on normal lung fibroblast cells MRC5 which did not show alterations in growth until high concentrations of APS8 were used. Evidence of apoptosis activation by APS8 in NSCLC was quantitatively analyzed by FITC-labeled annexin V/propidium iodide uptake analysis with fluorescent cytometry. Cell morphology of APS8-induced apoptosis was investigated by staining the cells with a combination of the fluorescent DNA-binding dyes acridine orange and ethidium bromide. Based on observations, our results indicate that this molecule may be a new and innovative chemotherapeutic agent.

The histone deacetylase inhibitor trichostatin A augments cisplatin-induced apoptosis by exaggerating LMP and reducing lysosomal pH

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Modification of gene expression through deacetylation of histones is a common mechanism in cancer cells to silence genes and gain growth advantages. Histone deacetylase inhibitors are used to revert epigenetic modification and restore the activity of e.g., tumor suppressor genes. Using 8 different squamous cell carcinoma (SCC) cell lines from head and neck, we identified increased cisplatin sensitivity in 5 SCC cell lines after pretreatment with the pan-histone deacetylase inhibitor Trichostatin A. The UT-SCC-77 cell line was selected for further studies. Four hours of trichostatin A treatment caused reduction of lysosomal from 4.5 to 4.2 and sensitized the cells to cisplatin-induced apoptosis. If lysosomal pH was increased by NH4Cl treatment, apoptosis was prevented indicating that cisplatin-induced cell death is triggered by a lysosomal dependent mechanism. Cell death was preceded by lysosomal membrane permeabilization (LMP) and trichostatin A pretreatment enhanced the release lysosomal proteases to the cytosol. The pan-cysteine cathepsin inhibitor zFA-AMC reduced cell death. Interestingly, LAMP-2 was released from lysosomal membranes to the cytosol, and a corresponding decrease of LAMP-2 in the membrane fraction was detected. Overall protein level of LAMP-2 was decreased during cell death and, noteworthy, inhibition of cysteine cathepsins prevented loss of LAMP-2 protein level in the cell. However, we found no trichostatin A-induced alteration of protein levels when expression of Bcl-2 family proteins and lysosomal proteases were determined. We conclude that trichostatin A enhance cisplatin-induced cell death by decreasing lysosomal pH, which augments cathepsin activity resulting in enhanced LMP in UT-SCC-77 cells.