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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.

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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.

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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.

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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.

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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