2185-Pos Board B155

Extracellular ATP Mediates FasL-induced Necrosis of Lymphoid Cells Via P2X7 Activation

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We demonstrated previously that FasL triggers both necrotic and apoptotic death in lymphoid cells. The execution of apoptosis was linked to activation of caspase-8 and caspase-3, while necrotic cell death required only activation of caspase-8 associated with delayed increases in ceramides. However, the precise signaling mechanisms involved in deciding between FasL-induced apoptotic or necrotic cell death in lymphoid cells remained unknown. On the other hand, many studies have implicated extracellular ATP as a mediator of cell death by necrosis, as well as apoptosis. In particular, ATP-dependent activation of purinergic P2X7 receptors has been suggested to trigger both forms of cell death. Hence, the objective here was to evaluate whether ATP/P2X7 participated in cell death induced by FasL in lymphoid cells. Flow cytometric analysis following staining with propidium iodide, in human Jurkat cells demonstrated that oxidized ATP, a specific P2X7 antagonist, selectively inhibited necrotic, but not apoptotic cell death induced by Fas-L. ATP, the physiological P2X7 ligand, was released from Jurkat cells following incubation with FasL. Furthermore, FasL-stimulated intracellular calcium-transients in Jurkat cells (measured using the Fluo3-AM probe) were blocked either when extracellular calcium was chelated or when cells were preincubated with oxidized ATP. The presence of P2X7 receptors in Jurkat cells was corroborated by Western blotting.

This study represents the first demonstration of cross-talk between the two cell death receptors Fas and P2X7 in lymphoid cells.

2186-Pos Board B156

T Cell Receptor Regulation Of Fas-mediated Apoptotic Calcium Release

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Although alterations in calcium levels are known to play an important role in a variety of physiological processes, only in past few years has the role of calcium signaling in regulation of apoptosis been well recognized. It has been shown that apoptotic stimuli provoke rapid elevation of cytosolic calcium concentration, which in turn orchestrates the release and activation of multiple pro-apoptotic factors. Our previous results in T cells demonstrated that Fas-mediated apoptosis requires calcium release, which was dependent upon phospholipase C-γ1 (PLC-γ1) activation and calcium release from inositol 1,4,5-trisphosphate receptor (IP_3R) channels. Here, we show that PLC-γ1 activation after Fas receptor ligation requires canonical components of the T cell receptor complex. Specifically, the active form of the Src family tyrosine kinase Lck and PLC-γ1 become associated with the death-inducing signaling complex (DISC) in a stimulus-dependent manner. We found that Lck activates PLC-γ1 indirectly *via* Zap70 tyrosine kinase and other members of the T cell receptor signaling pathway. Moreover, in absence of a functional T cell receptor complex, Fas stimulation failed to induce calcium release. This led to significantly inhibited effector caspase activation and delayed cell death. These findings strongly suggest a vital interplay between Fas and the T cell receptor complex, which has significant implications for T cell regulation.

