Journal of Cellular Physiology

FasL-Triggered Death of Jurkat Cells Requires Caspase 8-Induced, ATP-Dependent Cross-Talk Between Fas and the Purinergic Receptor $P2X_7$

ADAM AGUIRRE,[1] KENJI F. SHOJI,[3] JUAN C. SÁEZ,[3,4] MAURICIO HENRÍQUEZ,[1,2]* AND ANDREW FG QUEST[1]*

[1]*Facultad de Medicina, Centro de Estudios Moleculares de la Célula, Universidad de Chile, Santiago, Chile*

[2]*Facultad de Medicina, Programa Fisiología y Biofísica (ICBM), Universidad de Chile, Santiago, Chile*

[3]*Departamento de Fisiología, Pontificia Universidad Católica de Chile, Santiago, Chile*

[4]*Instituto Milenio, Centro Interdisciplinario de Neurociencias de Valparaíso, Valparaíso, Chile*

Fas ligation via the ligand FasL activates the caspase-8/caspase-3-dependent extrinsic death pathway. In so-called type II cells, an additional mechanism involving tBid-mediated caspase-9 activation is required to efficiently trigger cell death. Other pathways linking FasL–Fas interaction to activation of the intrinsic cell death pathway remain unknown. However, ATP release and subsequent activation of purinergic $P2X_7$ receptors ($P2X_7$Rs) favors cell death in some cells. Here, we evaluated the possibility that ATP release downstream of caspase-8 via pannexin1 hemichannels (Panx1 HCs) and subsequent activation of $P2X_7$Rs participate in FasL-stimulated cell death. Indeed, upon FasL stimulation, ATP was released from Jurkat cells in a time- and caspase-8-dependent manner. Fas and Panx1 HCs colocalized and inhibition of the latter, but not connexin hemichannels, reduced FasL-induced ATP release. Extracellular apyrase, which hydrolyzes ATP, reduced FasL-induced death. Also, oxidized-ATP or Brilliant Blue G, two $P2X_7$R blockers, reduced FasL-induced caspase-9 activation and cell death. These results represent the first evidence indicating that the two death receptors, Fas and $P2X_7$R connect functionally via caspase-8 and Panx1 HC-mediated ATP release to promote caspase-9/caspase-3-dependent cell death in lymphoid cells. Thus, a hitherto unsuspected route was uncovered connecting the extrinsic to the intrinsic pathway to amplify death signals emanating from the Fas receptor in type II cells.

J. Cell. Physiol. 228: 485–493, 2013.

Apoptosis is an active process that depends on a defined sequence of signaling events and can occur via two different routes, referred to as the extrinsic (death receptor mediated) and the intrinsic (endogenous) pathways. The extrinsic pathway is activated in a manner dependent on ligand binding to death receptors. Fas ligand (FasL) binding to the Fas/CD95 receptor promotes formation of the death-induced signaling complex (DISC) by recruiting Fas-associating death domain (DD) [FADD-containing protein] through its DD domain, which then recruits death-effector domain (DED)-containing initiator caspases, such as caspase-8 and -10. Proximity of pro-caspase molecules is required for autocleavage and activation of initiator caspases, which then go on to proteolytically cleave and activate executioner caspases, including caspase-3, -6, -7 (Scaffidi et al., 1998; Peter and Krammer, 2003).

In some cells, referred to as Type I, this sequence of events is sufficient to trigger apoptosis. Alternatively in Type II cells, caspase-8 activation is weak and signaling downstream of the DISC needs to be amplified via a loop involving cleavage of Bid to generate tBid and activation of the mitochondrial pathway, which generally responds to intracellular stress signals. Changes in mitochondrial membrane potential and mitochondrial membrane permeability induced downstream of tBid formation culminate in release of mitochondrial molecules to the cytosol. Mitochondrial cytochrome c in conjunction with cytosolic apoptosis protease activating factor 1 (Apaf-1) and adenosine triphosphate (ATP) assemble into a multi-protein complex, called the apoptosome, which catalyzes proteolytic auto-activation of the initiator caspase-9 and activation downstream of the executioner caspases (Hengartner, 2000; Samraj et al., 2006).

The purinergic receptor $P2X_7$ ($P2X_7$R), whose ligand is ATP, can trigger either necrosis and/or apoptosis in various cell types, including myeloid cells, dendritic cells, thymocytes, macrophages and lymphocytes (Di Virgilio et al., 1998).

The authors declare no conflict of interest.

Additional supporting information may be found in the online version of this article.

Contract grant sponsor: FONDECYT-FONDAP;
Contract grant number: 15010006.
Contract grant sponsor: FONDECYT;
Contract grant number: 1090071.
Contract grant sponsor: FONDECYT-Postdoctoral Fellowship;
Contract grant number: 3070045.
Contract grant sponsor: ANILLO;
Contract grant number: ACT-71.

*Correspondence to: Mauricio Henríquez and Andrew FG Quest, Facultad de Medicina, Universidad de Chile, Independencia 1027 Santiago, Chile. E-mail: mhenriqu@med.uchile.cl; aquest@med.uchile.cl

Manuscript Received: 24 April 2012
Manuscript Accepted: 3 July 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 17 July 2012.
DOI: 10.1002/jcp.24159

Previous studies have shown that prolonged stimulation of $P2X_7R$ receptor with high concentrations of ATP induces apoptosis (Tsukimoto et al., 2006; Yoon et al., 2007). However, apoptosis induced by $P2X_7R$ activation in CD4+ and CD8+ T cells occurs only when the extracellular ATP concentrations in membrane proximity exceeds 100 μM (Aswad and Dennert, 2006). Alternatively, transient stimulation with low concentrations of ATP, secreted as an autocrine or paracrine signal, may stimulate proliferation of T cells (Adinolfi et al., 2002; Elliott et al., 2009). Futhermore, extracellular nucleotides have been identified as critical signals for the removal of apoptotic cells during the early stages of apoptosis. Specifically, caspase-dependent ATP release was observed during Fas-induced apoptosis of Jurkat cells (Elliott et al., 2009).

The mechanisms of extracellular ATP release are highly variable and, depending on the cell type, connexin-43 (Cx43) hemichannels (HCs), the volume-regulated anion channel (VRAC), or the purinergic $P2X_7R$ (Maroto and Hamill, 2001; Bal-Price et al., 2002; Parpura et al., 2004) have been implicated. More recently, ATP release through HCs formed by Panx1 following P2X receptor stimulation was implicated as an essential autocrine co-stimulatory signal in lymphocyte activation (Schenk et al., 2008). Decreased nucleotide release was observed upon siRNA-mediated knockdown of Panx1 and overexpression of the protein increased nucleotide release from apopotic cells (Chekeni et al., 2010). Alternatively, in mouse T lymphocytes activated by anti-CD28 and anti-CD3 antibodies a second mechanism of ATP release was proposed involving exocytosis (Tokunaga et al., 2010).

The above observations led us to ask whether ATP-mediated cross-talk existed between Fas and $P2X_7R$ and how ATP release downstream of the Fas receptor is triggered. Here, we provide evidence indicating that indeed Fas-dependent caspase-8 activation promotes ATP release via Panx1 HCs, which in turn favors caspase-9 activation and cell death downstream of $P2X_7R$. These results identify a novel, hitherto unsuspected route connecting the extrinsic to the intrinsic pathway to amplify death signals emanating from Fas in type II cells.

Materials and Methods

Reagents

Culture medium RPMI and DMEM as well as the antibiotics penicillin and streptomycin were purchased from Gibco-BRL (Paisley, Scotland, UK). The fetal calf serum and the EZ-ECL kit were obtained from Biological Industries, (Kibbutz Beit Haemek, Israel). The polyclonal rabbit anti-β-actin antibodies and all caspase inhibitors were obtained from R&D System (Minneapolis, MN), the goat polyclonal anti-$P2X_7R$ antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and the monoclonal anti-human Fas conjugated with FITC were purchased from eBioscience (San Diego, CA). The goat anti-rabbit IgG (H + L)-HRP Conjugate was purchased to Bio-Rad (Hercules, CA).The human recombinant FasL were obtained from Apotech SA (Geneva, Switzerland). The kits Cell Titer Glo and CytoTox-ONE were purchased from Promega (Madison, WI). Ebselen were obtained from AppliChem (Darmstadt, Germany), carbenoxolone, probenecid, $LaCl_3$, heptanol, and oATP purchased from Sigma (St. Louis, MO) and brillant blue G (BBG) from American Bioanalytical (Natick, MA).

