Adenosine-induced caspase-3 activation by tuning Bcl-X$_L$/DIABLO/IAP expression in HuH-7 human hepatoma cells

**Dongqin Yang · Takahiro Yaguchi ·
Takashi Nakano · Tomoyuki Nishizaki**

Abstract Extracellular adenosine disrupted mitochondrial membrane potentials in HuH-7 cells, a Fas-deficient human hepatoma cell line, and the effect was inhibited by the adenosine transporter inhibitor dipyridamole or by overexpressing Bcl-X$_L$. Adenosine downregulated the expression of mRNAs and proteins for Bcl-X$_L$ and inhibitor of apoptosis protein 2 (IAP2) to directly inhibit caspase-3, -7, and -9, but it otherwise upregulated the expression of mRNA and protein for DIABLO, an inhibitor of IAPs. Those adenosine effects were attenuated by dipyridamole. Caspase-3 and -8 were implicated in adenosine-induced HuH-7 cell death, and adenosine actually activated caspase-3 without caspase-9 activation. The caspase-3 activation was inhibited by overexpressing Bcl-X$_L$ or IAP2. Taken together, the results of the present study indicate that intracellularly transported adenosine activates caspase-3 by neutralizing caspase-3 inhibition due to IAP as a result of decreased IAP2 expression and reduced IAP activity in response to increased DIABLO expression and perhaps DIABLO release from damaged mitochondria, in addition to caspase-8 activation. This represents further insight into adenosine-induced HuH-7 cell apoptotic pathway.

Keywords Adenosine · HuH-7 cell · Bcl-X$_L$ · DIABLO · Inhibitor of apoptosis protein

D. Yang · T. Yaguchi · T. Nishizaki (✉)
Division of Bioinformation, Department of Physiology,
Hyogo College of Medicine,
1-1 Mukogawa-cho,
Nishinomiya 663-8501, Japan
e-mail: tomoyuki@hyo-med.ac.jp

T. Nakano
Department of Thoracic Oncology,
Hyogo College of Medicine,
1-1 Mukogawa-cho,
Nishinomiya 663-8501, Japan

Present Address:
D. Yang
Laboratory of Molecular Cell Biology,
Institute of Biochemistry and Cell Biology,
Shanghai Institutes for Biological Sciences,
Chinese Academy of Sciences,
Shanghai, China

Abbreviations

IAP	Inhibitor of apoptosis protein
FADD	Fas-associated death domain protein
FLIP	FADD-like interleukin-1β-converting enzyme inhibitory protein
AIF	Apoptosis-inducing factor
Apaf-1	Apoptosis proteases activating factor-1
DMEM	Dulbecco's modified Eagle's medium
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
PBS	Phosphate-buffered saline
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ANOVA	Analysis of variance

Introduction

Apoptosis is induced via two major pathways bearing extrinsic (receptor-mediated) and intrinsic (nonreceptor-mediated) signaling. For extrinsic pathways, apoptotic signals, i.e., ligands for death receptors, induce apoptosis via a death receptor/Fas-associated death domain protein (FADD)/FADD-like interleukin-1β-converting enzyme inhibitory protein (FLIP)/caspase pathway (Irmler et al. 1997; Krueger et al. 2001; Scaffidi et al. 1998). For intrinsic pathways, the mitochondria play a central role, and the Bcl family of proteins such as Bcl-2, Bcl-X_L, Bax, and Bad expressed in the mitochondria promotes or prevents apoptosis (Kroemer 1997; Green and Reed 1998). Mitochondrial damage in response to intrinsic apoptotic signals releases cytochrome c that forms an oligomeric complex with deoxyadenosine triphosphate (dATP) or apoptosis proteases activating factor-1 (Apaf-1). The complex, in turn, activates caspase-9 followed by activation of the effector caspases, caspase-3, -6, and -7, to induce apoptosis (Earnshaw et al. 1999; Li et al. 1997; Zou et al. 1999). Smac/DIABLO and Omi/HtrA2, alternatively, promote caspase activation by neutralizing inhibitors of apoptosis proteins (IAPs; Verhagen et al. 2000, 2002; Du et al. 2000) to directly inhibit caspase-3, -7, and -9 (Salvesen and Duckett 2002; Gao et al. 2007).

The purine nucleoside adenosine, a metabolite of adenosine triphosphate, is abundantly present inside and outside cells. Accumulating studies have shown that extracellular adenosine induces apoptosis in a variety of cell types via an extrinsic pathway linked to A_1, A_{2a}, A_{2b}, and A_3 adenosine receptors and via an intrinsic pathway linked to adenosine transporters (Barry and Lind 2000; Bar-Yehuda et al. 2008; Grbovic et al. 2002; Peyot et al. 2000; Sai et al. 2006; Saitoh et al. 2004; Wen and Knowles 2003; Yang et al. 2007; Yasuda et al. 2009). In our earlier study, extracellular adenosine induced apoptosis of HuH-7 cells, a Fas-deficient human hepatoma cell line (Yang et al. 2007). The adenosine action was inhibited by dipyridamole, an adenosine transporter inhibitor, or 5′-amino-5′-deoxyadenosine, an inhibitor of adenosine kinase to convert from adenosine to adenosine monophosphate (AMP), but it was not affected by inhibitors for adenosine A_1, A_{2a}, A_{2b}, and A_3 adenosine receptors. Adenosine activated caspase-3 and -8 and the caspase-8 activation was prevented

by overexpressing c-FLIP short. Additionally, adenosine downregulated the expression of the c-FLIP mRNA and protein. In this context, these results indicated that intracellularly transported adenosine followed by conversion to AMP activates caspase-8 by neutralizing caspase-8 inhibition due to c-FLIP as a consequence of decreased c-FLIP expression and, in turn, activates the effector caspase caspase-3 to induce apoptosis.

The present study was conducted to gain further insight into the pathway underlying adenosine-induced caspase-3 activation in HuH-7 cells. In the present study, intracellularly transported adenosine downregulated Bcl-X_L expression to cause mitochondrial damage allowing Smac/DIABLO release. Adenosine also downregulated IAP2 expression, but upregulated DIABLO expression to reduce IAP activity. Those adenosine actions may neutralize caspase-3 inhibition due to IAPs, thereby activating caspase-3 responsible for HuH-7 cell apoptosis. The results of the present study, thus, extend our understanding of adenosine-induced molecular apoptotic pathways in HuH-7 cells.