2187-Pos Board B157

Novel K^+ Channel Blocker Induces Apoptosis Via Ca^{2+} Release From ER Stores

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In search of novel K^+ channel modulators we have undertaken HTS of chemical libraries. The primary screen using yeast whose growth in low $[K^+]$ medium depends on expression of the inwardly rectifying K^+ channel Kir2.1 was followed by K^+ current measurements in mammalian cells. One of the identified compounds, 22G ({1-[3-(4-chloro-phenyl)-adamantan-1-yl]-ethyl}-(1-ethyl-piperidin-4-yl)-amine), inhibits Kir2.1-dependent yeast growth, decreases $^{86}Rb^+$ flux via Kir2.1 and acutely blocks the whole-cell Kir2.1 current (EC_{50}=25 μM) in transfected HEK293 cells. As a part of specificity characterization of 22G, we examined its effect on $^{86}Rb^+$ efflux via recombinant and native Ca^{2+}-activated K^+ channel (IKCa) in HEK293 cells and IEC-6 enterocytes, respectively. Surprisingly, 22G induced rapid K^+ efflux via IKCa. Because previously we have established that IKCa-dependent K^+ efflux is essential for apoptosis, we tested whether 22G is capable of inducing apoptosis. When applied at 25 μM for 10 min, 22G caused activation of caspases in 90% of IEC-6 cells, as determined by staining with the fluorescent pan-caspase inhibitor FAM-VAD-FMK. Blockade of 22G-induced K^+ current by the IKCa inhibitor clotrimazole prevented the induction of apoptosis. Thus, 22G is a potent inducer of IKCa-mediated apoptosis. To test whether 22G activates pro-apoptotic IKCa current by elevating the cytosolic $[Ca^{2+}]$, we measured Ca^{2+} levels in 22G-treated HEK293, CHO and IEC-6 cells loaded with the Ca^{2+} indicator fluo-4. At 50 μM, 22G induced increase in cytosolic Ca^{2+} in Ca^{2+}-free solution in all three cell lines. Depletion of ER Ca^{2+} stores with thapsigargin and cyclopiazonic acid abolished 22G-induced surge of cytosolic $[Ca^{2+}]$ and IKCa current. Therefore, 22G activates IKCa current via Ca^{2+} release from ER stores. We suggest using compound 22G as a reliable apoptogen, a tool for modulation of intracellular $[Ca^{2+}]$ and inhibitor of Kir2.1 current in patch-clamp experiments.

2188-Pos Board B158

The Amino-terminal Peptide Of Bax Perturbs Intracellular Ca^{2+} Homeostasis To Enhance Apoptosis In Prostate Cancer Cells

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Targeting the interconnected cellular pathways controlling apoptosis and regulation of Ca^{2+} homeostasis are two avenues for treatment of human cancers. During apoptosis, proteolytic cleavage of Bax at the amino-terminus generates a truncated Bax of ~18 kDa (p18Bax) and an amino-terminal peptide of ~3 kDa (p3Bax). Extensive studies have shown that p18Bax behaves like a BH3 protein with enhanced pro-apoptotic function over the full-length Bax (p21Bax), little is known about the function of p3Bax in apoptosis. We have previously shown that Bax and Ca^{2+} synergistically amplifying apoptosis signaling (Pan, et al. *J Biol Chem* **276**: 32257, 2001), and that store-operated Ca^{2+} entry (SOCE) contributes to Bax-mediated apoptosis in prostate cancer cells (Li, et al. *J Cell Physiol* **216**: 172, 2008). Here we test if p3Bax can contribute to regulation of Ca^{2+} signaling during apoptosis, through a membrane penetrating peptide (TAT) to facilitate delivery of recombinant p3Bax into NRP-154 cells, a prostate epithelial cell line with tumorigenic capacity. We find that TAT-p3Bax fusion peptide can enhance thapsigargin-induced apoptosis in NRP-154 cells, elevate SOCE activity and increase IP_3 sensitive intracellular Ca^{2+} stores. Our data indicates that p3Bax can modulate the entry of extracellular Ca^{2+}, and thus regulate the amplification of apoptosis in prostate cancer cells. Another unique observation of this study is that TAT-p3Bax is not toxic to NRP-Bax cells under resting conditions, it only enhances the process of apoptosis initiated by exposure to TG. This is particularly important when considering the exogenous p3Bax peptide as a therapeutic agent for prostate cancer. In such a case, p3Bax would not produce cytotoxic effects in cells with normal Ca^{2+} homeostasis, it could be used in combination with other cytotoxic agents to amplify apoptosis in targeted cancer cells.

2189-Pos Board B159

The Anti-apoptotic Mitochondrial Membrane Protein Bcl-2; An Achilles Heel Of Cancer Cells?

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Escape from programmed cell death, apoptosis, is one of the main hallmarks of cancer.. The anti-apoptotic protein Bcl-2 belongs to the Bcl-2 protein family, which function as a major gatekeeper in the mitochondrial pathway. Bcl-2 is found to a great extent in many breast cancers and is highly involved in the inherent resistance to anti-cancer drugs. This protein is mitochondrial membrane-associated and we will use NMR spectroscopy to provide structural information of the membrane-mediated mechanism underlying the action of Bcl-2 as a potent inhibitor of cell death.