Cell culture

Jurkat human leukemia T cells, as well as Ramos and Raji human lymphoma B cell lines were cultured in RPMI (1.8 g glucose/L) supplemented with 10% fetal calf serum and antibiotics (10,000 U/ml penicillin, 10 μg/ml streptomycin) at 37°C and 5% CO_2. A20 and A20R mouse B lymphoma cells were cultured in DMEM (4.5 g glucose/L) supplemented with 10% fetal calf serum, antibiotics (10,000 U/ml penicillin, 10 μg/ml streptomycin) and 50 μM ethanol-2-thiol at 37°C and 5% CO_2.

Viability assays

Cell viability was analyzed by FACS as previously described (Hetz et al., 2002; Villena et al., 2008). In this assay, cells impermeable to propidium iodide (PI negative) are considered as viable. After setting the baseline to exclude cell debris, two populations of PI-permeable (PI positive) dead cells are distinguished based on fluorescence intensity, corresponding to either hypodiploid apoptotic cells or necrotic cells with intact DNA. Here, Jurkat, Ramos, and Raji cells were incubated with increasing concentrations of FasL (0–80 ng/ml) for 16 h at 37°C. For inhibition experiments, cells were preincubated for 1 h with either oATP (600 μM) or BBG (50 μM). Alternatively, cells were preincubated for 15 min with apyrase (10 U) or simultaneously treated with one of the caspase inhibitors zVAD (20 μM), zIETD (20 or 50 μM), zLEHD (20 or 50 μM) or zDEVD (20 or 50 μM), and FasL (0–80 ng/ml). Cells were harvested and stained with 10 μg/ml of propidium iodide to determine cell viability. Samples containing roughly 1×10^4 cells were analyzed by flow cytometry (FACScanto; Becton Dickinson, Mountain View, CA) using the software program FCS Express.

$P2X_7R$ expression

Total RNA from human Jurkat and mouse A20 cell was extracted using Trizol® and the $P2X_7R$ mRNA levels were evaluated using primers specific for human $P2X_7R$ sequence (sense 5′-TCCGAGAAACAGGCGATAA-3′, antisense 5′-ACTCGCACTTCTTCCTGTA-3′). As a control, actin mRNA levels were evaluated in a parallel reaction using appropriate primers (sense 5′-GCATTGTAACCAACTGGGACG-3′, antisense 5′-CATGAGGTAGTCTGT CAG GTC-3′) After 25–30 cycles (1 min at 94°C, 56°C (T_m) and 72°C) RT-PCR amplified products specific for $P2X_7R$ (456 bp) and β-actin (380 bp) were analyzed in a 2% agarose gel containing ethidium bromide (EtBr). For Western blots, whole Jurkat cell lysates were prepared in ice-cold lysis buffer (20 mM Tris, pH 7.6, 1% Triton X-100, 300 mM NaCl) and protein extracts (50 μg/lane) were separated by SDS–PAGE in 10% gels and transferred to PVDF membranes. Membranes were blocked with TBS-5% gelatin and incubated with either goat anti-$P2X_7R$ or rabbit anti-actin antibody followed by an HRP-coupled secondary antibody. The peroxidase activity was detected by enhanced chemiluminescence EZ-ECL.

Measurement of ATP

ATP in cell supernatants was measured using a commercially available luminescence assay (Cell-Titer Glo Kit; Promega). Briefly, Jurkat, Ramos, and Raji cells were pre-incubated 10 min with ebselen (30 μM) and stimulated 0–5 h with FasL (80 ng/ml). For inhibition experiments, Jurkat cells were incubated with caspase inhibitors zVAD (20 μM), zIETD (20 μM), zDEVD (20 μM), added at the same time as FasL or pre-incubated for 10 min with carbenoxolone (10 μM), probenecid (1 mM), or heptanol (500 μM). Final luminescence was measured in a TopCount NXT microplate luminescence counter (Perkin-Elmer, Waltham, MA) and expressed in nM of ATP determined using a standard curve.

Lactate dehydrogenase release assay

Supernatant lactate dehydrogenase (LDH) concentrations were determined using the CytoTox-One™ kit (Promega) following instructions provided by the manufacturer. Jurkat cells were incubated with FasL (80 ng/ml) for 0–5 h at 37°C. After the indicated time of incubation, a volume of CytoTox-ONE™ reagent (Promega) was added to the equivalent volume of cell culture medium present in each well. To determine maximum LDH release possible, lysis solution (0.9% weight/volume solution of Triton® X-100 in water) was added to the cells. LDH release was

standardized to total LDH content and results were expressed in percent (%).

Confocal microscopy

Jurkat cells cultured on glass cover slips were fixed in 4% formaldehyde at room temperature for 30 min. PBS solution, containing 1% IgG free BSA, 50 mM NH_4Cl, 0.05% Triton X-100 (blocking solution), was used to permeabilize and block non-specific binding sites. To block Fc receptors, samples were incubated for 45 min in a solution containing Fc-Block (1:100; Becton Dickinson) at room temperature. Then, samples were washed twice in PBS and incubated with anti-Panx1 $F(ab')_2$ fragments for 12 h in blocking solution at 4°C. Finally, samples were washed three times with blocking solution, incubated with Cy2 conjugated goat anti-rabbit IgG $F(ab')_2$ fragments (1:300) for 30 min at room temperature and mounted with fluoromount G (Electron Microscopy Sciences, Washington, PA). Also, Jurkat cells were stained with the monoclonal anti-human Fas-FITC antibody (eBioscience). Images were examined with a confocal laser-scanning microscope (Olympus, Fluoview FV1000, Tokio, Japan). Co-localization was analyzed using ImageJ (NIMH, Bethesda, MD) and the ImageJ JACoP plugin (Bolte and Cordelieres, 2006). Cytofluorograms were generated by plotting the gray values of pixels in the red and green channel of images against each other, in order to obtain Person's and Manders coefficients in segmented images. Manders coefficients for Panx1 overlap with Fas were obtained by analyzing 30 cells in each condition using segmented images from at least four independent experiments. The cross-correlation function method was also used to evaluate the overall co-localization between red and green channel signals (van Steensel et al., 1996).

Time-lapse fluorescence imaging

For time-lapse imaging experiments, Jurkat cells were plated on No. 1 glass poly-L-lysine (0.01%) coated coverslips for 1 h and then washed twice with recording solution (HANKS-HEPES solution: 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose and 10 mM HEPES, pH 7.4). Cells were then incubated in recording solution containing 5 μM EtBr. Jurkat cells were pretreated 10 min with $LaCl_3$ (500 μM) or probenecid (1 μM) and subsequently stimulated with FasL (80 ng/ml) up to 180 min. As controls, cells were treated with PBS. Fluorescence was recorded in regions of interest of different cells with a water immersion Olympus BX51WI upright microscope (Melville, NY). Images were captured with a Q Imaging model Retiga 13001 fast cooled monochromatic digital camera (12-bit; Qimaging, BC, Canada) every 5 min (exposure time = 30 ms, gain = 0.5). Metafluor software (version 6.2R5; Universal Imaging, Downingtown, PA) was used for image analysis and fluorescence quantification. Three independent background fluorescence intensity values were obtained at each time point (BF, expressed as arbitrary units, AU), averaged and then subtracted from the fluorescence intensity determined for each cell at the same time point interval (CF). Results of this calculation (CF–BF) at each time point for each of 40 cells were averaged and plotted against time (expressed in minutes) over a period of 20 min. EtBr uptake rates were calculated using Microsoft Excel software (Redmond, WA) and expressed as AU/min. Cells with high fluorescence intensities before stimulation were excluded from the analysis.

Determination of active caspase-9

Jurkat cells were incubated with FasL (80 ng/ml) for 16 h at 37°C. For inhibition experiments, cells were preincubated for 1 h with BBG (50 μM) or 15 min with apyrase (10 U). Then the inhibitors of caspase-8- (zIETD, 50 μM), caspase-3- (zDEVD, 50 μM) or caspase-9-like activity (zLEHD, 50 μM) were added to cells together with FasL. Cells were harvested as indicated and the specific activity of caspase-9 was assessed by staining cells with LEHD-FMK conjugated to FITC (Abcam, Cambridge, UK) for 1 h. Samples were analyzed by flow cytometry (FACScanto; Becton Dickinson) using the software program FCS Express.

