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Intracellularly transported adenosine induces apoptosis in HuH-7 human hepatoma cells by downregulating c-FLIP expression causing caspase-3/-8 activation

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ABSTRACT

Extracellular adenosine induced apoptosis of HuH-7 cells, a Fas-deficient human hepatoma cell line. 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 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, but not caspase-9, in HuH-7 cells, and the activation was abolished by dipyridamole. In the real-time RT-PCR and Western blot analysis, extracellular adenosine downregulated mRNA and protein levels for c-FLIP, and the effect was suppressed by dipyridamole. Furthermore, overexpression of c-FLIP short in HuH-7 cells inhibited adenosine-induced caspase-8 activity. Taken together, these results suggest that intracellularly transported adenosine, perhaps converted AMP as the ensuing event, activates caspase-8 and the downstream effector caspase caspase-3 by neutralizing caspase-8 inhibition due to c-FLIP as a consequence of decreased c-FLIP expression, leading to apoptosis. This extends our understanding of adenosine-induced molecular apoptotic pathways.

1. Introduction

Apoptotic processes, to remove harmful or unnecessary cells, are indispensable for ongoing of cell development, differentiation, proliferation, and protection. Apoptotic cell death is initiated via a variety of extracellular and intracellular death signals [1,2]. For well-recognized extrinsic apoptotic pathways, Fas ligand (FasL) and tumor necrosis factor (TNF)-α induce apoptosis by activating the cell surface death receptors, Fas and TNF receptor 1 (TNFR1), respectively [3]. Activated Fas

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Abbreviations: FasL, Fas ligand; TNF-α, tumor necrosis factor-α; TNFR1, TNF receptor 1; FADD, Fas-associated death domain protein; TRADD, TNFR1-associated death domain protein; RIP1, receptor-interacting protein 1; FLIP, FADD-like interleukin-1β-converting enzyme inhibitory protein; c-FLIP, cellular FLIP; PKA, protein kinase A; AMPK, AMP-activated protein kinase; CHA, N^6-cyclohexyladenosine; DMPX, 3,7-dimethyl-1 propargylxanthine; CGS21680, 2-*p*-(2-carboxyethyl) phenetylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride; MRS1706, *N*-(4-acethylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]acetamide; NECA, 5′-(*N*-ethylcarboxamido)adenosine; MRS1523, 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate; AMDA, 5′-amino-5′-deoxyadenosine; PI, propidium iodide; DMEM, Dulbecco's modified Eagle's medium; 8-CPT, 8-cyclopentyltheophylline; EHNA, erythro-9 (2-hydroxy-3-nonyl)-adenosine; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylurinamide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; SDS, sodium dodecylsulfate; PBS, phosphate-buffered saline; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; c-FLIPS, c-FLIP short; ANOVA, analysis of variance

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recruits the adaptor protein Fas-associated death domain protein (FADD) that aggregates procaspase-8 [4] and activated TNFR1 forms a complex of TNFR1-associated death domain protein (TRADD)/receptor-interacting protein 1 (RIP1)/FADD/procaspase-8 [5], each of which cleaves procaspase-8 one other to initiate an active form of caspase-8, that activates the downstream effector caspase caspase-3, leading to apoptosis [3]. FADD-like interleukin-1β-converting enzyme inhibitory protein (FLIP) is known to specifically inhibit caspase-8 in the receptor-mediated apoptotic pathways [6,7]. c-Myc, alternatively, mediates apoptotic cell death by enhancing death receptor signals [8–10] or repressing transcription of cellular FLIP (c-FLIP) [11].

Adenosine, a metabolite of ATP, is abundantly present inside and outside cells, and exerts its diverse biological actions in a wide range of cell types. Accumulating studies have shown the implication of adenosine in apoptotic cell death as mediated via different signaling pathways. Adenosine receptors are classified as three major classes of receptors, denoted A_1, A_2 and A_3 adenosine receptors, and A_2 adenosine receptors are further divided into A_{2a} and A_{2b} receptors [12,13]. Extracellular adenosine induces apoptosis via A_2 adenosine receptors linked to G_s proteins involving adenylate cyclase activation/cAMP production/protein kinase A (PKA) activation, as found in a variety of cell types such as human arterial smooth muscle cells, glioma cells, myeloid leukemia cells, mammary carcinoma cells, embryonic epithelial cells, granulosa cells, thymocytes, B lymphocytes, and neutrophils [14–23]. A_3 adenosine receptors participate in the apoptotic process of breast cancer cells, leukemia cells, Li-7A hepatoma cells, and glomerular mesangial cells [24–26]. Moreover, adenosine may induce apoptosis in RCR-1 rat astrocytoma cells in part via A_1 adenosine receptors [27]. Interestingly, the adenosine derivative Cl-IB-MECA induces apoptosis in HL-60 and MOLT-4 human leukemia cells via a Fas-mediated pathway [28]. Distinct from adenosine receptor-mediated pathways, extracellular adenosine induces apoptosis by uptake into cells through adenosine transporters and the ensuing conversion to AMP by adenosine kinase, as found in neuroblastoma cells and human epithelial cancer cells originated from the breast, the colon, and the ovary [29,30]. Recent evidence shows that AMP-activated protein kinase (AMPK) may function downstream of AMP in the apoptotic pathway [31].

The present study focused upon adenosine-induced apoptosis of HuH-7 cells, a human Fas-deficient hepatoma cell line, and probed the underlying intracellular signaling pathways. The results of the present study suggest that extracellular adenosine induces apoptosis of HuH-7 cells via an intracellularly transported adenosine/AMP signal, possibly involving activation of caspase-8 by neutralizing caspase-8 inhibition due to c-FLIP as a result of decreased expression of c-FLIP, followed by activation of the downstream effector caspase caspase-3.