Materials and methods

Cell culture

HuH-7 cells, SH-N-SH human neuroblastoma cells, and HepG2 human hepatoma cells were obtained from the RIKEN Cell Bank (Ibaraki, Japan). DU145 human prostate carcinoma cells and HEK293 human embryo kidney cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured in a culture medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Assay of cell viability

Cell viability was evaluated by a dye staining method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as previously described (Yang et al. 2007). MTT-reactive cells were quantified at an absorbance of 570 nm using a microplate

reader (SPECTRAmax PLUS384, Molecular Devices, Sunnyvale, CA, USA), and the percentage of independent basal levels (MTT intensities of cells untreated with any drug) was calculated.

Assay of mitochondrial membrane potentials

Mitochondrial membrane potentials were measured using a DePsipher™ kit. HuH-7 cells were untreated and treated with adenosine (10 mM) for 48 h. After washing with cold phosphate- buffered saline (PBS), cells were incubated in a DePsipher™ solution at 37°C for 20 min. Then, cells were washed with 1 ml of a reaction buffer containing a stabilizer solution. The fluorescent signals were observed with a laser scanning microscope (LSM 510, Carl Zeiss Co., Ltd, Germany) equipped with an epifluorescence device using a fluorescein long-pass filter (fluorescein and rhodamine) at an absorbance of 590 nm for red aggregations and at an absorbance of 530 nm for green aggregations.

Reverse transcription-polymerase chain reaction

Total RNAs of HuH-7 cells, SK-N-SH cells, HepG2 cells, A549 cells, DU145 cells, and HEK293 cells were purified by an acid/guanidine/thiocyanate/ chloroform extraction method using a Sepasol-RNA I Super kit. After purification, total RNAs were treated with RNase-free DNase I (2 U) at 37°C for 30 min to remove genomic DNAs, and 10 μg of RNAs were resuspended in water. Then, random primers, dNTP, 10× reverse transcription (RT) buffer, and MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) were added to an RNA solution and incubated at 25°C for 10 min followed by 37°C for 120 min to synthesize the first-strand cDNA. Subsequently, 2 μl of the reaction solution was diluted with water and mixed with 10× polymerase chain reaction (PCR) reaction buffer, dNTPs, MgCl$_2$, oligonucleotide, dimethyl sulfoxide (final concentration, 5% v/v), and 1 U of Taq polymerase (Fermentas, St. Leon-Roth, Germany; final volume, 20 μl). Reverse transcription-polymerase chain reaction (RT-PCR) was carried out with a GeneAmp PCR system model 9600 DNA thermal cycler (Applied Biosystems, Indianapolis, IN, USA) programmed as follows: the first one step, 94°C for 2 min and the ensuing 30 cycles, 94°C for 1 s, 62°C

for 15 s, and 72°C for 30 s. The primers as shown in Table 1 were used for RT-PCR. PCR products were stained with ethidium bromide and visualized by 2% agarose electrophoresis.

Real-time RT-PCR

Total cellular RNA from HuH-7 cells was prepared using a Sepasol-RNA I Super kit. The first-strand cDNAs were synthesized using a High-Capacity cDNA Archive Kit. Each cDNA (2 μl) was amplified in a SYBR Green Realtime PCR Master Mix (final volume, 20 μl) and loaded on the Applied Biosystems 7900 Real-time PCR Detection System (ABI, Foster City, CA, USA). Thermal cycling conditions were as follows: the first one step, 94°C for 4 min and the ensuing 40 cycles, 94°C for 1 s, 65°C for 15 s, and 72°C for 30 s. A standard curve was made by amplifying 0.5, 1, 2, 4, and 8 μl of the GAPDH mRNA diluted at 1:250. The mRNA quantity for each target gene was calculated from the standard curve using an SDS 2.1 Software (Applied Biosystems, Foster City, CA, USA).

Plasmid construction and transfection

DNAs for Bcl-X$_L$ and c-IAP2 were cloned in the EcoRI site with pcDNA6/V5. The plasmid DNAs for Bcl-X$_L$ and c-IAP2 were transfected into cells using an electroporation system (Optimizor500, BTX, San Diego, CA, USA).

Western blotting

After centrifugation of homogenates from HuH-7 cells at 12,000×g for 10 min, supernatants (30 μg protein) were loaded on 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked with TTBS (150 mM NaCl, 0.05% Tween20, and 20 mM Tris, pH 7.5) containing 5% bovine serum albumin and subsequently reacted with antibodies against Bcl-X$_L$, DIABLO, and c-IAP2, followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and visualized using a chemiluminescence detection system (FUJIFILM, Tokyo, Japan). Signal density was measured with an Image Gauge software (FUJIFILM, Tokyo, Japan).

Table 1 Primers used for RT-PCR

Primer		Base pair	Number
Bax-α	CAGAGGATGATTGCCGCCGTG AGATGGTGAGTGAGGCGGTGAGC	334	BC014175
Bax-β	CAGAGGATGATTGCCGCCGTG GGGGTGAGGAGGCTTGAGGAGTC	271	NM_004324
Bax-γ	CGGACCCGGCGAGAGGC TCAGCTTCTTGGTGGACGCATCC	162	NM_138763
Bax-δ	AGGGGATGATTGCCGCCGT GTGAGCGAGGCGGTGAGCA	328	NM_138762
Bax-σ	CAGAGGATGATTGCCGCCGTG AGATGGTGAGTGAGGCGGTGAGC	292	NM_138765
Bax-ε	CAGAGGATGATTGCCGCCGTGx AGATGGTGAGTGAGGCGGTGAGC	198	NM_138764
Bid	TAAAAGATGAAAGAGCAGTTGGACTTTTTAAAA TGAACAATATAGAGTTTGTTTTTCCTTTCTGATGA	378	NM_197966
Bcl-X$_L$	AGGGAGGCAGGCGACGAGTTT TGAAGAGTGAGCCCAGCAGAACCA	420	Z23115
Bcl-2α	GAACTGGGGGAGGATTGTGGCC TCGACGTTTTGCCTGAAGACTGTTAA	485	M13994
Bcl-2β	GAACTGGGGGAGGATTGTGGCC TGGGAGTGAACGCTTTGTCCAGAG	332	M13995
DIABLO-L	CTGTGACGATTGGCTTTGGAGTAACC TCCTCCCCTTCCTCCTGTGTTTTC	530	AF262240
DIABLO-S	GCGGTCGTGCCTTATGAAATCTAG TCCTCCCCTTCCTCCTGTGTTTTC	543	AK001399
XIAP	TATTGCTATCGTTTTTGTTCCTTGTGGA AAATGGACAGAATCCTAAAACACAACAACC	633	U45880
IAP1	TTAGAGAAATGGCAGGAAGGTGATGACC CCCTCGTCTGGTCTGGTGGAACTAAG	577	NM_004536
IAP2	GCCTGATGCTGGATAACTGGAAACTAGG CGACCCACATAATAAAAACCAGCACTTG	639	NM_001166
IAP3	AATGCTTTTGCTGTGATGGTGGACTC TCTCTCCTCTTCCCTTATTTCATCTTCTGC	424	NM_001165
IAP4	CTGATGCTGTGAGTTCTGATAGGAATTTCC GTCCACAAGGAACAAAAACGATAGCAATA	677	BC032729
IAP5	CATCCACTGCCCCACTGAGAACG CCTTCTTCCTCCCTCACTTCTCACCT	606	NM_001168
IAP6	CAGGAAATGGAAAGGTCAGTAGTTGCAC GTGAGTGGTGGAGTCATAAAAAGAGGTGTT	513	NM_016252
IAP7	AGGTGAGGTGCTTCTTCTGCTATGGG CGGCACAAAGACGATGGACACG	436	NM_139317
IAP8	TCTGATGTTGCGAGTTCTGATAGGAATTTC CGTTTTATAGTTGCTCCCAGATGTTTGAAT	497	BC039318
GAPDH	GAACATCATCCCTGCCTCTACTGGC GTGCTCTTGCTGGGGCTGGTG	428	BC023632