For this purpose we express currently the full length protein and carry out various preliminary studies of the membrane dependent behaviour of synthesized segments of the proteins by a range of biophysical methods ranging from CD (Circular Dichroism), ATR (Attenuated Total Reflection), Calorimetry to solid state NMR spectroscopy.At present, we study the impact of the unique BH4 domain of the pro-survival Bcl-2 protein on the mitochondrial membranes, since this interaction seems to be essential to block any apoptotic activation with its connected pore-formation and cytochrome c release. Our first results reveal that the BH4 domain requires cardiolipin to be able to convert into an α-helix conformation. In contact with neutral membranes, the peptide seems to aggregate on the surface. Bcl-2's counterplayer is Bax protein which upon activation translocates to the mitochondrial membrane. In this process the first helix localized at the N-terminus of Bax (Bax-α1) can act as an addressing sequence, which upon activation directs the protein from the cytosol towards the mitochondrial surface. Our biophysical approach provided insight into the molecular

mechanism behind the recognition of the mitochondrial membrane system by the Bax-α1 sequence.

2190-Pos Board B160

Direct Interactions Between Tbid And Bcl-xlΔXl Are Enhanced In Lipid Membranes

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The proteins of the Bcl-2 family are key regulators of apoptosis, but their molecular mechanisms remains controversial. Two important aspects that center the debate involve the interaction network between the pro- and antiapoptotic family members and the role of their translocation to the mitochondrial outer membrane (MOM) during apoptosis. We have used FCCS to examine quantitatively the dynamic interactions of Bid and tBid with Bcl-xLΔCt in solution and in lipid membranes. We found that only the active form tBid binds to Bcl-xLΔCt and that the membrane strongly promotes binding between them. Importantly for drug design, a BH3 peptide from Bid disrupts the tBid/Bcl-xL complex in solution but not in lipid bilayers. Our findings convincingly suggest that the most relevant interaction between tBid and Bcl-xL happens in the membrane and reveal its significance as an additional regulatory stage for MOM permeabilization.

2191-Pos Board B161

Membrane Changes during Apoptosis: Part of the Process or Characteristics of the Corpse?

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Thapsigargin-induced apoptosis in S49 lymphoma cells causes biophysical changes in the plasma membrane. Thapsigargin (TG) is a sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor that causes depletion of intracellular calcium stores and increased cytosolic calcium levels. It is used to model endoplasmic reticulum stress-induced apoptosis. This study focuses on membrane physical changes involved in this specific apoptotic pathway and possible cellular mechanisms that could account for these alterations. The fluorescent probes merocyanine 540 (MC540), laurdan, patman, and diphenylhexatriene (DPH) were used to assess lipid spacing, order, and fluidity using fluorescence spectroscopy, two-photon excitation microscopy, and confocal microscopy. MC540 fluorescence intensity increased throughout the apoptotic process, suggesting an increase in interlipid spacing. Two-photon microscopy images with laurdan showed a transient reduction in membrane order. Alexa Fluor-labeled annexin V was used to assess phosphatidylserine exposure in the outer leaflet of the plasma membrane. Flow cytometry experiments showed a sharp increase in the population showing this flip-flop after 2.5 h incubation with TG. Susceptibility of the cells to secretory phospholipase A2 (sPLA2), a hydrolytic enzyme that can distinguish apoptotic membranes from healthy ones, was also evaluated by measuring membrane permeability to propidium iodide. Two hours after addition of TG, a small population of cells became susceptible to sPLA2, and that population increased steadily with longer incubations. All of these alterations in the plasma membrane were compared temporally with caspase activation using a fluorescently labeled caspase inhibitor, FAM-VAD-fmk. This comparison suggested that caspase activation, susceptibility to sPLA2, and decreased lipid order detected by laurdan precede PS exposure, elevated MC540 fluorescence, and eventual cellular demise. Experiments are in progress with multiple pharmacological agents to assess cause and effect relationships among these events and a possible role of ceramide.

2192-Pos Board B162

Chemotherapeutic Apoptosis: Who Assailed The Membrane, The Inducer Or The Induced?