2. Materials and methods

2.1. Materials

N^6-Cyclohexyladenosine (CHA), 3,7-dimethyl-1 propargylxanthine (DMPX), 2-*p*-(2-carboxyethyl) phenetylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS21680), *N*-(4-acethylphenyl)-2-[4-(2,3,67-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]acetamide (MRS1706), 5′-(*N*-ethylcarboxamido)adenosine (NECA), 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate (MRS1523), 5′-amino-5′-deoxyadenosine (AMDA), propidium iodide (PI), an annexin V-FITC apoptosis detection kit, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma (St. Louis, USA). 8-Cyclopentyltheophylline (8-CPT) was from Biomol Research Laboratories (Philadelphia, USA). Erythro-9 (2-hydroxy-3-nonyl)-adenosine (EHNA) was from Calbiochem (San Diego, USA). 2-Chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylurinamide (Cl-IB-MECA) was from Tocris (Ellisville, USA). Dipyridamole was from MP Biomedicals (Aurora, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was from Dojindo (Kumamoto, Japan). Dimethylformamide were from Wako (Osaka, Japan). Adenosine, sodium dodecylsulfate (SDS), and a Sepasol-RNA I Super kit were from Nacalai

Table 1 – Primers used for RT-PCR

Gene name	Sense primer	Anti-sense primer	Base pair	Number
AdensineA_1 receptor	GGTGCTCATCGCCCTGGTCTC	CGCTCCACCGCACTCAGATTG	413	NM_0064
AdenosineA_{2a} receptor	GCTCGCCATCCCCTTTGCC	GCAGTCGGGGCAGAAGAAAGTGAA	615	BC013780
AdenosineA_{2b} receptor	GCCATCCCCTTTGCCATCACC	GGTAACCAGCACAGGGCAAAAATCC	568	NM_000676
AdenosineA_3 receptor	CAAAGGCTGGGTATCGGCTGTG	CCAACTTGCTCATTCCTACCCTTTTCTG	631	NM_000677
CNT1	CATCCCCGCCTGCTGCTCTG	CCCTGACTTAGTCTTTGGGTATCTTTGGTC	525	BC039898
CNT2	TTTGTCCTGGTTCACTCGTTTTTGAAA	GGTAGGGACGGATGAGCAGAGGTG	627	NM_004212
CNT3	GGGGCATTTTATTAGCAGGTTATCTGGTTA	GCATGATCCATCCAACCTTTCTAATAATCC	641	NM_022127
ENT1	GCCCACCAATGAAAGCCACTCTATC	TGCCAGACCCAGACACAGGAAGAA	507	NM_004955
ENT2	CTGGTCTGCCTGCGGTTCCTG	TTTCTTCCCCGCAGCACTCCA	462	NM_001532
ENT3	CCCCCTCTTCGTGCTCTGTAACTACC	GGAGTACCCCTGATTTGTGCTATTTCTTC	513	NM_018344
ENT4	GCTTCCCCCTTCTGTCCTCATTCTTAGAG	GCAGGGGTGAAACAGGGCAGTC	542	NM_153247
Fas	ATTATCGTCCAAAAGTGTTAATGCCCAA	TGCACTTGGTGTTGCTGGTGAGTG	313	X89101
FasL	CACTACCGCTGCCACCCCTGA	CATCATCTTCCCCTCCATCATCACC	496	BC017502
FADD	GCCTGGGGAAGAAGACCTGTGTG	CTGGCTTCCTGCTGGGTCTTCAC	459	BC000334
c-FLIPL	CAGAGATTGGTGAGGATTTGGATAAATCTG	CCTAGGGGCTTGCTCTTCATCTTGTA	462	U97074
c-FLIPS	CAGAGATTGGTGAGGATTTGGATAAATCTG	GGAACAATTTCCAAGAATTTTCAGATCAGG	367	U97075
GAPDH	GAACATCATCCCTGCCTCTACTGGC	GTGCTCTTGCTGGGGCTGGTG	428	BC023632

Tesque (Kyoto, Japan). A DePsipher™ kit was from Trevigen (Gaithersburg, USA). A caspase fluorometric assay kit and a cell lysis buffer were from BioVision Research Products (Mountain View, USA). SuperScript III reverse transcriptase was from Invitrogen (Carlsbad, USA). Taq polymerase was from Fermentas (Burlington, USA). A SYBR Green Realtime PCR Master Mix was from TOYOBO (Osaka, Japan). A High-Capacity cDNA Archive Kit was from Applied Biosystems (Austin, USA).

2.2. Cell culture

HuH-7 cells were obtained from RIKEN cell bank (Ibaraki, Japan). Cells were cultured in a culture 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.

2.3. MTT assay

Cell viability was evaluated by a dye staining method using MTT. After several sets of treatments in the culture medium, HuH-7 cells were rinsed twice with phosphate-buffered saline (PBS) and incubated in 100 μl of MTT solution (250 μg/ml diluted with the culture medium deleting serum) at 37 °C for 3 h. After adding 20% (w/v) SDS and 50% (v/v) dimethylformamide, the mixtures stood at room temperature for 24 h, and MTT-reactive cells were quantified at an absorbance of 570 nm using a micro-plate reader (SPECTRAmax PLUS384, Molecular Devices, Sunnyvale, USA). MTT is taken only into viable cells, and therefore, the MTT intensity corresponds to the number of viable cells. To assess cell viability, percentage of independent basal levels (MTT intensities of cells untreated with any drug) was calculated.

2.4. Cell cycle analysis

After non-treatment and treatment with adenosine (10 mM) for 48 h, HuH-7 cells were harvested by a trypsinization, centrifuged at 1500 × *g* for 5 min, washed with PBS, and fixed in 70% ethanol at 4 °C overnight. Fixed cells were washed twice with PBS and incubated in PBS containing 1.5 μg/ml RNase A for 1 h at 37 °C, followed by staining with 5 μl PI for 20 min on ice. Then, cells were collected on a nylon mesh filter (pore size, 40 μm), and cell cycles including the sub-G_0 phase (apoptosis) were assayed in 2×10^4 cells with a flowcytometer (FACSCalibur, Becton Dickinson, USA) at an excitation of 488 nm and an emission of 585 nm, and analyzed using a Mod Fit LT software (Verity Software House Inc., Topshan, USA).

2.5. Apoptosis assay

After non-treatment and treatment with adenosine (10 mM) for 12–48 h, HuH-7 cells were harvested by adding trypsin and then washed twice with PBS. Cells were resuspended in a binding buffer and stained with both PI and annexin V-FITC, and loaded on a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, USA) available for FL1 (annexin V) and FL2 (PI) bivariate analysis. Data from 20,000 cells/sample were collected, and the quadrants were set according to the population of viable, unstained cells in untreated samples. CellQuest analysis of the data was used to calculate the percentage of the cells in the respective quadrants.