Data analysis

Data sets were generally compared using Student's *t*-test. Comparisons yielding $P \leq 0.05$ or less were considered indicative of statistically significant differences. For group statistical analysis, each treatment was compared with its respective control and significance was determined using a one-way ANOVA followed by a Kruskall–Wallis post hoc test. Differences were considered significant at $P < 0.05$. Statistics were performed using Microsoft Excel and Graph Pad Prism 4 software (2007 and 2003 versions, respectively).

Results

Sensitivity of different cell lines to FasL

To evaluate sensitivity to FasL, Jurkat, Raji, and Ramos cells were treated with different concentrations of FasL (0–80 ng/ml) for 16 h. Raji and Ramos cells were completely resistant to the effect of FasL at the different concentrations used. Alternatively, in Jurkat cells, FasL induced a dose-dependent decrease in viability (Fig. 1A) and the prevalent type of cell death

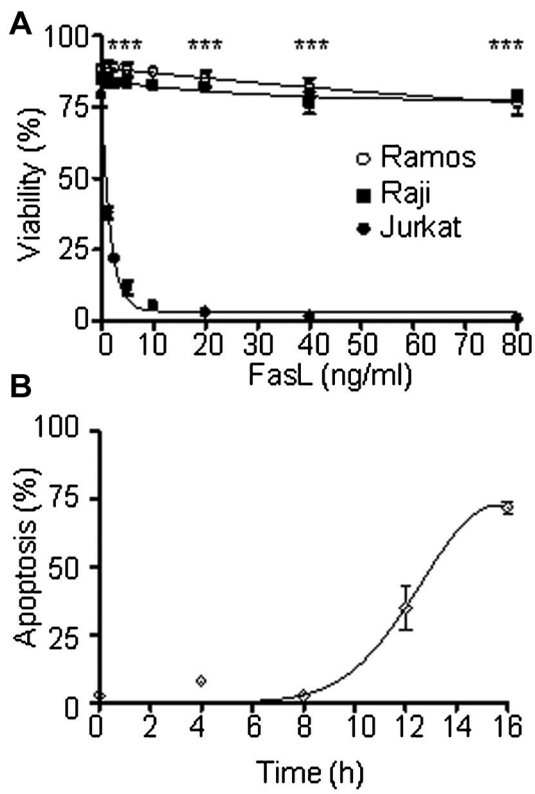


Fig. 1. Viability in lymphoid cells treated with FasL. Cells were stimulated for 16 h with increasing concentrations of FasL (0–80 ng/ml). The cells were then stained with propidium iodide (PI; 20 μg/ml) and cell viability was evaluated by flow cytometry in Jurkat (●), Ramos (○), and Raji cells (■) (A). Time dependence of FasL-induced apoptosis. Jurkat cells were treated with FasL (80 ng/ml) and apoptosis was evaluated at 4 h intervals after stimulation (B). The results represent averages from three independent experiments (n = 3). Statistically significant differences are indicated (*$P < 0.001$).**

was apoptosis (Fig. 1B). Interestingly, surface expression of the Fas receptor was comparable in Jurkat and Ramos cells, and even significantly higher in Raji cells (Supplementary Fig. 1), suggesting that the observed differences in susceptibility to FasL-induced death reflected a problem in signaling downstream of the receptor. For Jurkat cells, FasL-induced cell death became detectable within about 8–10 h following FasL (80 ng/ml) addition and reached maximum levels after 14–16 h (Fig. 1B).

P2X$_7$R antagonists and ATP hydrolysis inhibited FasL-induced cell death in Jurkat cells

Since P2X$_7$Rs have been implicated in events leading to cell death, we hypothesized that FasL-induced ATP release might activate P2X$_7$Rs and thereby promote cell death in Jurkat cells. Indeed, for Jurkat cells pre-incubated with the P2X$_7$R antagonists BBG (50 μM) or oATP (600 μM) and then stimulated with FasL (0–80 ng/ml) for 16 h, the decrease in cell viability was less pronounced (Fig. 2A). Since ATP is considered the physiological P2X$_7$R ligand, we also treated cells with apyrase, an enzyme that degrades ATP. As anticipated, presence of the enzyme reduced cell death observed following FasL stimulation (Fig. 2B). Consistent with these pharmacological observations, the presence of P2X$_7$R mRNA and protein was confirmed in Jurkat cells (Fig. 2C).

Extracellular ATP accumulation in response to FasL

Recent evidence indicates that ATP is released from lymphocytes upon triggering cell death (Elliott et al., 2009; Yip et al., 2009). To evaluate this possibility in our system, a highly sensitive luminescence assay was employed to quantify accumulation of ATP in the cell medium. Following FasL stimulation, ATP levels increased in the medium of Jurkat cells and significant differences compared to cells treated with PBS (control) were detectable after 3, 4, and 5 h (Fig. 3A). In contrast, for Raji (Fig. 3B) and Ramos (Fig. 3C) cells no differences between FasL- and PBS-treated cells were observed. This increase in ATP was not attributable to reduced Jurkat viability, since no loss of plasma membrane integrity was detectable during the same period of time, as assessed using the LDH release assay (Fig. 3D). These results suggested that ATP was liberated in a FasL-dependent manner from Jurkat, but not Ramos and Raji cells.

Effect of Cx HC and Panx1 HC inhibitors on ATP release

To evaluate more specifically the mechanism of FasL-induced ATP release, the effects of different pharmacological inhibitors were tested. Prior to FasL (80 ng/ml) addition, a 10 min pretreatment of Jurkat cells with a low concentration of carbenoxolone (10 μM) that inhibits Panx1 HCs but not Cx HCs, was sufficient to significantly reduce ATP accumulation in culture supernatants almost to levels detected for PBS-treated cells (Fig. 4A). In the second set of experiments, another Panx1 HC inhibitor, probenecid (1 mM), was compared with the Cx HC inhibitor heptanol (500 μM). For Jurkat cells pretreated 10 min with probenecid, ATP accumulation in culture supernatants was significantly reduced compared to levels observed for cells treated with FasL alone. On the other hand, pretreatment with heptanol had essentially no effect (Fig. 4B). These experiments directly implicated Panx1 HCs in FasL-induced ATP release from Jurkat cells. Consistent with this interpretation, we detected Panx1, but not Cx43 expression in Jurkat cells (Supplementary Fig. 2).

Expression and localization of Panx1 and Fas following FasL stimulation

As a next step, we asked whether FasL induced changes in the expression and/or distribution of Fas and Panx1 occurred. To this end, Jurkat cells were stimulated with 80 ng/ml FasL for up to 5 h and changes in fluorescence were visualized by confocal microscopy (Fig. 5A). For Panx1, a significant increase was detected within 3 h of stimulation with FasL as compared to untreated cells (control; Fig. 5B). This was corroborated by western blot analysis where FasL exposure was also found to augment Panx1 protein levels (Supplementary Fig. 3). By contrast, no statistically significant changes were observed for Fas at the different time points (Fig. 5C). Interestingly, also