In a different set of experiments, mitochondrial and cytosol components were separated. After treatment with adenosine (10 mM) for 2–48 h, HuH-7 cells were harvested and centrifuged at 600×g for 10 min. After washing out with 1 ml of PBS, the pellet was resuspended in 300 μl of buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose, pH7.5) and homogenized. The lysate was centrifuged at 1,000×g for

10 min, and the supernatant was further centrifuged at 10,000×g for 1 h. The pellet and supernatant were used as mitochondria- and cytosol-enriched component, respectively, followed by Western blot analysis.

Enzymatic assay of caspase-3 and caspase-9 activity

Caspase activation was measured using a caspase fluorometric assay kit (Ac-Asp-Glu-Val-Asp-MCA for a caspase-3 substrate peptide and Ac-Leu-Glu-His-Asp-MCA for a caspase-9 substrate peptide; Yang et al. 2007). After 24 h treatment with adenosine (10 mM), HuH-7 cells were harvested, pelleted, and frozen on dry ice. The pellet was resuspended in a cell lysis buffer and incubated on ice for 10 min, and the lysates were centrifuged at 15,000×g for 10 min at 4°C. A total of 50 µg of cell lysates was incubated with 5 µl of 1 mM stock of the respective fluorescently labeled tetrapeptide at 37°C for 2 h. The fluorescence was measured at an excitation of 400 nm and at an emission of 505 nm with a fluorometer (Fluorescence Spectrometer, F-4500, HITACHI, Tokyo, Japan).

Statistical analysis

Statistical analysis was carried out using analysis of variance (ANOVA) and unpaired t test.

Results

Intracellularly transported adenosine disrupts mitochondrial membrane potentials in HuH-7 cells

To examine whether extracellular adenosine-induced HuH-7 cell apoptosis is mediated via a mitochondrial pathway, we monitored mitochondrial membrane potentials using DePsipher™. DePsipher™, a mitochondrial activity marker, is detected as an orange–red fluorescence at an absorbance of 590 nm for normal mitochondrial membrane potentials, but in case of disruption, the dye turns into green fluorescence at an absorbance of 530 nm. For untreated cells, the mitochondria exhibited orange–red fluorescent signals alone without green fluorescent signals (Fig. 1a, b). In contrast, 24 h treatment with adenosine (10 mM) accumulated green fluorescent signals (Fig. 1d), and no orange–red fluorescent signal was

found (Fig. 1c). The adenosine effect was inhibited by the adenosine transporter inhibitor dipyridamole (10 µM; Fig. 1e, f). This indicates that intracellularly transported adenosine perturbs mitochondrial membrane potentials in HuH-7 cells.
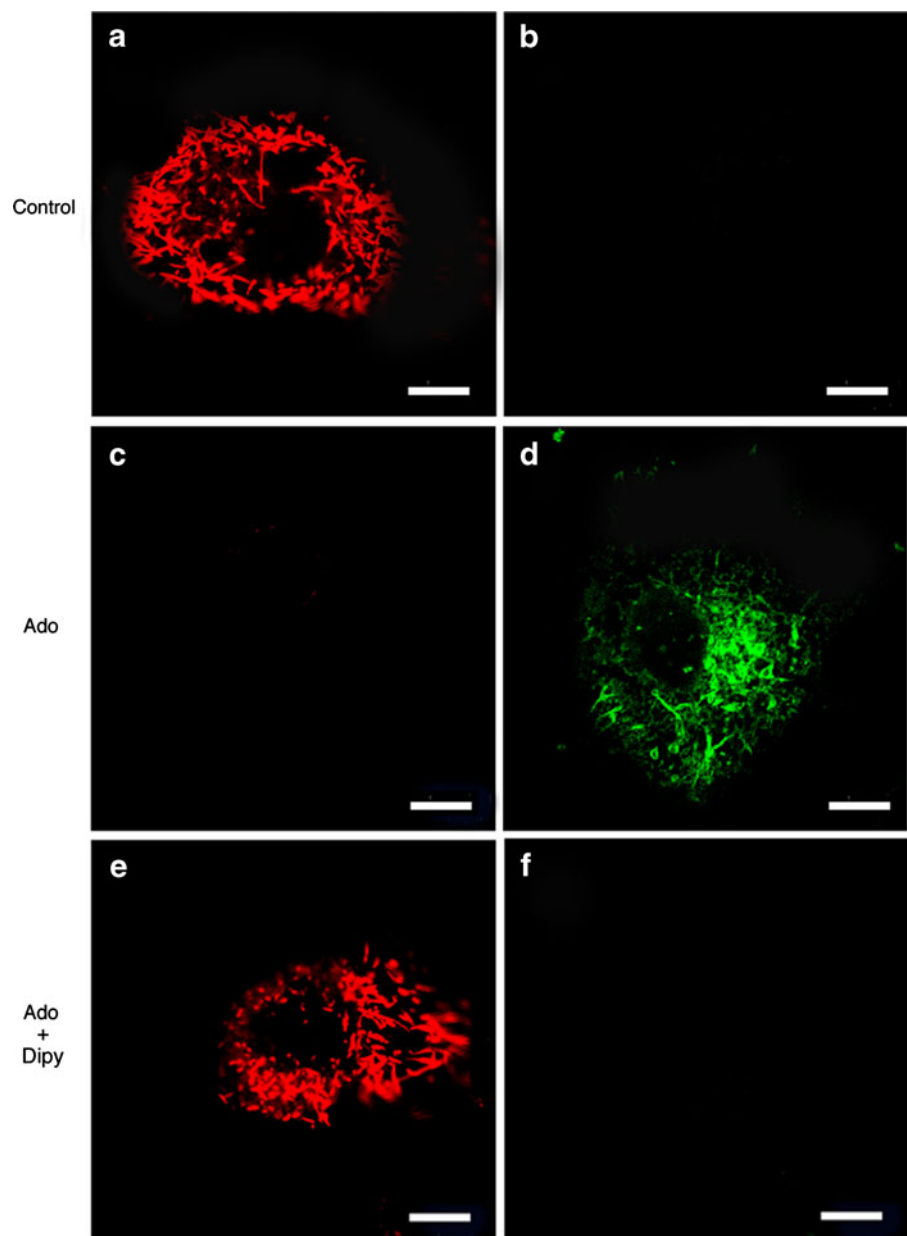
Intracellularly transported adenosine downregulates expression of mRNAs for Bcl-X$_L$, IAP2, and IAP3 but upregulates expression of the DIABLO-S mRNA in HuH-7 cells