2.6. DNA laddering

HuH-7 cells untreated or treated with adenosine (10 mM) for 12, 36, and 48 h were harvested and centrifuged at 1500 × *g* for 5 min. After washing twice with PBS/ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), cells were incubated in a lysis buffer [0.5% (v/v) Triton X-100, 10 mM EDTA, 0.4 μg/μl proteinase K, and 10 mM Tris–HCl, pH 7.4] at 56 °C for 1 h. Cell lysate was treated with 0.4 μg/μl DNase-free RNase A at 37 °C for 30 min. The genomic DNAs were purified by phenol/chloroform

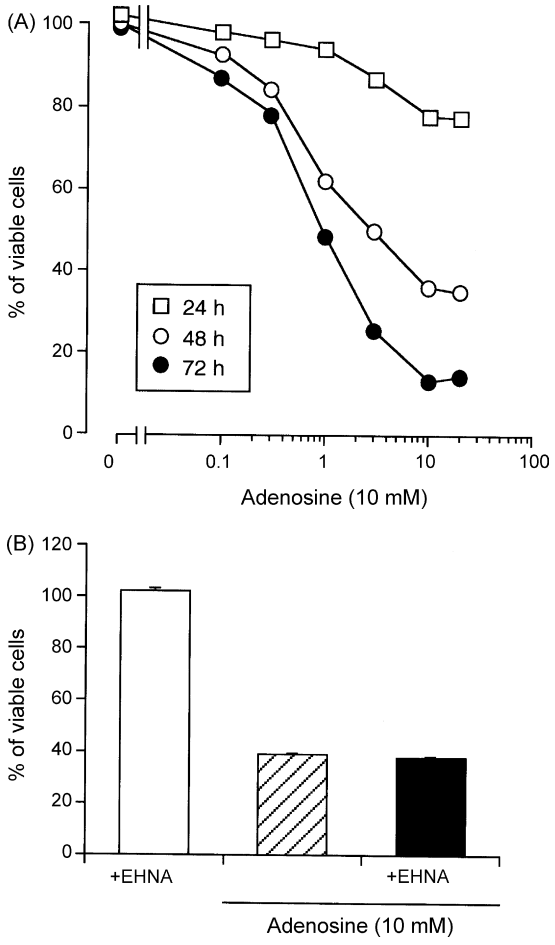

Fig. 1 – Effect of extracellular adenosine on HuH-7 cell viability. (A) HuH-7 cells were treated with adenosine at concentrations as indicated for 24–72 h, and the cell viability was quantified with an MTT assay. Data represent the mean (±S.E.M.) percentage of basal levels (MTT intensities of cells untreated with adenosine) (*n* = 4). (B) MTT assay was carried out in cells treated with adenosine (10 mM) for 48 h in the presence of EHNA (10 μM). Data represent the mean (±S.E.M.) percentage of basal levels (MTT intensities of cells untreated with adenosine) (*n* = 4). Note that EHNA by itself did not affect HuH-7 cell viability.

extraction and ethanol precipitation, and resuspended in a Tris–EDTA buffer. DNA fragments were stained with ethidium bromide and visualized by 2.5% agarose electrophoresis.

2.7. Enzymatic assay of caspase-activity

Caspase activation was measured using a caspase fluorometric assay kit (Ac-Asp-Glu-Val-Asp-MCA for a caspase-3 substrate peptide; Ac-Ile-Glu-Thr-Asp-MCA for a caspase-8 substrate peptide; and Ac-Leu-Glu-His-Asp-MCA for a caspase-9 substrate peptide) [32]. After treatment with adenosine (10 mM) in the presence and absence of dipyridamole (10 μM) for 0–48 h, 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).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs of HuH-7 cells were purified by an acid/guanidine/thiocyanate/chloroform extraction method using a Sepasol-RNA I Super kit. After purification, total RNAs were treated

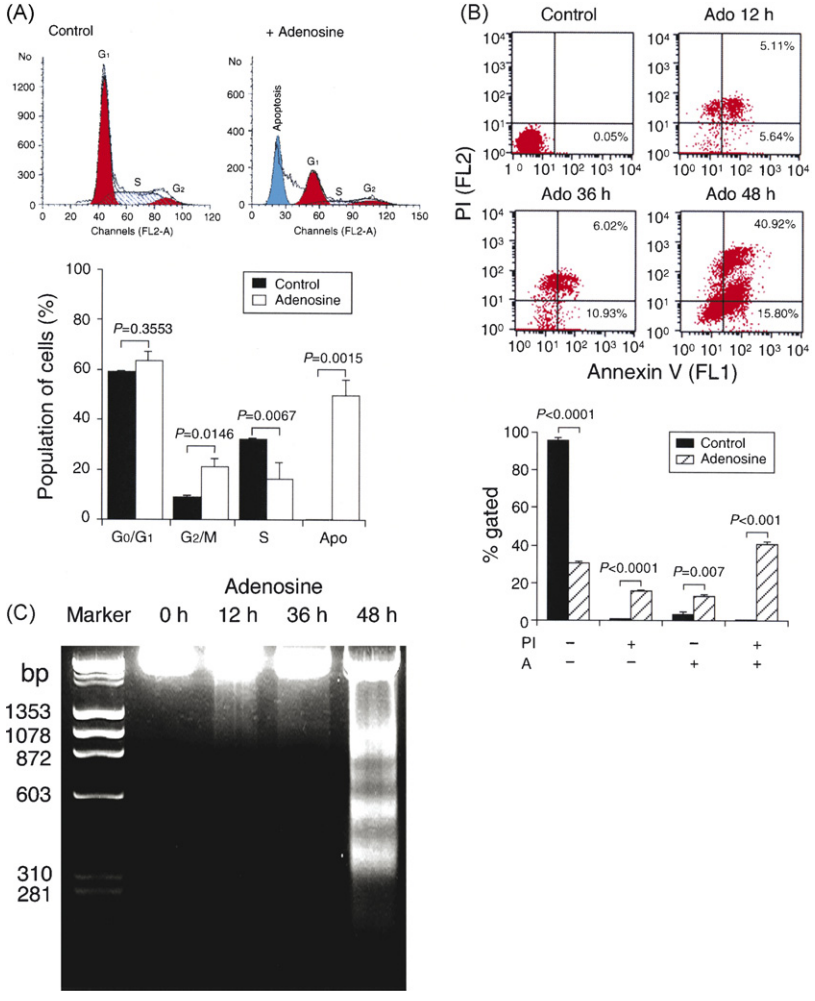

Fig. 2 – Extracellular adenosine-induced apoptosis of HuH-7 cells. (A) Cell cycle analysis. HuH-7 cells were untreated (control) and treated with adenosine (10 mM) (+adenosine) for 48 h, and proportion of cells in each phase of cell cycle was assayed. In the graphs, each column represents the mean (±S.E.M.) percentage (n = 4). P values shown were obtained from unpaired t-test. (B) Flow cytometry using PI and annexin V-FITC. After non-treatment (control) and treatment with adenosine (10 mM) for 12 h (Ado 12 h), 36 h (Ado 36 h), and 48 h (Ado 48 h), the number of cells in four fractions (PI-negative/annexin V-negative, PI-positive/annexin V-negative, PI-negative/annexin V-positive, and PI-positive/annexin V-positive) was counted and its distribution was analyzed. Typical profiles are shown in the upper panel. Graph shows the results from 48 treatment with adenosine, and data represent the mean (±S.E.M.) percentage (n = 4). P values shown were obtained from unpaired t-test. (C) Electrophoretic analysis of internucleosomal DNA fragmentation. DNA fragments from HuH-7 cells before (0 h) and after treatment with adenosine (10 mM) for 12, 36, and 48 h were stained with ethidium bromide. Note that similar results were obtained in four independent experiments.