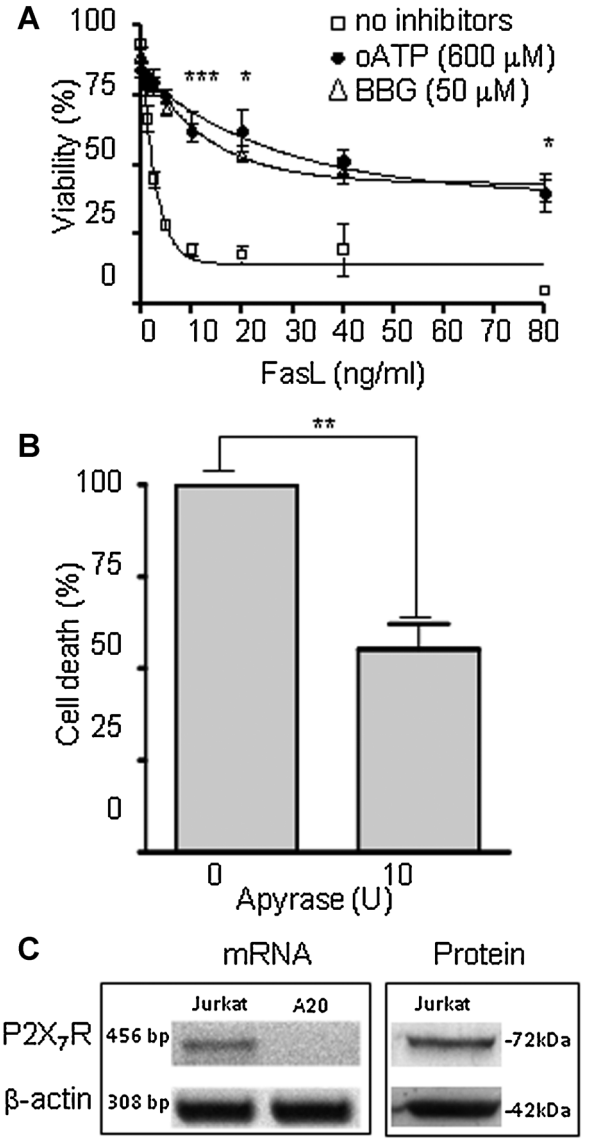


Fig. 2. Effects of P2X$_7$R-antagonists on cell viability and apyrase on cell death. Jurkat cells were pretreated 1 h with oATP (600 μM) (●) or BBG (50 μM) (Δ) before adding FasL (0–80 ng/ml) for 16 h. Then cells were stained with propidium iodide (PI) and cell viability was evaluated by flow cytometry (A). Jurkat cells were pretreated 15 min with apyrase (10 U) before adding FasL (80 ng/ml) for 16 h. Then, cells were stained with propidium iodide (PI) and dead cells were identified by flow cytometry (B). The results shown were averaged from three independent experiments (n = 3). Statistically significant differences are indicated (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). The expression of P2X$_7$R in Jurkat cells was corroborated by RT-PCR and Western blot analysis. Actin expression is shown as a loading control. A20 mouse cells were used as negative control for RT-PCR (C).

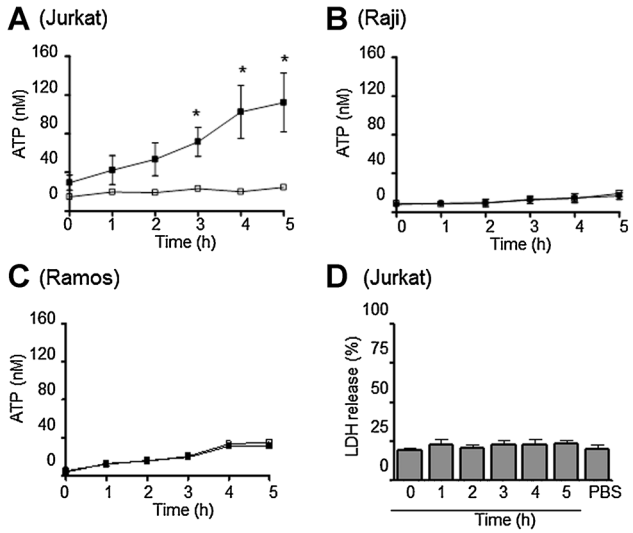


Fig. 3. ATP release induced by stimulation of lymphoid cells. ATP release to the medium was measured using a luminescence assay for Jurkat (A), Raji (B), and Ramos (C) cells treated with FasL (80 ng/ml) (■) or PBS (□) for different periods of time (0–5 h). All cells were pretreated with ebselen (30 μM) to block ectonucleotidase activity and then stimulated 10 min later. For Jurkat cells, integrity of the plasma membrane was monitored up to 5 h using the lactate dehydrogenase (LDH) release assay (D). The results shown were averaged from three independent experiments (n = 3). Statistically significant differences in (A) are indicated (*$P < 0.05$).

co-localization between Panx1 and Fas increased up to 3 h post-stimulation (Fig. 5D).

Time-lapse fluorescence imaging of EtBr uptake of Jurkat cells stimulated with FasL

Results obtained previously in the ATP release assays using inhibitors (Fig. 4) implicated Panx1 HCs in the sequence of events downstream of Fas. To corroborate these findings, we measured the incorporation of EtBr in response to FasL (Fig. 6). This compound is known to enter cells via Panx1 channels once they open and this event can be registered as an increase in intracellular fluorescence (Pelegrin and Surprenant, 2009). Thus, Jurkat cells were seeded on coverslips pretreated with poly-L-lysine in the presence of HANK'S solution with 5 μM EtBr. Fluorescence intensity of cells associated with EtBr uptake was recorded for up to 3 h (see Fig. 6A). Jurkat cells were pretreated or not either with 1 mM probenecid or 200 μM $LaCl_3$ (an inhibitor of Cx HCs) 10 min before the addition of FasL (80 ng/ml). In Jurkat cells, the basal uptake rate was similar for cells that were pretreated or not with inhibitors before adding FasL (data not shown). In cells stimulated with FasL alone or following pretreatment with $LaCl_3$ (Fig. 6B), fluorescence intensity increased steadily in a similar manner. However, pretreatment with probenecid reduced significantly the increase in fluorescence triggered by FasL. These results link FasL-induced ATP release to the opening of Panx1 HCs.

Caspase inhibitors blocked FasL-stimulated ATP release from Jurkat cells and treatment with Apyrase or the $P2X_7R$ antagonist BBG reduced caspase-9 activity in FasL-stimulated Jurkat cells

We then asked whether caspases might be involved in triggering ATP release in response to stimulation with FasL. Thus, ATP release from Jurkat cells was measured in the presence of a

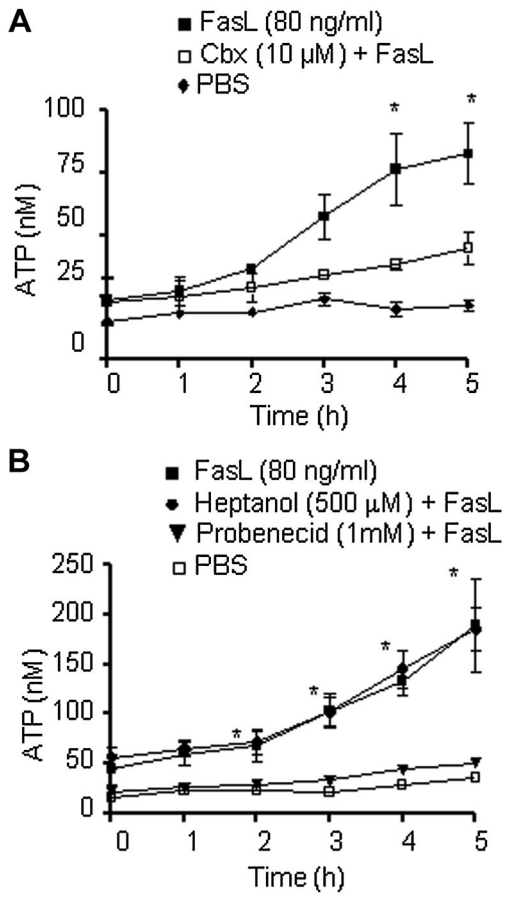


Fig. 4. Effect of Cx HC and Panx1 HC inhibitors on ATP accumulation in medium. ATP release from Jurkat cells treated with FasL (80 ng/ml) alone for up to 5 h (■) or following pretreatment with carbenoxolone (10 μM) (□) (A), probenecid (1 mM) (▼) or heptanol (500 μM) (●) (B) was measured. As a control, cells were treated with PBS (□) for the same periods of time. The results shown were averaged from three independent experiments (n = 3). Statistically significant differences are indicated (*$P < 0.05$).

general caspase inhibitor (zVAD, 20 μM), an inhibitor of caspase-8- (zIETD, 20 μM), or caspase-3-like activity (zDEVD, 20 μM), for up to 5 h (Fig. 7A). For cells treated with either zVAD or zIETD FasL-induced ATP release was similar to that observed for PBS-treated cells, while zDEVD had no significant effect on ATP release even at 50 μM (data not shown). In parallel, cell viability was evaluated by flow cytometry. For cells treated with zVAD (20 μM), viability was essentially maintained even in the presence of FasL. When cells were treated with zIETD, zLEHD (an inhibitor of caspase-9-like activity), and zDEVD (20 μM), viability was partially maintained at low FasL concentrations (Fig. 7B). However, when the inhibitors zDEVD, zLEHD, or zIETD were used at a higher concentrations (50 μM), FasL-induced loss of viability was largely prevented (Fig. 7C).