The Bcl-2 family is recognized to engage mitochondrial apoptosis and anti-apoptosis. In the RT-PCR analysis, HuH-7 cells expressed mRNAs for Bax-α, Bax-β, Bax-γ, Bax-δ, Bax-σ, Bid, and Bcl-X$_L$ (Fig. 2a). In contrast, mRNAs for Bcl-2α, Bcl-2β, and Bax-ε were not expressed in HuH-7 cells (Fig. 2a), while the Bcl-2α and Bcl-2β mRNA were detected in HEK293 cells and the Bax-ε mRNA was detected in HepG2 cells (Fig. 2b). In the real-time RT-PCR analysis, adenosine (10 mM) downregulated the expression of the Bcl-X$_L$ mRNA ($P=0.0007$ compared with the Bcl-X$_L$ mRNA quantity before treatment with adenosine, ANOVA), the expression reaching almost 0 at 48 h treatment, and the effect was prevented by dipyridamole (10 µM; $P<0.0001$ between two groups: adenosine alone and adenosine plus dipyridamole, ANOVA; Fig. 3a), but the expression of the Bax and Bid mRNAs was not affected by adenosine (10 mM; data not shown).

In the RT-PCR analysis, HuH-7 cells expressed mRNAs for DIABLO short (DIABLO-S) and DIABLO long (DIABLO-L) to inhibit IAP, an inhibitor of caspase-3, -7, and -9 (Salvesen and Duckett 2002; Gao et al. 2007; Fig. 2a). In the real-time RT-PCR analysis, adenosine (10 mM) upregulated the expression of the DIABLO-S mRNA ($P<0.0001$ compared with the DIABLO-S mRNA quantity before treatment with adenosine, ANOVA) that was inhibited by dipyridamole (10 µM; $P<0.0001$ between two groups: adenosine alone and adenosine plus dipyridamole, ANOVA; Fig. 3b). Adenosine (10 mM) also upregulated the expression of the DIABLO-L mRNA during the initial 2 h, but the expression eventually decreased, reaching below basal levels at 48 h treatment (Fig. 3c).

As concerned with IAPs, mRNAs for XIAP, IAP2, IAP3, IAP4, IAP5, and IAP6 were expressed in HuH-7 cells (Fig. 2a), while those for IAP1, IAP7, and IAP8 were detected in HEK293 cells, DU145 cells,

Fig. 1 Effect of extracellular adenosine on mitochondrial membrane potentials in HuH-7 cells. Mitochondrial membrane potentials were assayed using a DePsipher™ kit before (control) and after 24 h treatment with adenosine (10 mM) in the absence (*Ado*) and presence of dipyridamole (10 μM; *Ado + Dipy*). *Orange–red* fluorescent signals at an absorbance of 590 nm (**a**, **c**, **e**) and *green* fluorescent signals at an absorbance of 530 nm (**b**, **d**, **f**). Scale bars, 10 μm. Note that similar results were obtained with five independent experiments

and SK-N-SH cells, respectively (Fig. 2b). In the real-time RT-PCR analysis, adenosine (10 mM) down-regulated the expression of mRNAs for XIAP (Fig. 3d), IAP2 (Fig. 3e), IAP3 (Fig. 3f), IAP4 (Fig. 3g), and IAP6 (Fig. 3i; $P<0.0001$ compared with each IAP mRNA quantity before treatment with adenosine, ANOVA), but not for IAP5 (Fig. 3h). The decrease in the IAP2 and IAP3 mRNA was significantly inhibited by dipyridamole (10 μM; $P=0.0013$ and 0.0103, respectively, between two groups: aden-

osine alone and adenosine plus dipyridamole, ANOVA; Fig. 3e, f).

Intracellularly transported adenosine downregulates expression of proteins for Bcl-X_L and IAP2 but upregulates expression of DIABLO protein in HuH-7 cells

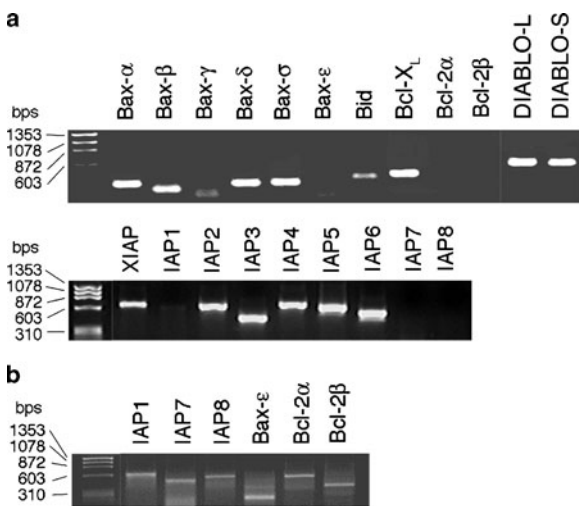In the Western blot analysis, adenosine (10 mM) decreased the expression of Bcl-X_L protein in a

Fig. 2 RT-PCR analysis. **a** PCR products from total RNAs of HuH-7 cells with RT (30 cycles). **b** PCR products for IAP1, Bcl-2α, and Bcl-2β from total RNAs of HEK293 cells, for IAP7 from total RNAs of DU145 cells, for IAP8 from total RNAs of SK-N-SH cells, and for Bax-ε from total RNAs of HepG2 cells with RT (30 cycles)

treatment time (0–48 h)-dependent manner ($P<$ 0.0001 compared with Bcl-X_L protein levels before treatment with adenosine, ANOVA), in parallel with decreased Bcl-X_L mRNA that is also inhibited by dipyridamole (10 µM; $P<0.0001$ between two groups: adenosine alone and adenosine plus dipyridamole, ANOVA; Fig. 4a).