with RNase free-DNase I (2 units) at 37 °C for 30 min to remove genomic DNAs, and 10 μg of RNAs were resuspended in water. Then, random primers, dNTP, 10× RT buffer, and Multiscribe Reverse Transcriptase (Applied Biosystems, 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× PCR reaction buffer, dNTPs, $MgCl_2$, oligonucleotide, dimethylsulfoxide (final concentration, 5%, v/v) and 1 unit of Taq polymerase (Fermentas, St. Leon-Roth, Germany) (final volume, 20 μl). RT-PCR was carried out with a GeneAmp PCR system model 9600 DNA thermal cycler (Applied Biosystems, Indianapolis, USA) programmed as follows: the first one step, 94 °C for 4 min and the ensuing 30 cycles, 94 °C for 1 s, 67 °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.

2.9. *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 Real-time PCR Master Mix (final volume, 20 μl) and loaded on the Applied Biosystems 7900 Real-time PCR Detection System (ABI, Foster City, 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

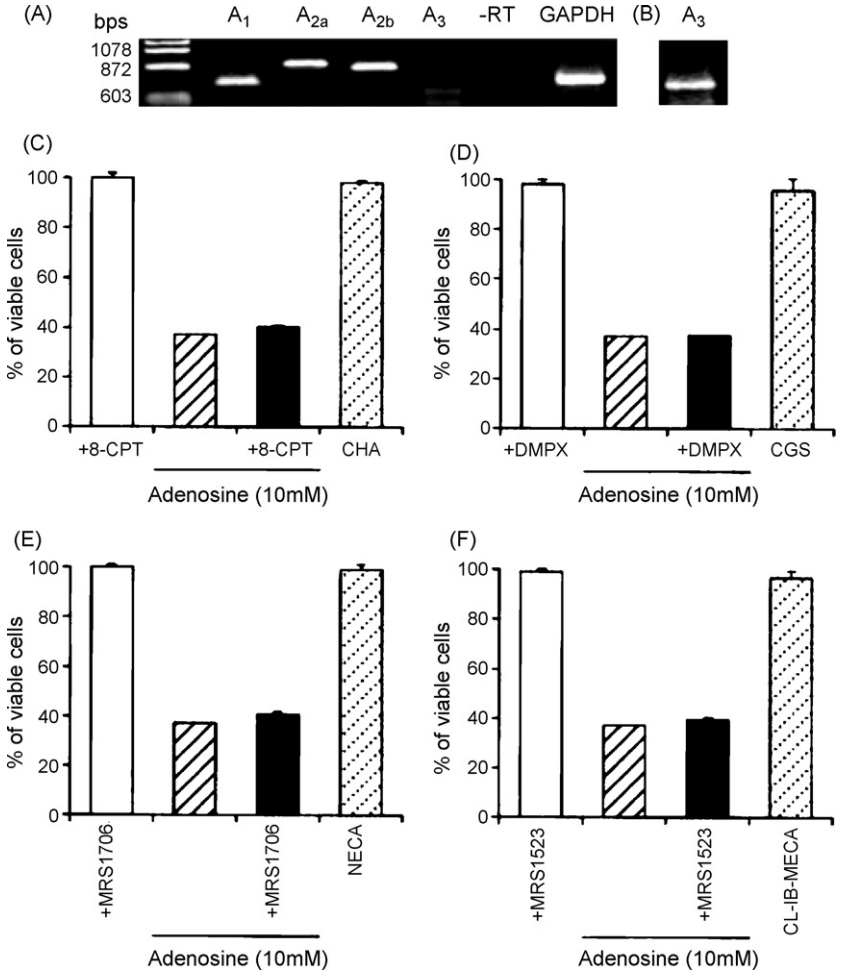

Fig. 3 – Extracellular adenosine-induced HuH-7 cell death, independently of adenosine receptors. (A) RT-PCR analysis of adenosine receptors expressed in HuH-7 cells. Lane 1, size markers; PCR products for A_1 adenosine receptors (lane 2), A_{2a} adenosine receptors (lane 3), A_{2b} adenosine receptors (lane 4), and A_3 adenosine receptors (lane 5) from total RNAs of HuH-7 cells with reverse transcription, PCR product from total RNAs of HuH-7 cells without reverse transcription (−RT) (lane 6) (30 cycles), and PCR product for GAPDH from total RNAs of HuH-7 cells with reverse transcription as a positive control (25 cycles). (B) PCR product for A_3 adenosine receptors from total RNAs of HepG2 cells with reverse transcription (30 cycles). HuH-7 cells were treated with adenosine (10 mM) in the presence and absence of 8-CPT (10 μM) (C), DMPX (100 μM) (D), MRS1706 (2.5 μM) (E), or MRS1523 (10 μM) (F) for 48 h. In another sets of experiments, cells were treated with CHA (10 μM), CGS21680 (CGS) (100 μM), NECA (100 μM), or Cl-IB-MECA (100 μM) for 48 h. Data show the mean (±S.E.M.) percentage of basal levels (MTT intensities of cells untreated with adenosine or each adenosine receptor agonist in the absence of each adenosine receptor inhibitor) ($n = 4$). Note that each adenosine receptor inhibitor had no effect on cell viability.

made by amplifying 0.5, 1, 2, 4, and 8 μl of the GAPDH cDNA diluted at 1:250. mRNA quantity for each target gene was calculated from the standard curve using an SDS 2.1 Software (Applied Biosystems, Foster City, USA).

2.10. Western blotting

After treatment with adenosine (10 mM) in the presence and absence of dipyridamole (10 μM) for 4–48 h, HuH-7 cells were lysed on ice in a lysis buffer [50 mM Tris–HCl, 150 mM NaCl, 0.1% (w/v) sodium deoxycholate, 0.1% (v/v) Triton X-100, and protease inhibitor cocktail, pH 7.4] and sonicated for 15 s. Cell lysates were centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant (20 μg protein/well), boiled for 5 min, was subjected to 12% (w/v) sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes stood overnight in Tris-buffered saline containing 0.1% (v/v) Tween 20 supplemented with 0.1% bovine serum albumin and reacted with an anti-FLIP antibody (eBioscience, San Diego, USA) overnight followed by the secondary antibody horseradish peroxidase-conjugated anti-rabbit IgG. Proteins were detected with an ECL Plus system (GE Healthcare, Buckinghamshire, UK). Signal density was measured with an Image Gauge software (FUJIFILM, Tokyo, Japan).