In our model, ATP-dependent activation of $P2X_7R$ plays a key role in FasL-induced death of Jurkat cells (see Fig. 2), which is known to be caspase-9 dependent. In order to implicate more directly caspase-9 downstream of $P2X_7R$, the kinetics of FasL-dependent caspase-9 activation was evaluated in the absence or presence of the $P2X_7R$ inhibitor BBG or the inhibitor of casapase-9-like activity zLEHD. Indeed, caspase-9 activation after stimulation with FasL was substantially delayed

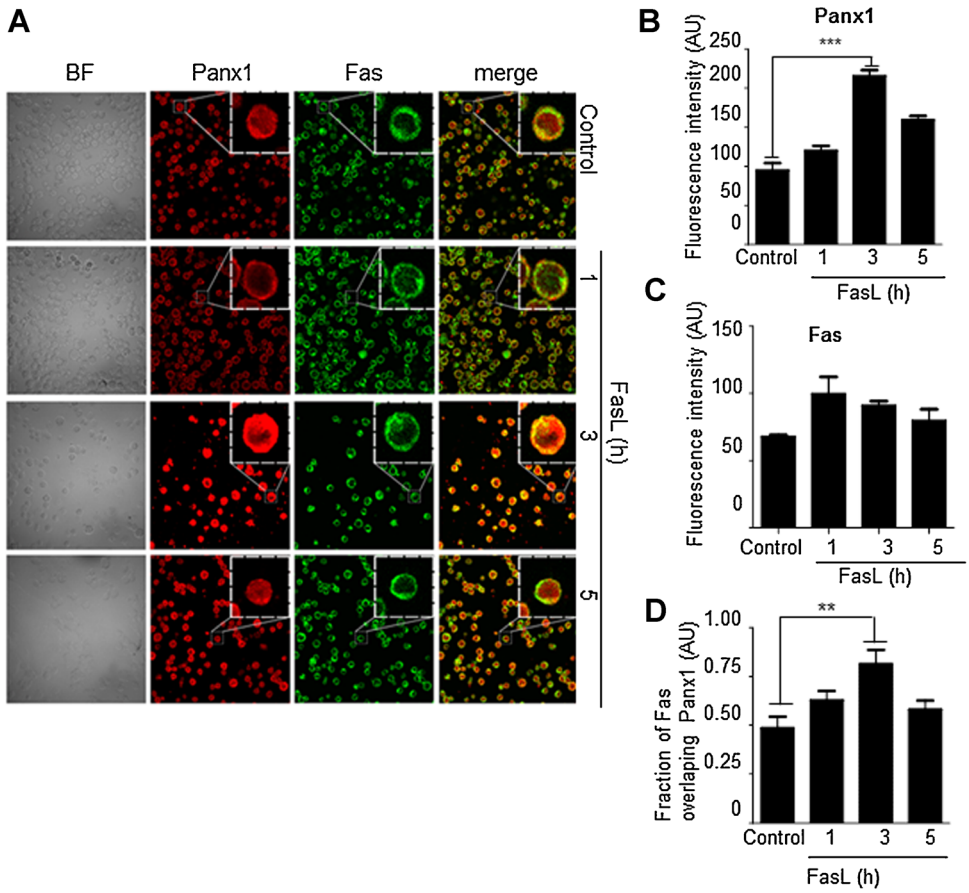


Fig. 5. Confocal microscopy of FasL-treated Jurkat cells. (A) Jurkat cells were stimulated for 1, 3, and 5 h with 80 ng/ml FasL. Then, cells were fixed and stained with anti-Panx1 or anti-human Fas antibody followed by PE- or FITC-coupled secondary antibodies, respectively (B,C) expressed as fluorescence intensity (F.I.) in arbitrary units (UA). Additionally, the fraction of Fas overlapping with Panx1 is shown (D). Means of the relative fluorescence intensity for 120 cells in every condition are shown. Statistically significant differences (Kruskall–Wallis analysis) are indicated ($^{**}P < 0.01$ and $^{***}P < 0.001$).

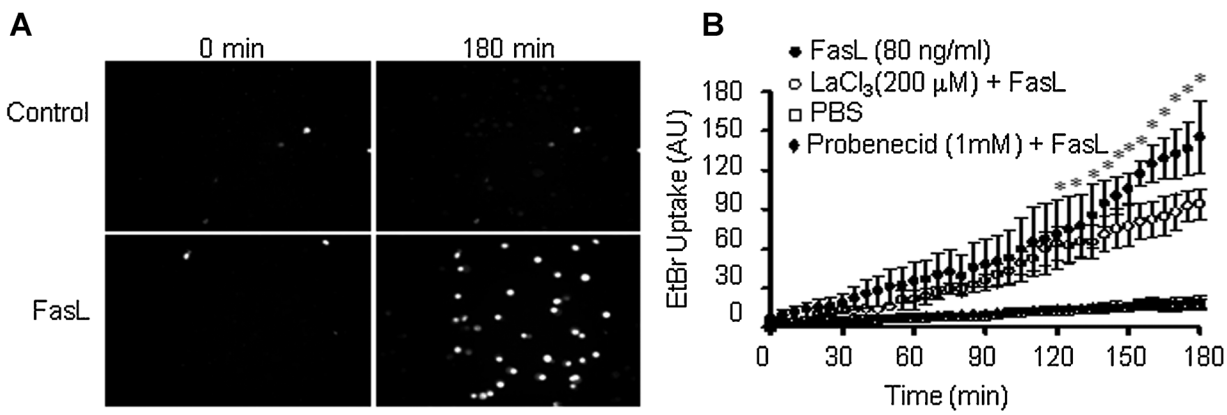
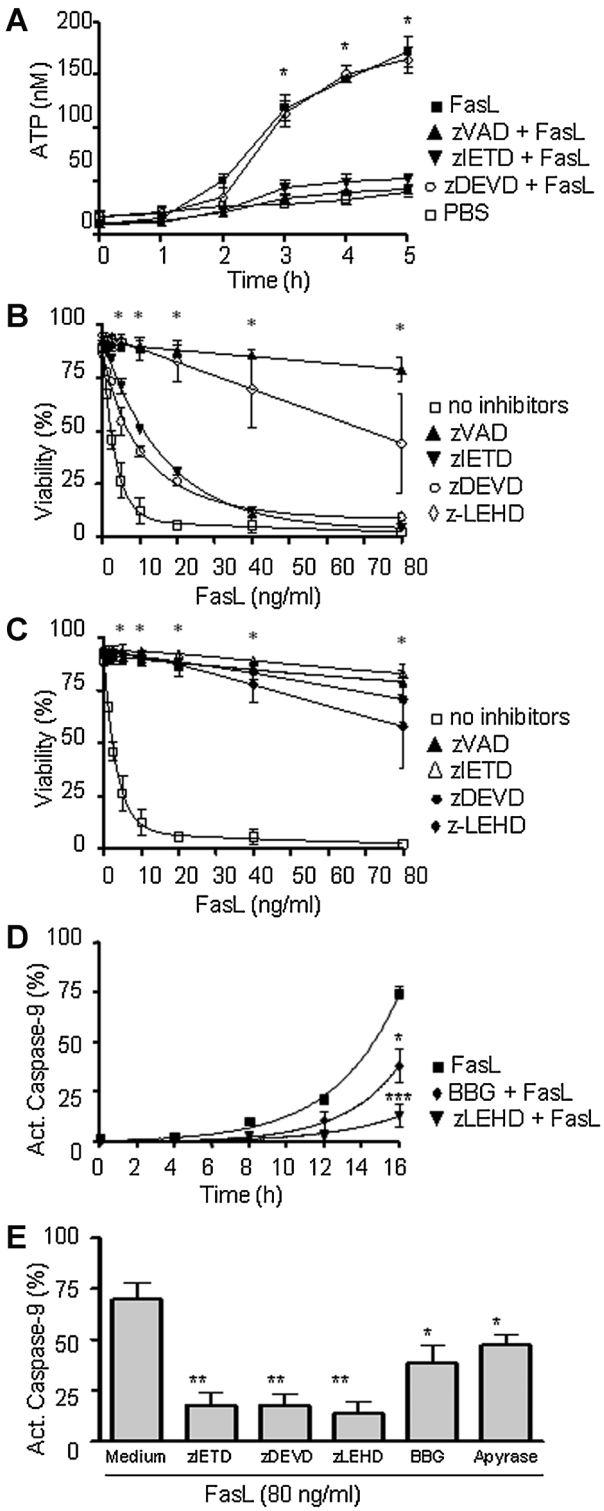