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The ability of certain chemotherapeutic agents to partition into the cell membrane raises the possibility that some of their effects on cells may involve direct disruption of normal bilayer function. Moreover, previous studies on hormone-stimulated apoptosis indicate a variety of indirect membrane changes that accompany the death process including changes in membrane fluidity and order, increases in interlipid spacing, and susceptibility to hydrolysis by secretory phospholipase A2 (sPLA2). To compare the relative roles of potential direct and indirect effects of chemotherapeutic agents on cell membrane properties, we treated S49 lymphoma cells with daunorubicin (partitions in membrane) or methotrexate (non-membrane perturbing). An additional difference between the two drugs relates to their involvement of caspase-3 in the apoptotic process; daunorubicin requires it, and methotrexate does not. Membrane properties were assessed over time after addition of the drugs by fluorescence spectroscopy and microscopy using merocyanine 540, laurdan, diphenylhexatriene, and patman. The preliminary results of these studies showed commonalities between daunorubicin and methotrexate. For example, their effects on susceptibility to sPLA2 were nearly identical. Initially, the membrane remained resistant to hydrolysis for several hours. Thereafter, a sharp increase in sPLA2 activity was observed. These results suggested that changes that render the membrane vulnerable to hydrolytic attack are controlled by biochemical processes associated with apoptosis rather than reflecting direct effects of a chemotherapeutic drug on the cell membrane. Interestingly, activation of caspase-3 appeared not to be part of those processes.

2193-Pos Board B163

Designing Single Fluorescent Protein Based Caspase Sensor For Monitoring Apoptosis In Living Cells

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Intracellular apoptotic signals regulated by caspase-cascade systems are closely associated with human diseases such as cancer and neurodegenerative diseases. Monitoring the activation and inhibition of caspase 3 and other caspases with fluorescence spectrum changes in living cells is essential for further understanding these processes. Here, we report progress in the development of caspase sensors based on a single fluorescent protein. These developed sensors exhibit strong enzymatic selectivity as well as high sensitivity based on observed ratiometric fluorescence changes. Additionally, our sensors can be targeted to different subcellular locations, such as the ER and mitochondria. We have further applied these sensors to monitor caspase-dependant apoptosis in different cells. Our results indicate that different inducers and drugs have diversified effects on triggering cell death pathways.

2194-Pos Board B164

Binding of the Pro-Apoptotic protein Bid to Mitochondrial Membranes is a Two Step Process

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Apoptosis is an essential process for the development of all multi-cellular organisms, and Bcl-2 family proteins critically regulate most pathways of apoptosis at the level of mitochondria. Bid is a pro-apoptotic member of Bcl-2 family proteins that regulates the integrity of OMM (Outer Mitochondrial Membrane). Upon induction of apoptosis, Bid is cleaved into a p15 and a p7 fragment, a complex that is held together by strong non-covalent interactions. The separated p15 fragment, also known as tBid (truncated Bid) is a potent inducer of cell death, however, the mechanism of the fragments' separation and activation of Bid to tBid is unknown. The focus of this work was to develop an *in vitro* fluorescent assay system to elucidate the mechanism of activation of Bid using a recombinant liposomal system bearing physiological relevance along with isolated mitochondria. Single cysteine mutants of Bid were created, and labelled with fluorescent thiol-reactive molecules to study the individual steps of the activation of Bid. Using size exclusion chromatography, FCS (Fluorescent Correlation Spectroscopy), and FRET (Förster Resonance Energy Transfer), it was quantitatively determined that the two cleaved fragments of Bid spontaneously separate upon binding to the membrane without any additional post-translational modifications. After the initial binding to the membrane, p15 fragment undergoes a conformational change to adopt its active form. Taking together, we found that the activation of Bid is a two step process encompassing the separation of the cleaved fragments and a conformational change in the membrane.

2195-Pos Board B165

Protein BAX During Detergent Activation: Characterization by Fluorescence Correlation Spectroscopy and Fluorescence Intensity Distribution Analysis

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BAX is a pro-apoptotic member of the BCL-2 protein family. During apoptosis in mammalian cells cytoplasmic BAX is activated and translocates to the outer mitochondrial membrane (OMM), where it participates in formation of an