2.11. Overexpression of c-FLIP short (c-FLIPS)

The plasmid c-FLIPS DNA (6 μg) was transfected in HuH-7 cells using an electroporation system (Optimizor500, BTX, San Diego, USA). Cells were used for experiments 48 h after transfection.

2.12. Statistical analysis

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

3. Results

3.1. Extracellular adenosine induces apoptosis in HuH-7 cells

We initially examined the effect of extracellular adenosine on HuH-7 cell death. In the MTT assay, adenosine reduced HuH-7 cell viability in a concentration (0.1–20 mM)- and treatment time (24–72 h)-dependent manner ($P < 0.01$ for 24-h treatment and $P < 0.0001$ for 48 and 72-h treatment, as compared with viability of cells untreated with adenosine, ANOVA) (Fig. 1A). For the following experiments, cells were treated with 10 mM of adenosine, the concentration exhibiting the maximal effect at 48-h treatment. Adenosine-induced reduction of HuH-7 cell viability was not affected by EHNA (10 μM), an inhibitor of adenosine deaminase (Fig. 1B), indicating that the effect is caused by adenosine itself but not adenosine metabolites.

In the cell cycle analysis, 48-h treatment with adenosine (10 mM) markedly increased proportion of apoptotic cells accompanied by an increase in the proportion of cells in the G_2/M phase of cell cycle and a decrease in that in the S phase without affecting that in the G_0/G_1 phase (Fig. 2A).

Dead cells are stained with PI, and apoptotic cells are reactive to annexin V-FITC detecting externalized phosphatidylserine residues [33]. In the flow cytometry using PI and

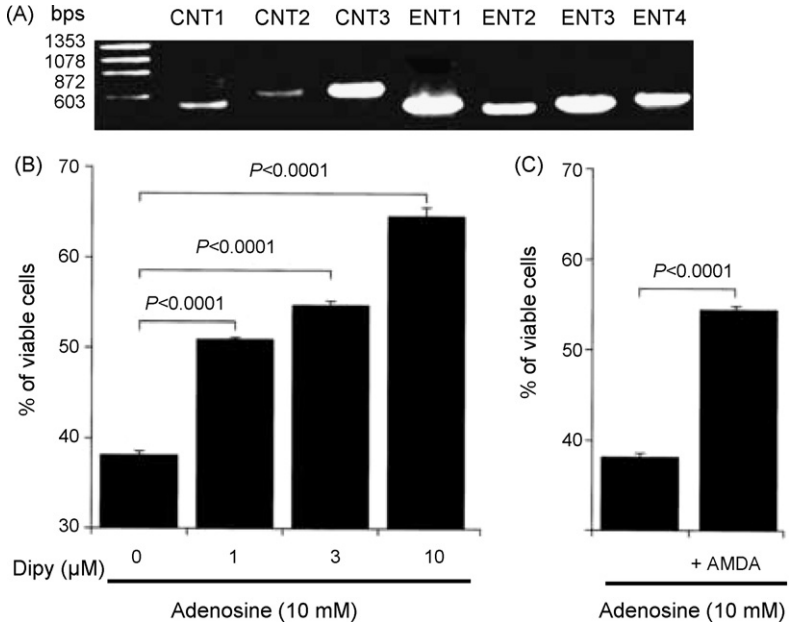

Fig. 4 – Extracellular adenosine-induced HuH-7 cell death by adenosine uptake and the ensuing conversion to AMP. (A) RT-PCR analysis of adenosine transporters expressed in HuH-7 cells. Lane 1, size markers; PCR products for CNT1 (lane 2), CNT2 (lane 3), CNT3 (lane 4), ENT1 (lane 5), ENT2 (lane 6), ENT3 (lane 7), and ENT4 (lane 8) from total RNAs of HuH-7 cells with reverse transcription (30 cycles). HuH-7 cells were treated with adenosine (10 mM) in the presence and absence of dipyridamole (Dipy) (1, 3, and 10 μM) (B) or AMDA (100 μM) (C) for 48 h. In the graphs, data show the mean (±S.E.M.) percentage of basal levels (MTT intensities of cells untreated with adenosine in the absence of each inhibitor) ($n = 4$). *P* values shown were obtained from unpaired *t*-test.

annexin V-FITC, treatment with adenosine (10 mM) significantly increased the population of PI-negative and annexin V-positive cells and that of PI-positive and annexin V-positive cells in a treatment time (24–48 h)-dependent manner (Fig. 2B), each population corresponding to early apoptosis and late apoptosis/secondary necrosis [34]. Adenosine also increased the population of PI-positive and annexin V-negative cells, that reflects necrotic cell death.

In the internucleosomal DNA fragmentation analysis, a DNA ladder formation was found with cells treated with adenosine (10 mM) for 48 h (Fig. 2C). Taken together, these results indicate that adenosine reduces HuH-7 cell viability due to apoptotic cell death, in part to necrotic cell death.

3.2. *Extracellular adenosine induces apoptosis in HuH-7 cells via an intrinsic pathway*

Our next attempt was to see what pathway underlies adenosine-induced HuH-7 cell apoptosis. HuH-7 cells expressed mRNAs for A_1, A_{2a}, A_{2b} and A_3 adenosine receptors, although signal for the A_3 adenosine receptor mRNA was very shallow (Fig. 3A). Adenosine (10 mM)-induced cell death was not inhibited by 8-CPT (10 μM), an inhibitor of A_1 adenosine receptors, DMPX (100 μM), an inhibitor of A_{2a} adenosine receptors, MRS1706 (2.5 μM), an inhibitor of A_{2b} adenosine receptors, or MRS1523 (10 μM), an inhibitor of A_3 adenosine receptors (Fig. 3C–F). In addition, the A_1 adenosine receptor agonist, CHA (10 μM), the A_{2a} adenosine receptor agonist, CGS21680 (100 μM), the non-selective A_2 adenosine receptor agonist, NECA (100 μM), or the A_3 adenosine receptor agonist, Cl-IB-MECA (100 μM) had no effect on HuH-7 cell viability (Fig. 3C–F). These results indicate no participation of adenosine receptors in the HuH-7 cell apoptosis.