An increase in the expression of DIABLO protein, alternatively, was found with adenosine (10 mM) treatment, with a peak at 4 h treatment ($P<0.0001$ compared with DIABLO protein levels before treatment with adenosine, ANOVA), and the effect was also inhibited by dipyridamole (10 µM; $P<0.0001$ between two groups: adenosine alone and adenosine plus dipyridamole, ANOVA; Fig. 4b). In addition, adenosine (10 mM) increased DIABLO protein in the cytosolic fraction, but otherwise decreased it in the mitochondrial fraction (Fig. 5). This suggests that adenosine stimulates DIABLO release from the mitochondria to the cytosol.

Adenosine (10 mM) decreased the expression of IAP2 protein in a treatment time (0–48 h)-dependent manner ($P<0.0001$ compared with DIABLO protein levels before treatment with adenosine, ANOVA; Fig. 4c), and dipyridamole (10 µM) attenuated the decrease ($P=0.0003$ between two groups: adenosine

alone and adenosine plus dipyridamole, ANOVA; Fig. 4c).

Collectively, these results indicate that intracellularly transported adenosine downregulates the expression of both the mRNAs and proteins for Bcl-X_L and IAP2 but upregulates expression of the DIABLO mRNA and protein. Moreover, adenosine might increase cytosolic DIABLO concentrations by stimulating DIABLO release from the mitochondria.

Intracellularly transported adenosine activates caspase-3 via a pathway linked to Bcl-X_L/DIABLO/IAP2, independently of caspase-9 activation

Adenosine-induced HuH-7 cell death was inhibited by Z-VAD-FMK, a pancaspase inhibitor, Z-DEVD-FMK, a caspase-3 inhibitor, or Z-IETD-FMK, a caspase-8 inhibitor, but not by Z-YVAD-FMK, a caspase-4 inhibitor, or Z-LEHD, a caspase-9 inhibitor (Fig. 6). This suggests that adenosine induces HuH-7 cell death by activating caspase-8 and -3, regardless of caspase-9.

Decreased Bcl-X_L expression might cause the disruption of mitochondrial membrane potentials. To address this point, Bcl-X_L was overexpressed in HuH-7 cells (Fig. 7a). Adenosine (10 mM) produced orange–red fluorescent signals alone and no accumulation of green fluorescent signals after 24 h treatment in the mitochondria of cells overexpressing Bcl-X_L (Fig. 7b). This raises the possibility that intracellularly transported adenosine disrupts mitochondrial membrane potentials by downregulating Bcl-X_L expression. In the enzymatic assay of caspase activity, adenosine (10 mM) did not activate caspase-9 in HuH-7 cells, but it otherwise activated caspase-3 (Fig. 7c). Caspase-9 activity in the presence of adenosine was not affected by overexpressing Bcl-X_L or IAP2 (Fig. 7c). In contrast, adenosine-induced caspase-3 activation was significantly prevented by overexpressing Bcl-X_L or IAP2 compared with the activation for control cells transfected with MOCK (Fig. 7c).