Fig. 6. EtBr uptake by Jurkat cells stimulated with FasL. Time lapse images are shown representing different conditions: untreated (control) Jurkat cells at 0 min and 180 min and Jurkat cells treated with FasL (80 ng/ml) at time 0 and 180 min (A). EtBr uptake was quantified over time. For some experiments, cells were pretreated with probenecid (1 mM) or $LaCl_3$ (200 µM) 10 min before adding FasL. As a control, cells were treated only with PBS (B). Statistically significant differences are indicated ($^{*}P < 0.05$).

in the presence of the $P2X_7R$ inhibitor BBG, as was also observed in the presence of zLEHD (Fig. 7D). Furthermore, treatment of Jurkat cells for 1 h with either BBG (50 μM) or apyrase (10 U) prior to FasL (80 ng/ml) stimulation for 16 h significantly reduced caspase-9 activation (Fig. 7E). This decrease was more pronounced with the inhibitors of caspase-like activity zIETD, zDEVD, or zLEHD (50 μM).



Discussion

Fas–FasL interaction is known to activate caspase-8 followed by caspase-3 induced apoptotic cell death (Peter and Krammer, 2003; Strasser et al., 2009). In type II cells, successful execution of this program requires intermediate participation of a mitochodria-dependent pathway that leads to caspase-9 and subsequently caspase-3 activation (Scaffidi et al., 1998; Samraj et al., 2006). Alternatively, activation of the purinergic $P2X_7R$ via ATP triggers necrotic or apoptotic cell death (Di Virgilio et al., 1998). Moreover, ATP-induced caspase activation via the $P2X_7R$ was also previously reported (for review see Adinolfi et al., 2002). Whether a connection exists between Fas and $P2X_7R$ was, however, not known. In this study, we demonstrate, for the first time, that cross-talk observed between the two cell death receptors, Fas and $P2X_7$, is required to trigger cell death. Specifically, FasL-induced activation of caspase-8 leads to ATP release mediated by Panx1 HCs. Extracellular ATP then activates the $P2X_7R$ and promotes cell death in a caspase-9-dependent manner.

Previously, we observed that sensitivity of lymphoid cells to stimulation with FasL or ceramide analogs lead to execution of variable death programs (Hetz et al., 2002; Villena et al., 2008). Here, we evaluated the sensitivity to FasL in different lymphoid cell lines and observed striking differences. For instance, Raji, and Ramos cell lines were highly resistant to FasL-induced death, although surface Fas expression was highest in Raji cells and somewhat lower but similar levels were detected in Jurkat and Ramos cells (Supplementary Fig. 1). This observation is in agreement with previous studies showing that cell "sensitization" (Kim et al., 2007) or addition of substantially higher FasL concentrations (Irmler et al., 1997) are required to induce cell death by Fas activation in Burkitt's lymphoma cell lines. On the other hand, Jurkat cells were highly sensitive to FasL-induced death (Fig. 1).

ATP can activate $P2X_7R$ and stimulate a variety of responses including Ca^{2+} and Na^{+} influx, K^{+} efflux, activation of mitogen-activated protein kinases (MAPKs) ERK1/2, p38, and JNKs, the NADPH oxidase complex, phospholipase D, and several caspases. Additionally, formation of reactive oxygen species (ROS), a non-specific pore permeable to small molecules (<900 Da), as well as ATP dependent cell death have been reported (Burnstock, 2006; Lenertz et al., 2009; Hill et al., 2010; Lenertz et al., 2011). ATP-induced effects in cells are often linked to $P2X_7R$, activation. Recent studies in thymocytes and Jurkat cells have shown that ATP is released by cells undergoing apoptosis and that the molecule serves as a chemoattractant for macrophages, which then eliminate the cell debris (Elliott et al., 2009; Chekeni et al., 2010). We suspected however, that ATP release might also serve other purposes and, for instance, help to connect the two death receptors, Fas and $P2X_7$. In agreement with this possibility, ATP release was only observed

Fig. 7. Effect of different inhibitors on ATP release, viability and caspase-9 activation in Jurkat cells stimulated with FasL. ATP presence in supernatants from Jurkat cells treated with FasL (80 ng/ml) for up to 5 h either alone or together with caspase inhibitors zVAD (20 μM), zIETD (20 μM), or zDEVD (20 μM) were measured using the luminescence assay. As controls, cells were treated with PBS (A). In parallel, cell viability was evaluated by flow cytometry after 16 h of treatment with FasL either alone or together with zVAD, zIETD, zDEVD, or zLEHD (20 μM) (B) or at higher concentrations (50 μM) (C). Time course of FasL-dependent caspase-9 activation in Jurkat cells was measured either in the absence or presence of BBG (50 μM) or zLEHD (50 μM) (D). Cells were pretreated with BBG (50 μM), zLEHD (50 μM), zIETD (50 μM), zDEVD (50 μM), and apyrase (10 U) before adding FasL (80 ng/ml) for 16 h (E). The results shown were averaged from three independent experiments (n = 3). Statistically significant differences are indicated (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$).

for Jurkat cells upon FasL stimulation. Neither for Raji nor Ramos cells was ATP release detectable, despite the use of an inhibitor (Fig. 3) to avoid ATP hydrolysis via CD39 ectonucleotidase abundantly expressed in B cells (Pulte et al., 2007). Moreover, ATP release from Jurkat cells occurred prior to initiation of cell death and was not attributable to loss of membrane integrity (Fig. 3). Thus, overall these results suggest that ATP release in the early phase of FasL-induced apoptosis may contribute to death observed in Jurkat cells.

ATP release mechanisms are highly variable and depending on the cell type, Cx43 based HCs, the volume-regulated anion channel (VRAC) and the purinergic $P2X_7R$, have been implicated (Maroto and Hamill, 2001; Bal-Price et al., 2002; Parpura et al., 2004; Anselmi et al., 2008). Recent evidence also suggests that Panx1 HCs participate in the release of ATP from activated T cells. In this model, the ATP released through Panx1 HCs is an essential autocrine signal that acts on P2XRs (Schenk et al., 2008). Consistent with the possibility that Panx1 might be involved, Western blot analysis revealed substantial expression of Panx1, while Cx43 was not detected (Supplementary Fig. 2). Panx1 HCs are relatively insensitive to $LaCl_3$ and heptanol, while Cx HCs are effectively blocked by these agents (Bruzzone et al., 2005; Pelegrin and Surprenant, 2006). Probenecid, a compound used clinically for the treatment of gout, is reportedly a non-specific inhibitor (at 7 mM) of membrane resistant protein (MRP) family transporters (Hammond et al., 2007). However, more recently probenecid was shown also to block Panx1 HC currents at 1 mM (IC50 of 150 μM) (Silverman et al., 2008). In our experiments in Jurkat cells, probenecid, also at 1mM, decreased cellular EtBr uptake induced by FasL to background levels, while $LaCl_3$ had no effect (Fig. 6). Similarly, carbenoxolone at the low concentrations, like those employed here, inhibits Panx1 (IC50 of 5 μM) and not Cx HCs (Bruzzone et al., 2005; Locovei et al., 2007). Also, carbenoxolone reduced FasL-induced ATP release in Jurkat cells, but heptanol had no effect (Fig. 4). Importantly Cx43 was essentially undetectable in Jurkat cells (Supplementary Fig. 2B). Taken together, the data strongly suggest that Panx1 HCs, but not Cx HCs are activated in Jurkat cells stimulated with FasL.