Extracellular adenosine is shown to induce apoptosis in some types of cells by being taken up into cells through adenosine transporters [29–31]. HuH-7 cells expressed mRNAs for all the adenosine transporters such as CNT1, CNT2, CNT3, ENT1, ENT2, ENT3, and ENT4 (Fig. 4A). Adenosine-induced HuH-7 cell death was significantly inhibited by dipyridamole, a broad inhibitor of adenosine transporters, in a concentration (1–10 μM)-dependent manner (Fig. 4B). In addition, the adenosine effect was also significantly inhibited by AMDA (100 μM), an inhibitor of adenosine kinase (Fig. 4C). Extracellular adenosine, accordingly, is likely to induce apoptosis of HuH-7 cells by being taken up into cells through adenosine transporters and converted to AMP by adenosine kinase.

3.3. *Intracellularly transported adenosine activates caspase-3 and -8*

We subsequently examined whether adenosine activates caspases in HuH-7 cells. Adenosine (10 mM) activated caspase-3 in a treatment time (4–48 h)-dependent manner ($P < 0.0001$ as compared with caspase-3 activity before treatment with adenosine, ANOVA), that was significantly inhibited by dipyridamole (10 μM) ($P < 0.0001$ between two groups; adenosine alone and adenosine plus dipyridamole, ANOVA) (Fig. 5A). Adenosine (10 mM) also activated caspase-8 in a bell-shaped treatment time (4–48 h)-dependent manner ($P < 0.0001$ as compared with caspase-8 activity before treatment with adenosine, ANOVA), with the maximum at 8-h treatment, and the activation was significantly inhibited by dipyridamole (10 μM) ($P < 0.0001$ between two groups; adenosine alone and adenosine plus dipyridamole, ANOVA) (Fig. 5B). In contrast, no significant activation of caspase-9 was obtained with adenosine (10 mM) (Fig. 5C). It is indicated from these results that intracellularly transported adenosine activates caspase-3 and

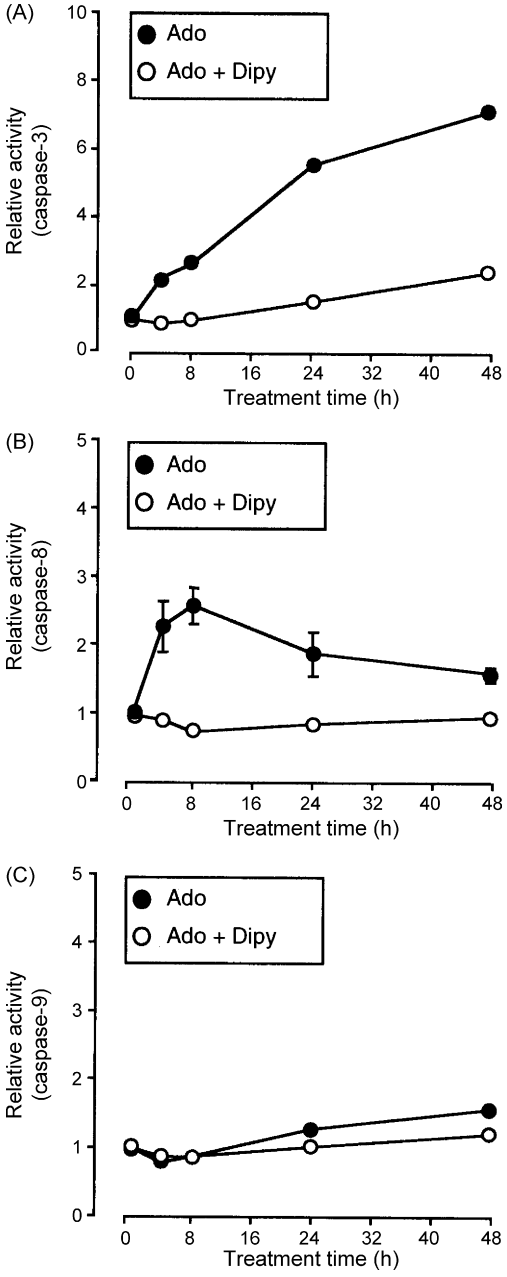

Fig. 5 – Extracellular adenosine-induced caspase activation in HuH-7 cells. Cells were treated with adenosine (Ado) (10 mM) in the presence and absence of dipyridamole (Dipy) (10 μM) for periods of time as indicated, and then activities of caspase-3 (A), caspase-8 (B), and caspase-9 (C) were assayed. The activities were normalized by regarding the average of independent basal caspase activities at 0 h as 1. In the graph, each point represents the mean (±S.E.M.) ratio ($n = 4$).