Mitochondrial damage would stimulate the release of DIABLO into the cytosol from the mitochondria. In addition, intracellularly transported adenosine increases the expression of the DIABLO mRNA and protein. These effects could inhibit IAP activity to neutralize caspase-3 inhibition due to IAP, leading to
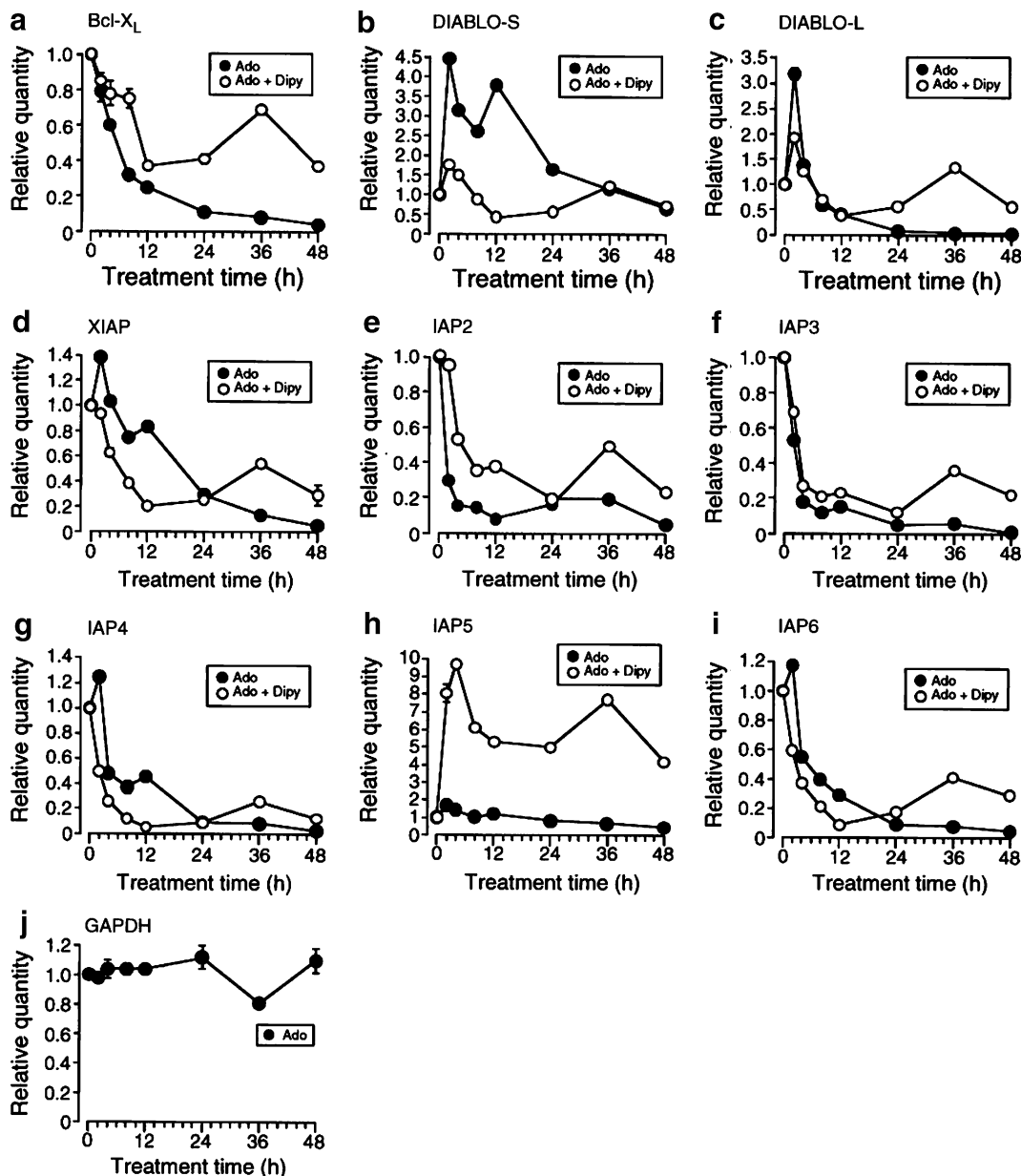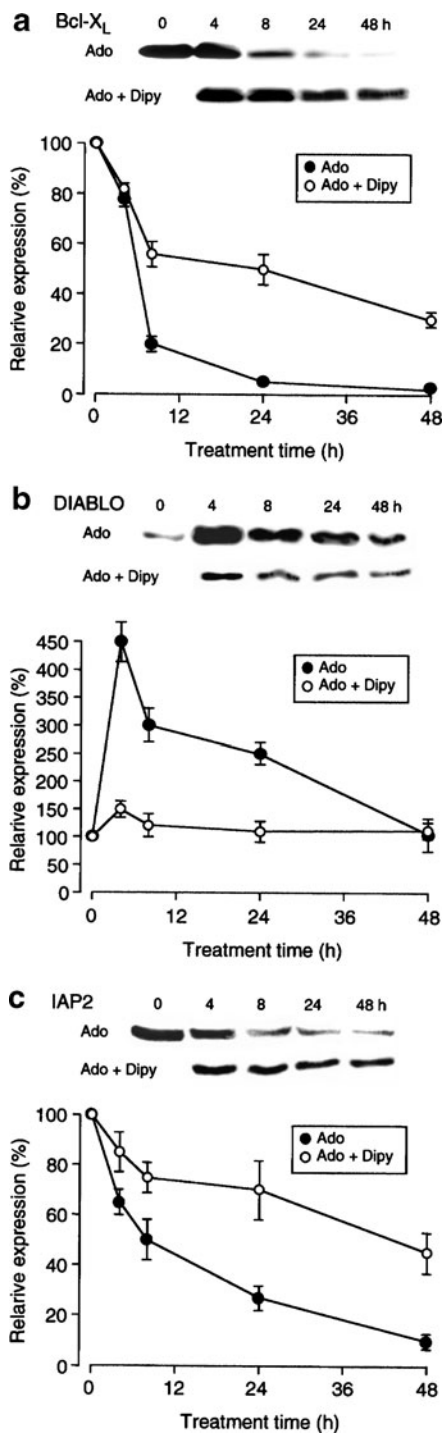
Fig. 3 Real-time RT-PCR analysis. HuH-7 cells were treated with adenosine (10 mM) in the absence (*Ado*) and presence of dipyridamole (*Ado + Dipy*; 10 μM) for 0–48 h, and then real-time RT-PCR for each gene as indicated was carried out. The mRNA quantity for each gene was calculated from the standard curve made by amplifying different amounts of the GAPDH mRNA and normalized by regarding the average of independent basal mRNA quantity at 0 h as 1. In the graphs, each point represents the mean (±SEM) ratio (*n*=4)

the activation of caspase-3. This may account for adenosine-induced caspase-3 activation in a caspase-9-independent manner. Intracellularly transported adenosine, thus, could engage caspase-3 activation by regulating the expression of Bcl-X$_L$, DIABLO, and IAP2 to induce HuH-7 cell apoptosis.

Discussion

Adenosine is recognized to induce apoptosis in a variety of cancer cells via diverse signaling pathways. Extracellular adenosine induces apoptosis in Caco-2 human colonic cancer cells by activating caspase-3

Extracellular adenosine also induces apoptosis in RCR-1 astrocytoma cells via both intrinsic and extrinsic pathways each relevant to AMPK activation and A$_1$ adenosine receptors (Sai et al. 2006). Extracellular adenosine, alternatively, induces hepatocellular carcinoma cell apoptosis as mediated via A$_3$ adenosine receptors (Bar-Yehuda et al. 2008).

In the present study, adenosine-induced HuH-7 cell death was inhibited by a pancaspase inhibitor, a caspase-3 inhibitor, and a caspase-8 inhibitor, but not a caspase-3 inhibitor. In the enzymatic assay of caspase activity, adenosine actually activated caspase-3 and -8, but not caspase-9 (Yang et al. 2007). These results imply that caspase-3 and -8 are responsible for adenosine-induced HuH7 cell apoptosis. The mitochondria are the central executioners of apoptosis. Extracellular adenosine here disrupted mitochondrial membrane potentials that is inhibited by the adenosine transporter inhibitor dipyridamole. This suggests that intracellularly transported adenosine disrupts mitochondrial membrane potentials.
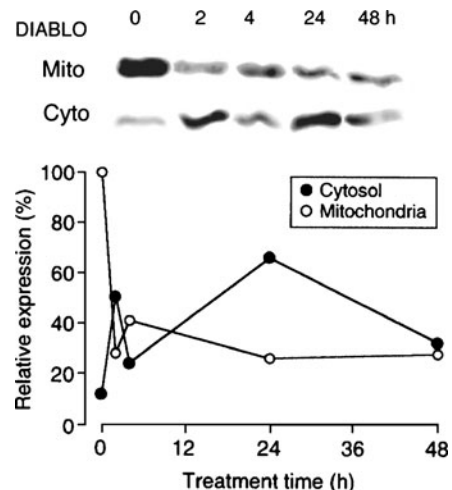