The kinetics of FasL-induced ATP release via Panx1 HCs favor the notion that caspase activation is required in Jurkat cells. Indeed, FasL-induced ATP release decreased in the presence of the generic caspase inhibitor zVAD and upon inhibition of caspase-8- (zIETD), but not caspase-3-like activity (Fig. 7), indicating that ATP release occurs upstream of caspase-3. Interestingly, elimination of extracellular ATP by apyrase effectively reduced cell death induced by FasL (Fig. 2). Consistent with our observations, caspase-dependent nucleotide release from apoptotic cells has been observed in primary thymocytes, Jurkat and epithelial cells (Elliott et al., 2009).

All caspase inhibitors employed in this study (zVAD, zDEVD, zIETD, and zLEHD) were expected to protect the cells against FasL-induced death to varying extents. However, caspase inhibitors are also known not to be entirely selective (Berger et al., 2006). Thus, while there was partial and differential inhibition at the lower concentrations of caspase inhibitors (Fig. 7B), the strong essentially complete inhibition observed using caspase inhibitors at higher concentrations, probably reflects the consequence of caspase cross-inhibition. Together these observations suggest that Jurkat cells require an additional ATP-dependent amplification mechanism to execute the apoptotic process downstream of Fas.

With this in mind, we hypothesized that ATP released from apoptotic Jurkat cells is an additional extracellular signal to $P2X_7R$ that amplifies the apoptotic response via caspase-9 activation. Indeed, we observed that inhibiting the $P2X_7R$ using the specific BBG inhibitor delayed rather than blocked caspase-9 activation upon stimulation with FasL (Fig. 7D). Moreover, ATP hydrolysis by apyrase treatment reduced caspase-9 activity to an extent similar to what was observed with the $P2X_7R$ specific inhibitor BBG (Fig. 7E) and both treatments partially protected against FasL-induced death (Fig. 2). This is in agreement with the interpretation that $P2X_7R$-dependent caspase-9 activation is part of an amplification process that parallels the existing t-Bid pathway in Jurkat type II cells.

Panx1 HCs have been suggested to be responsible for ATP release in human T lymphocytes (Schenk et al., 2008). Also, Panx1 HC participation in ATP release from apoptotic cells was corroborated by experiments using specific siRNA for Panx1 and patch-clamp techniques. These experiments revealed that Panx1 HC are basally inactive and that the induction of Panx1 HC-dependent currents only occurs during apoptosis (Chekeni et al., 2010). These observations are consistent with our results, in that only for Jurkat cells treated with FasL was ATP release and EtBr uptake observed (Figs. 3, 4 and 6).

Results from several laboratories suggest that ATP release via Panx1 HC is observed in a variety of cell types and occurs by different mechanisms that do not invoke irreversible Panx1 truncation (Qiu et al., 2011). Additionally, however, specific cleavage by caspase-3 has been implicated in the activation of Panx1 HC using anti-hFas antibodies in Jurkat cells, where Panx1 COOH-terminal degradation was detected 2 h after stimulation (Chekeni et al., 2010). Moreover, a recent publication specifically identified a COOH-terminal auto-inhibitory region of Panx1 that is cleaved by caspases to activate the channel and permit ATP release (Sandilos et al., 2012). We observed that stimulation of Jurkat cells with FasL lead to an increase in levels of Panx1 by both confocal microscopy (Fig. 5) and western blot analysis (Supplementary Fig. 3). Since, multiple bands were detected by western blotting for Panx1, caspase-mediated cleavage may also be relevant here

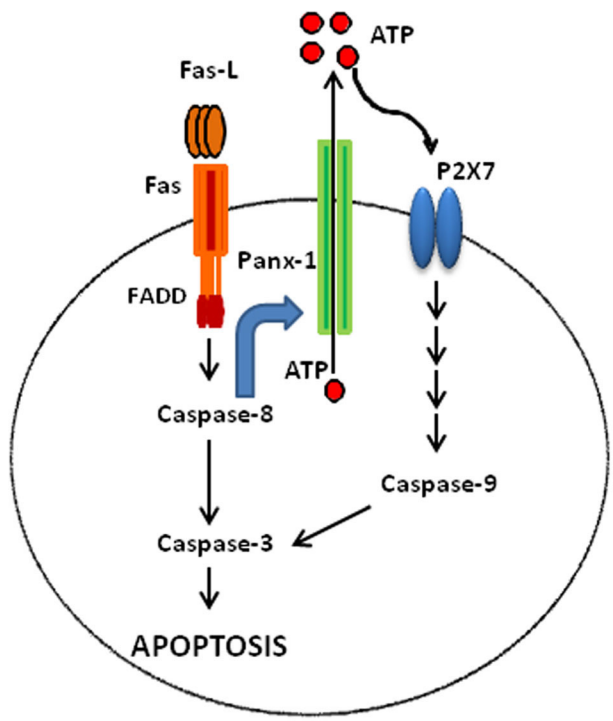


Fig. 8. A schematic summary of current findings. For details concerning this scheme, see discussion.

to Panx1 activation downstream of Fas. However, further experiments are required to evaluate this possibility.

Finally, we demonstrated a role for the $P2X_7R$ in FasL-induced cell death by $P2X_7R$ inhibition assays (Fig. 2). We observed an increase in cell viability and a clear inhibition of apoptosis in Jurkat cells when $P2X_7Rs$ were inhibited with oATP. To confirm these results we employed a more specific inhibitor for $P2X_7R$, BBG, which has been used as an antagonist of $P2X_7R$ in animal models of spinal cord trauma (Peng et al., 2009). Robust inhibition of FasL-induced caspase-9 activation and apoptosis was observed for Jurkat cells pretreated with BBG (see Figs. 2 and 7).

The events triggered downstream of $P2X_7R$ remain unclear. Activation of the $P2X_7R$ not only opens a typical ion channel, but also another independent channel permeable to large molecules up to 900 Da is recruited. Fluorescence imaging, electrophysiological recordings and Western blot analysis provided evidence suggesting that Panx1 HCs are likely to be the pore forming units activated by ATP stimulation of $P2X_7R$. This interpretation is sustained by experiments showing co-immunoprecipitation and colocalization of $P2X_7R$ subunit and Panx1 (North, 2002; Pelegrin and Surprenant, 2006; Locovei et al., 2007; Pelegrin, 2011). Together with our immunolocalization results showing enhanced colocalization between Fas and Panx1 (Fig. 5), it is tempting to speculate that Fas, Panx1, and $P2X_7R$ become functionally coupled in a complex after FasL stimulation.

Model to explain Fas and $P2X_7R$ coupling

In summary, FasL stimulation of Jurkat cells via Fas leads to the activation of caspase-8 and subsequently caspase-3. In addition, caspase-8 induces ATP release through Panx1 HCs, a process that coincides temporarily with enhanced colocalization between Fas and Panx1. Whether these two events are functionally linked remains to be established. Extracellular released ATP activates $P2X_7R$ and thereby favors caspase-9 activation, presumably via a mitochondria-dependent process. Thus, in addition to the classic mechanism described for type II cells connecting caspase-8 to caspase-9 activation and death via tBid, we identified here Panx1 HC-dependent ATP liberation and $P2X_7R$ activation as a further amplification loop to efficiently execute FasL-induced death in Jurkat cells (Fig. 8).

Acknowledgments

Parts of the results shown here have been presented previously in preliminary form as an abstract/poster at the 53rd Annual Meeting of Biophysical Society, Boston, MA, (USA, March 2009) and the XXIV Reunión Anual de la Sociedad de Biología Celular de Chile, Pucón (Chile, November 2010). This work was supported by FONDECYT-FONDAP (to A.F.G. Quest) 15010006, FONDECYT (to A.F.G. Quest) 1090071, FONDECYT-Postdoctoral fellowship (to M. Henriquez) 3070045, and ANILLO (to J.C. Sáez) ACT-71.

Literature Cited

Adinolfi E, Melchiorri L, Falzoni S, Chiozzi P, Morelli A, Tieghi A, Cuneo A, Castoldi G, Di Virgilio F, Baricordi OR. 2002. P2X7 receptor expression in evolutive and indolent forms of chronic B lymphocytic leukemia. Blood 99:706–708.

Anselmi F, Hernandez VH, Crispino G, Seydel A, Ortolano S, Roper SD, Kessaris N, Richardson W, Rickheit G, Filippov MA, Monyer H, Mammano F. 2008. ATP release through connexin hemichannels and gap junction transfer of second messengers propagate Ca2+ signals across the inner ear. PNAS 105:18770–18775.