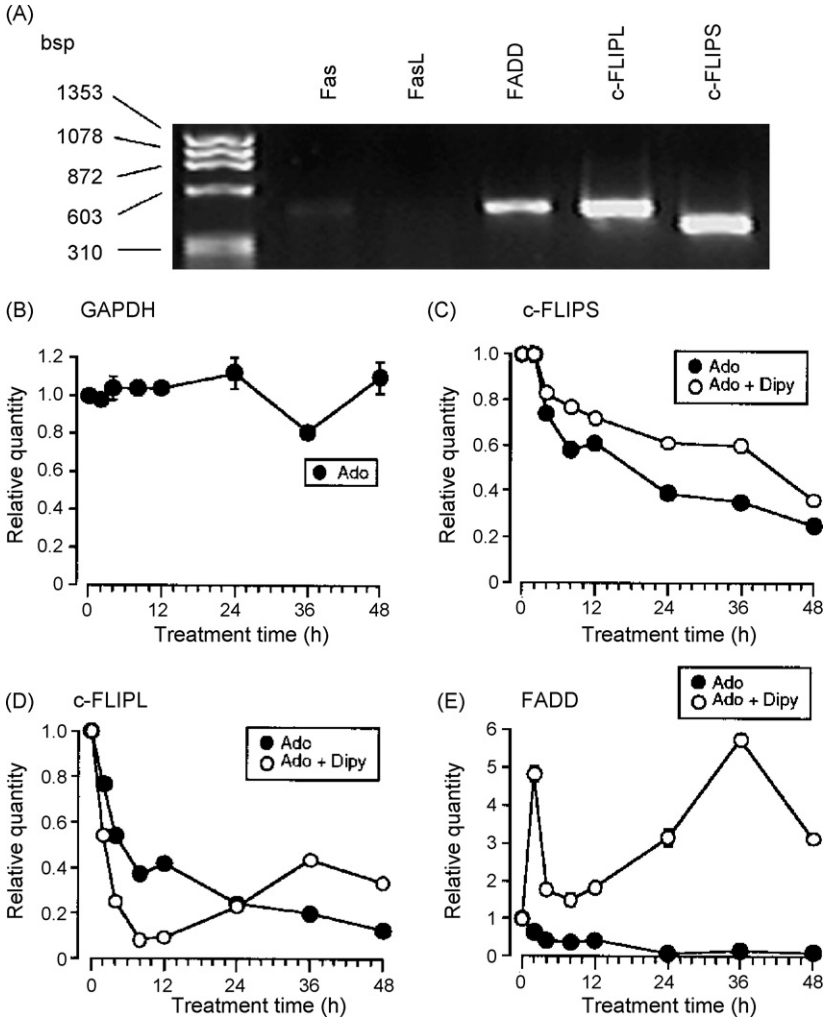

Fig. 6 – RT-PCR analysis of death regulatory molecule genes in HuH-7 cells. (A) PCR products for death regulatory molecules from total RNAs of HuH-7 cells with reverse transcription (30 cycles). We carried out real-time RT-PCR analysis of GAPDH (B), c-FLIPS (C), c-FLIPL (D), and FADD (E). Cells were treated with adenosine (10 mM) in the presence and absence of dipyridamole (Dipy) (10 μM) for 0–48 h, and then real-time RT-PCR for each gene as indicated was carried out. mRNA quantity for each gene was calculated from the standard curve made by amplifying different amount of the GAPDH first strand cDNA, and normalized by regarding the average of independent basal mRNA quantity at 0 h as 1. In the graphs, each point represents the mean (±S.E.M.) ratio (n = 4).

-8, but not caspase-9, leading to apoptotic cell death in HuH-7 cells.

3.4. *Intracellularly transported adenosine downregulates c-FLIP expression in HuH-7 cells, resulting in the activation of caspase-3/-8*

Our final question was addressed to how intracellularly transported adenosine activates caspase-3 and -8. HuH-7 cells are known to be Fas deficient [35]. RT-PCR indeed revealed no signal for the Fas mRNA and the FasL mRNA (Fig. 6A), ruling out a Fas-mediated caspase-8 activation pathway [3]. HuH-7 cells expressed mRNAs for c-FLIPS and c-FLIP long (c-FLIPL), to inhibit caspase-8 activation (Fig. 6A). In the real time RT-PCR analysis, a significant decrease in the c-FLIPS mRNA was obtained with adenosine (10 mM) ($P < 0.0001$ as compared with the mRNA quantity before treatment with adenosine, ANOVA), and the decrease was suppressed by dipyridamole (10 μM) ($P = 0.0522$ between two groups; adenosine alone and adenosine plus dipyridamole, ANOVA) (Fig. 6C). Adenosine (10 mM) did not affect expression of the GAPDH mRNA (Fig. 6B), indicating that a change in the expression of the mRNA induced by adenosine is not due to the non-specific effect of adenosine. Adenosine, in the light of the fact that c-FLIP is a specific inhibitor of caspase-8 activation [6,7], could activate caspase-8 by neutralizing inhibition of caspase-8 activation due to c-FLIPS as a result from downregulating expression of the c-FLIPS mRNA via an intracellularly transported adenosine/converted AMP signal. Adenosine (10 mM) decreased the c-FLIPL mRNA too ($P < 0.0001$ as compared with the mRNA quantity before treatment with adenosine, ANOVA), yet dipyridamole (10 μM) did not significantly inhibit the effect, instead further decreased expression of the c-FLIPL mRNA within 24-h treatment (Fig. 6D). This

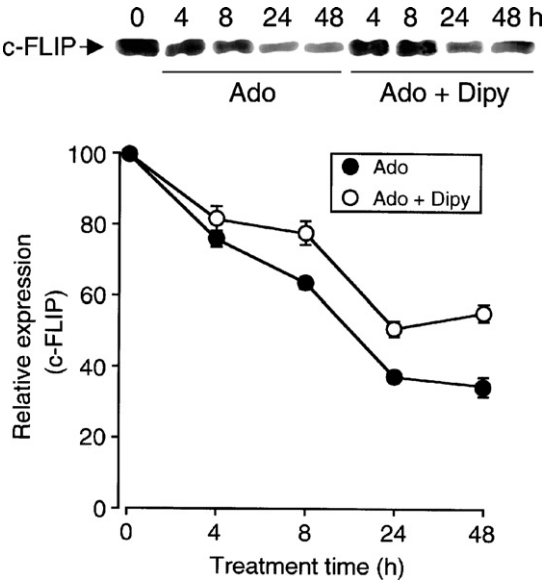

Fig. 7 – Effect of extracellular adenosine on c-FLIP protein expression level in HuH-7 cells. HuH-7 cells were treated with adenosine (Ado) (10 mM) in the presence and absence of dipyridamole (Dipy) (10 μM) for 0–48 h, and protein levels of c-FLIP expression were analyzed by Western blotting using an anti-FLIP antibody. Typical blotting was shown in the upper column. In the graph, each point represents the mean (±S.E.M.) percentage of signal density at 0 h ($n = 3$).

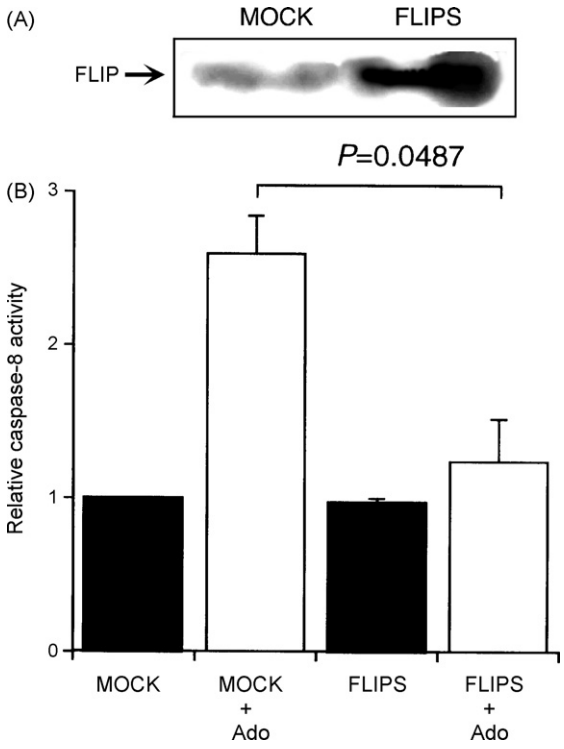

Fig. 8 – Effect of c-FLIP overexpression on adenosine-induced caspase-8 activity in HuH-7 cells. (A) Cells were transfected with MOCK and c-FLIPS, and Western blot using an anti-FLIP antibody was carried out. (B) Cells with 48-h incubation after transfection were treated with adenosine (Ado) (10 mM) for 8 h, and then caspase-8 activity was assayed. The activity was normalized by regarding the average of independent basal caspase activity at 0 h as 1. In the graph, each point represents the mean (±S.E.M.) ratio ($n = 3$). P values shown were obtained from unpaired t-test.

may explain that another unknown pathway underlies downregulation of expression of the c-FLIPL mRNA induced by adenosine. Adenosine (10 mM) also reduced expression of the FADD mRNA ($P < 0.0001$ as compared with the mRNA quantity before treatment with adenosine, ANOVA), and the effect was abolished by dipyridamole (10 μM); conversely, dipyridamole upregulated expression of the FADD mRNA (Fig. 6E). This indicates that intracellularly transported adenosine regulates mRNA expression of not only c-FLIPS but FADD.