Fig. 5 DIABLO distribution. HuH-7 cells were fractionated into the mitochondrial (*Mito*) and cytosolic component (*Cyto*) after treatment with adenosine (10 mM) for times as indicated, followed by Western blotting for DIABLO protein. In the graph, each point represents the mean (±SEM) percentage of basal levels (signal intensities at 0 h; $n=4$)
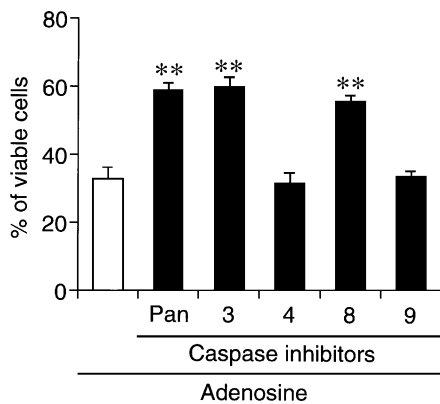
and -9 via A$_{2a}$ adenosine receptors linked to G$_s$ protein (Yasuda et al. 2009). Intracellularly transported adenosine induces apoptosis in GT3-TKB human lung cancer cells by activating AMP-activated protein kinase (AMPK; Saitoh et al. 2004).

Fig. 6 Effects of caspase inhibitors on adenosine-induced HuH-7 cell death. HuH-7 cells were treated with adenosine (10 mM) for 48 h in the presence and absence of caspase inhibitors such as Z-VAD-FMK, a pancaspase inhibitor (*Pan*), Z-DEVD-FMK, a caspase-3 inhibitor (*3*), Z-YVAD-FMK, a caspase-4 inhibitor (*4*), Z-IETD-FMK, a caspase-8 inhibitor (*8*), or Z-LEHD, a caspase-9 (*9*) at a concentration of 10 μM. Data represent the mean (±SEM) percentage of basal levels (MTT intensities of cells untreated with adenosine in the absence of caspase inhibitors; $n=4$). **$P<0.01$ compared with viability of cells treated with adenosine in the absence of caspase inhibitors, unpaired t test

The mitochondria express the Bcl-2 family of proteins such as Bcl-2, Bcl-X_L, Bax, and Bad that promotes or prevents apoptosis (Cory and Adams 2002; Kroemer 1997; Wang 2001). HuH-7 cell expressed mRNAs for Bax-α, Bax-β, Bax-γ, Bax-δ, Bax-σ, Bid, and Bcl-X_L, except for Bax-ε, Bcl-2α, or Bcl-2β. Adenosine downregulated the expression of the Bcl-X_L mRNA and protein in a treatment time (0–

Fig. 7 Effects of Bcl-X_L or IAP2 on the disruption of ▶ mitochondrial membrane potentials and activation of caspase-9 and -3 induced by extracellular adenosine. **a** HuH-7 cells were transfected with Mock and the DNAs for Bcl-X_L or IAP2 and 48 h later, Western blotting was carried out using an anti-Bcl-X_L antibody or an anti-IAP2 antibody, respectively. **b** HuH-7 cells were transfected with the Bcl-X_L DNA, and 48 h later, cells were treated with adenosine (10 mM) for 24 h followed by assay of mitochondrial membrane potentials. *Orange–red* fluorescent signals at an absorbance of 590 nm (*left column*) and *green* fluorescent signals at an absorbance of 530 nm (*right column*). Scale bars, 10 μm. Note that similar results were obtained with five independent experiments. **c** HuH-7 cells were transfected with Mock and the DNAs for Bcl-X_L or IAP2, and 48 h later, cells were untreated and treated with adenosine (*Ado*; 10 mM) for 24 h. Then, activities of caspase-9 and caspase-3 were enzymatically assayed. Each caspase activity was normalized by regarding the average of each independent basal caspase activities at 0 h as 1. In the graphs, each column represents the mean (±SEM) ratio ($n=4$). *P* values, unpaired *t* test

48 h)-dependent manner, and the effect was inhibited by dipyridamole. Amazingly, adenosine-induced disruption of mitochondrial membrane potentials was inhibited by overexpressing Bcl-X_L. Collectively, these results indicate that intracellularly transported adenosine downregulates Bcl-X_L expression, causing mitochondrial damage in HuH-7 cells.

For a mitochondrial apoptotic pathway, mitochondrial damage releases cytochrome *c* that forms an oligomeric complex with dATP or Apaf-1. The complex, in turn, activates caspase-9, followed by activation of the effector caspases, caspase-3, -6, and -7, responsible for DNA degradation, i.e., apoptotic cell death (Loeffler and Kroemer 2000; Li et al. 2001; Susin et al. 1999; Green and Reed 1998). Adenosine here, however, activated caspase-3 in a caspase-9-independent manner. Then, we speculated caspase-3
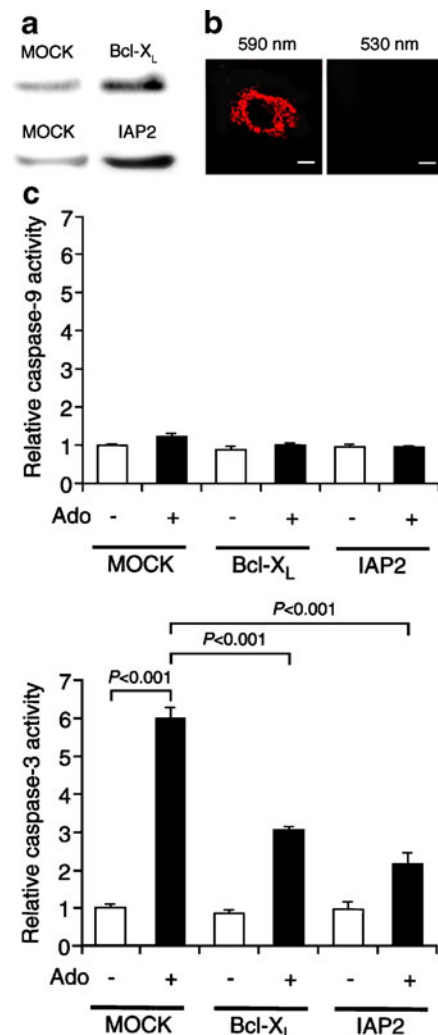
activation via a pathway relevant to DIABLO and IAPs. Caspase-3 is partially cleaved by caspase-8 or caspase-9, thereby producing the p20 fragment as a partial active form, followed by the p17 fragment as a fully active form through autoprocessing (Chen et al. 2003; Sun et al. 2002). DIABLOs exert a suppressing action on IAP activity (Verhagen et al. 2000, 2002; Du et al. 2000). IAPs, on the other hand, prevent the autoprocessing from the p20 fragment of caspase-3 to the p17 fragment by forming a complex with the p20 fragment; in other words, IAPs inhibit full caspase-3 activation (Salvesen and Duckett 2002; Gao et al. 2007). In the present study, intracellularly transported adenosine upregulated DIABLO expression but downregulated IAP2 expression in HuH-7 cells. These effects could result in reduced IAP activity. In addition, caspase-8-mediated partial processing of caspase-3 coupled with mitochondrial perturbations allowing Smac/DIABLO release and the subsequent neutralization of IAPs could lead to full caspase-3 activation to induce HuH-7 cell apoptosis. In support of this note, adenosine-induced caspase-3 activation was suppressed by overexpressing Bcl-X_L or IAP2.