Aswad F, Dennert G. 2006. P2X7 receptor expression levels determine lethal effects of a purine based danger signal in T lymphocytes. Cell Immunol 243:58–65.

Bal-Price A, Moneer Z, Brown GC. 2002. Nitric oxide induces rapid, calcium-dependent release of vesicular glutamate and ATP from cultured rat astrocytes. Glia 40:312–323.

Berger A, Sexton K, Bogyo M. 2006. Commonly used caspase inhibitors designed based on substrate specificity profiles lack selectivity. Cell Res 16:961–963.

Bolte S, Cordelieres FP. 2006. A guided tour into subcellular colocalization analysis in light microscopy. J Microsc 224:213–232.

Bruzzone R, Barbe MT, Jakob NJ, Monyer H. 2005. Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in Xenopus oocytes. J Neurochem 92:1033–1043.

Burnstock G. 2006. Pathophysiology and therapeutic potential of purinergic signaling. Pharmacol Rev 58:58–86.

Chekeni F, Elliott M, Sandilos J, Walk S, Kinchen J, Lazarowski E, Armstrong A, Penuela S, Laird D, Salvesen G, Isakson B, Bayliss D, Ravichandran K. 2010. Pannexin 1 channels mediate "find-me" signal release and membrane permeability during apoptosis. Nature 467:863–867.

Di Virgilio F, Chiozzi P, Falzoni S, Ferrari D, Sanz JM, Venketaraman V, Baricordi OR. 1998. Cytolytic P2X purinoceptors. Cell Death Differ 5:191–199.

Elliott M, Chekeni F, Trampont P, Lazarowski E, Kadl A, Walk S, Park D, Woodson R, Ostankovich M, Sharma P, Lysiak J, Harden T, Leitinger N, Ravichandran K. 2009. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature 461:282–286.

Hammond CL, Marchan R, Krance SM, Ballatori N. 2007. Glutathione export during apoptosis requires functional multidrug resistance-associated proteins. J Biol Chem 282:14337–14347.

Hengartner MO. 2000. The biochemistry of apoptosis. Nature 407:770–776.

Hetz CA, Hunn M, Rojas P, Torres V, Leyton L, Quest AF. 2002. Caspase-dependent initiation of apoptosis and necrosis by the Fas receptor in lymphoid cells: Onset of necrosis is associated with delayed ceramide increase. J Cell Sci 115:4671–4683.

Hill LM, Gavala ML, Lenertz LY, Bertics PJ. 2010. Extracellular ATP may contribute to tissue repair by rapidly stimulating purinergic receptor X7-dependent vascular endothelial growth factor release from primary human monocytes. J Immunol 185:3028–3034.

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer J, Schroter M, Burns K, Mattmann C, Rimoldi D, French L, Tschopp J. 1997. Inhibition of death receptor signals by cellular FLIP. Nature 388:190–195.

Kim YS, Park GB, Song HK, Hur I, Lee HK, Kang JS, Hahm E, Lee WJ, Hur DY. 2007. Cross-linking of CD54 on Burkitt lymphoma cell line Raji and Ramos induces FasL expression by reactive oxygen species and apoptosis of adjacent cells in Fas/FasL interaction. J Immunother 30:727–739.

Lenertz LY, Gavala ML, Hill LM, Bertics PJ. 2009. Cell signaling via the P2X(7) nucleotide receptor: Linkage to ROS production, gene transcription, and receptor trafficking. Purinergic Signal 5:175–187.

Lenertz LY, Gavala ML, Zhu Y, Bertics PJ. 2011. Transcriptional control mechanisms associated with the nucleotide receptor P2X7, a critical regulator of immunologic, osteogenic, and neurologic functions. Immunol Res 50:22–38.

Locovei S, Scemes E, Qiu F, Spray D, Dahl G. 2007. Pannexin1 is part of the pore forming unit of the P2X(7) receptor death complex. FEBS Lett 581:483–488.

Maroto R, Hamill OP. 2001. Brefeldin A block of integrin-dependent mechanosensitive ATP release from Xenopus oocytes reveals a novel mechanism of mechanotransduction. J Biol Chem 276:23867–23872.

North RA. 2002. Molecular physiology of P2X receptors. Physiol Rev 82:1013–1067.

Parpura V, Scemes E, Spray DC. 2004. Mechanisms of glutamate release from astrocytes: Gap junction "hemichannels," purinergic receptors and exocytotic release. Neurochem Int 45:259–264.

Pelegrin P. 2011. Many ways to dilate the P2X7 receptor pore. Br J Pharmacol 163:908–911.

Pelegrin P, Surprenant A. 2006. Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. EMBO J 25:5071–5082.

Pelegrin P, Surprenant A. 2009. The P2X(7) receptor-pannexin connection to dye uptake and IL-1beta release. Purinergic Signal 5:129–137.

Peng W, Cotrina ML, Han X, Yu H, Bekar L, Blum L, Takano T, Tian GF, Goldman SA, Nedergaard M. 2009. Systemic administration of an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. Proc Natl Acad Sci USA 106:12489–12493.

Peter ME, Krammer PH. 2003. The CD95(APO-1/Fas) DISC and beyond. Cell Death Differ 10:26–35.

Pulte D, Olson KE, Broekman MJ, Islam N, Ballard HS, Furman RR, Olson AE, Marcus AJ. 2007. CD39 activity correlates with stage and inhibits platelet reactivity in chronic lymphocytic leukemia. J Transl Med 5:23.

Qiu F, Wang J, Dahl G. 2011. Alanine substitution scanning of pannexin1 reveals amino acid residues mediating ATP sensitivity. Purinergic Signal 8:81–90.

Samraj AK, Keil E, Ueffing N, Schulze-Osthoff K, Schmitz I. 2006. Loss of caspase-9 provides genetic evidence for the type I/II concept of CD95-mediated apoptosis. J Biol Chem 281:29652–29659.

Sandilos JK, Chiu Y-HH, Chekeni FB, Armstrong AJ, Walk SF, Ravichandran KS, Bayliss DA. 2012. Pannexin 1, an ATP release channel, is activated by caspase cleavage of its pore-associated C terminal autoinhibitory region. J Biol Chem M111:323378.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli K, Debatin K, Krammer P, Peter M. 1998. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 17:1675–1687.

Schenk U, Westendorf AM, Radaelli E, Casati A, Ferro M, Fumagalli M, Verderio C, Buer J, Scanziani E, Grassi F. 2008. Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels. Sci Signal 1:ra6.

Silverman W, Locovei S, Dahl G. 2008. Probenecid, a gout remedy, inhibits pannexin 1 channels. Am J Physiol Cell Physiol 295:C761–C767.

Strasser A, Jost PJ, Nagata S. 2009. The many roles of FAS receptor signaling in the immune system. Immunity 30:180–192.

Tokunaga A, Tsukimoto M, Harada H, Moriyama Y, Kojima S. 2010. Involvement of SLC17A9-dependent vesicular exocytosis in the mechanism of ATP release during T cell activation. J Biol Chem 285:17406–17416.

Tsukimoto M, Maehata M, Harada H, Ikari A, Takagi K, Degawa M. 2006. P2X7 receptor-dependent cell death is modulated during murine T cell maturation and mediated by dual signaling pathways. J Immunol 177:2842–2850.

van Steensel B, van Binnendijk EP, Hornsby CD, van der Voort HT, Krozowski ZS, de Kloet ER, van Driel R. 1996. Partial colocalization of glucocorticoid and mineralocorticoid receptors in discrete compartments in nuclei of rat hippocampus neurons. J Cell Sci 109:787–792.

Villena J, Henriquez M, Torres V, Moraga F, Diaz-Elizondo J, Arredondo C, Chiong M, Olea-Azar C, Stutzin A, Lavandero S, Quest AF. 2008. Ceramide-induced formation of ROS and ATP depletion trigger necrosis in lymphoid cells. Free Radic Biol Med 44:1146–1160.

Yip L, Woehrle T, Corriden R, Hirsh M, Chen Y, Inoue Y, Ferrari V, Insel PA, Junger WG. 2009. Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. FASEB J 23:1685–1693.

Yoon M, Lee H, Lee Y, Kim J, Park J, Chang W, Shin H, Kim D. 2007. Extracellular ATP is involved in the induction of apoptosis in murine hematopoietic cells. Biol Pharm Bull 30:671–676.