In the Western blot analysis, adenosine (10 mM) decreased expression of c-FLIP protein in a treatment time (4–48 h)-dependent manner, and this effect was significantly suppressed by dipyridamole (10 μM) ($P < 0.0001$ as compared with the protein quantity before treatment with adenosine, ANOVA) (Fig. 7). This provides evidence that intracellular transported adenosine downregulates c-FLIP protein following the c-FLIPS mRNA.

We thought that overexpression of c-FLIPS should reduce adenosine-induced caspase-8 activation. To address this point, the plasmid c-FLIPS DNA was transfected in HuH-7 cells and caspase-8 activity was assayed in cells overexpressing c-FLIPS (Fig. 8A). Caspase-8 activation induced by 8-h treatment with adenosine (10 mM) was significantly inhibited as compared with the activation for control cells transfected with MOCK (Fig. 8B). Overall, these results lead to a conclusion that intracellularly transported adenosine activates caspase-8 by releasing caspase-8 inhibition due to c-FLIP through decreased expression of c-FLIP, followed by activation of the effector caspase caspase-3 to induce apoptosis.

4. Discussion

The results of the present study clearly demonstrate that extracellular adenosine induces HuH-7 cell death, mainly apoptosis. The adenosine effect was not influenced by EHNA, an inhibitor of adenosine deaminase, indicating that adenosine by itself not adenosine metabolites induces HuH-7 cell death. Adenosine-induced HuH-7 cell death was not inhibited by the A_1 adenosine receptor inhibitor 8-CPT, the A_{2a} adenosine receptor inhibitor DMPX, the A_{2b} adenosine receptor inhibitor MRS1706, or the A_3 adenosine receptor inhibitor MRS1523, suggesting no implication of adenosine receptors in the cell death. In further support of this, CHA, an A_1 adenosine receptor agonist, CGS21680, an A_{2a} adenosine receptor agonist, NECA, a non-selective A_2 adenosine receptor agonist, or Cl-IB-MECA, an A_3 adenosine receptor agonist, did not induce HuH-7 cell death. The adenosine effect, alternatively, was significantly prevented by the adenosine trans-

porter inhibitor dipyridamole and the adenosine kinase inhibitor AMDA. This implies that extracellular adenosine induces HuH-7 cell apoptosis by being taken up into cells through adenosine transporters and subsequently converted to AMP by adenosine kinase. Extracellular adenosine activated caspase-3 and -8, but not caspase-9, in HuH-7 cells and the activation was abolished by dipyridamole. This implies that intracellularly transported adenosine/converted AMP may produce signals to activate caspase-3 and -8, causing apoptotic cell death. Then, the central question to address is what events participate in the caspase activation.

TNF-α, FasL, VEGI, and TRAIL are recognized to induce apoptosis by activating death receptors such as TNFR1, Fas/Apo1/CD95, DR3/Apo3/WSL-1/LARD/TRAMP, DR4/TRAIL-R1, DR5/TRAIL-R2/TRICK2/KILLER, and DR6 [36]. Fas and TNFR1 are endowed with caspase-8 activation [3]. Fas is activated by binding FasL, recruiting the adaptor protein FADD to aggregate procaspase-8, that cleaves one other to initiate an active form of caspase-8 [4]. This pathway, however, is unlike here, since Fas and FasL are not expressed in HuH-7 cells. TNFR1, on the other hand, is activated by TNF-α, forming a complex of TRADD/RIP1/FADD/procaspase-8 to activate caspase-8 followed by caspase-3 [5]. No evidence, however, has been shown that adenosine acts as a ligand for TNFR1. Furthermore, adenosine here downregulated expression of the FADD mRNA. Adenosine-induced caspase-8 activation, therefore, appears not to be mediated via a TNFR1 signaling pathway.

Notably, extracellular adenosine here decreased expression of the c-FLIPS mRNA and c-FLIP protein, and the effect was inhibited by dipyridamole. Overexpression of c-FLIPS attenuated adenosine-induced caspase-8 activation. These findings, in the light of the fact that c-FLIP is a specific inhibitor of caspase-8 activation [6,7], allow us the explanation that adenosine could activate caspase-8 by neutralizing caspase-8 inhibition due to c-FLIP as a result of decreased c-FLIP expression, as mediated via an intracellularly transported adenosine/AMP signal, which follows the ensuing activation of caspase-3 responsible for apoptotic cell death in HuH-7 cells. Adenosine-induced caspase-8 activation was abolished by dipyridamole, while dipyridamole partially inhibited adenosine-induced HuH-7 cell death. This may explain that adenosine-induced HuH-7 cell death includes necrotic cell death or caspase-independent apoptosis. Moreover, dipyridamole did not fully inhibit adenosine-induced decrease in the expression of the c-FLIPS mRNA and c-FLIP protein, suggesting additional unknown pathways for caspase-8 activation relevant to an intracellularly transported adenosine signal. To address these questions, we are currently carrying out further experiments.

Unlike for HuH-7 cells, extracellular adenosine is shown to induce Li-7A hepatoma cell death via A_3 adenosine receptors [25]. So far as HepG2 hepatoma cells are concerned, adenosine induces apoptosis by activating caspase-3, -8 and -9 and increasing p53 expression, regulated by multi-signaling pathways as mediated via all the adenosine receptors, regardless of adenosine transporters (unpublished data). Adenosine, thus, appears to induce hepatoma cell death via diverse signaling pathways. It remains an open question why adenosine-induced apoptosis is mediated via different signaling pathways, depending upon hepatome cell lines.

In conclusion, the results of the present study show that extracellular adenosine induces HuH-7 cell death, dominantly apoptosis, via an intrinsic pathway relevant to adenosine uptake into cells and conversion to AMP. Intracellularly transported adenosine and/or AMP may produce signals to downregulate expression of the c-FLIPS mRNA and c-FLIP protein, thereby neutralizing inhibition of caspase-8 activation due to c-FLIP, that triggers activation of caspase-8 and the effector caspase caspase-3 to cause apoptotic HuH-7 cell death, regardless of death receptors. The results presented here, thus, may provide further insight into adenosine-induced molecular apoptotic pathways.

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