In conclusion, intracellularly transported adenosine damaged the mitochondria by downregulating Bcl-X_L expression in HuH-7 cells, allowing Smac/DIABLO release from the mitochondria. Intracellularly transported adenosine, alternatively, increased DIABLO expression and decreased IAP2 expression. Those adenosine actions could neutralize caspase-3 inhibition due to IAPs, leading to caspase-3 activation. This accounts for the pathway underlying extracellular adenosine-induced HuH-7 cell apoptosis as well as adenosine-engaged caspase-8 activation and the ensuing caspase-3 activation. The results of the present study, thus, provide further insight into the pathway for extracellular adenosine-induced HuH-7 cell apoptosis.

References

Barry CP, Lind SE. Adenosine-mediated killing of cultured epithelial cancer cells. Cancer Res. 2000;60:1887–94.

Bar-Yehuda S, Stemmer SM, Madi L, Castel D, Ochaion A, Cohen S, et al. The A_3 adenosine receptor agonist CF102 induces apoptosis of hepatocellular carcinoma via deregulation of the Wnt and NF-κB signal transduction pathways. Int J Oncol. 2008;33:287–95.

Chen L, Smith L, Wang Z, Smith JB. Preservation of caspase-3 subunits from degradation contributes to apoptosis evoked by lactacystin: any single lysine or lysine pair of the small subunit is sufficient for ubiquitination. Mol Pharmacol. 2003;64:334–45.

Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer. 2002;2:647–56.

Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. 2000;102:33–42.

Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem. 1999;68:383–424.

Gao Z, Tian Y, Wang J, Yin Q, Wu H, Li YM, et al. A dimeric Smac/diablo peptide directly relieves caspase-3 inhibition by XIAP. Dynamic and cooperative regulation of XIAP by Smac/Diablo. J Biol Chem. 2007;282:30718–27.

Grbovic O, Jovic Y, Ruzdijic S, Pejanovic V, Rakic L, Kanazir S. 8-Cl-cAMP affects glioma cell-cycle kinetics and selectively induces apoptosis. Cancer Invest. 2002;20:972–82.

Green DR, Reed JC. Mitochondria and apoptosis. Science. 1998;281:1309–12.

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, et al. Inhibition of death receptor signals by cellular FLIP. Nature. 1997;388:190–5.

Kroemer G. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat Med. 1997;3:614–20.

Krueger A, Baumann S, Krammer PH, Kirchhoff S. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. Mol Cell Biol. 2001;21:8247–54.

Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature. 2001;412:95–9.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 1997;91:479–89.

Loeffler M, Kroemer G. The mitochondrion in cell death control: certainties and incognita. Exp Cell Res. 2000;256:19–26.

Peyot ML, Gadeau AP, Dandre F, Belloc I, Dupuch F, Desgranges C. Extracellular adenosine induces apoptosis of human arterial smooth muscle cells via A_{2b}-purinoceptor. Circ Res. 2000;86:76–85.

Sai K, Yang D, Yamamoto H, Fujikawa H, Yamamoto S, Nagata T, et al. A_1 adenosine receptor signal and AMPK involving caspase-9/-3 activation are responsible for adenosine-induced RCR-1 astrocytoma cell death. Neurotoxicology. 2006;27:458–67.

Saitoh M, Nagai K, Nakagawa K, Yamamura T, Yamamoto S, Nishizaki T. Adenosine induces apoptosis in the human gastric cancer cells via an intrinsic pathway relevant to activation of AMP-activated protein kinase. Biochem Pharmacol. 2004;67:2005–11.

Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol. 2002;3:401–10.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, et al. Two CD95 (APO-1/Fas) signaling pathways. EMBO J. 1998;17:1675–87.

Sun XM, Bratton SB, Butterworth M, MacFarlane M, Cohen GM. Bcl-2 and Bcl-xL inhibit CD95-mediated apoptosis by preventing mitochondrial release of Smac/DIABLO and subsequent inactivation of X-linked inhibitor-of-apoptosis protein. J Biol Chem. 2002;277:11345–51.

Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature. 1999;7:441–6.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell. 2000;102:43–53.

Verhagen AM, Silke J, Ekert PG, Pakusch M, Kaufmann H, Connolly LM, et al. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. J Biol Chem. 2002;277:445–54.

Wang X. The expanding role of mitochondria in apoptosis. Genes Dev. 2001;15:2922–33.

Wen LT, Knowles AF. Extracellular ATP and adenosine induce cell apoptosis of human hepatoma Li-7A cells via the A_3 adenosine receptor. Br J Pharmacol. 2003;140:1009–18.

Yang D, Yaguchi T, Yamamoto H, Nishizaki T. Intracellularly transported adenosine induces apoptosis in HuH-7 human hepatoma cells by downregulating c-FLIP expression causing caspase-3/-8 activation. Biochem Pharmacol. 2007;73:1665–75.

Yasuda Y, Saito M, Yamamura T, Yaguchi T, Nishizaki T. Extracellular adenosine induces apoptosis in Caco-2 human colonic cancer cells by activating caspase-9/-3 via A_{2a} adenosine receptors. J Gastroenterol. 2009;44:56–65.

Zou H, Li Y, Liu X, Wang X. An APAF-1. Cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem. 1999;274:11549–56.