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Molecular mechanisms of adenosine-induced apoptosis in human HepG2 cells[1]

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Key words

adenosins; adenosine receptor; apoptosis; caspase; $HepG_2$; mitochondria

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Abstract

Aim: To investigate effects of adenosine on cell proliferation and apoptosis in human HepG2 cells. **Methods:** HepG2 cells were incubated in the presence of adenosine (0.1–5 mmol/L) for 12–48 h, and the effect of adenosine on cell proliferation was evaluated by using 3-(4,5-dimethyl-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Hoechst 33342 fluorescent staining, dUTP-fluorescein isothiocyanate (FITC) fluorescence and flow cytometric analysis techniques were used to observe cell apoptosis. The effects of adenosine receptor (A1, A2a, A3 and nonspecific receptor) antagonists (8-cpt, DMPX, MRS1191, and theophylline) and an adenosine transporter protein inhibitor (dipyridamole) on adenosine-induced cell apoptosis were observed. Mitochondrial membrane potential was analyzed using DePsipher fluorescent staining, and caspase activity was detected using a Fluorometric assay kit and a fluorescence microplate reader. **Results:** Adenosine significantly reduced cell viability in a dose- and time-dependent manner. The cytotoxicity of adenosine was related to the induction of cell apoptosis. Four adenosine receptor antagonists had no effect on cell apoptosis. However, dipyridamole significantly reduced the percentage of adenosine-induced apoptotic cells from 27.3% to 7.1% ($P<0.05$). At 48 h after treatment, 3 mmol/L adenosine increased caspase-3 activity 3.5-fold; dipyridamole markedly decreased caspase-3 activity 1.6-fold, and decreased apoptotic cell numbers. When HepG2 cells were treated with 3 mmol/L adenosine, mitochondrial membrane potential and the activity of caspase-8 or -9 remained unchanged. **Conclusion:** Our results suggest that adenosine-induced apoptosis in HepG2 cells is related to intracellular events rather than cell surface receptors, and that a caspase-3 cascade activation is required, which is not mediated via a mitochondrial pathway.

Introduction

China is a high-risk region for primary hepatocellular carcinoma. Treatment for primary hepatocellular carcinoma is still difficult, and depends on basic medical research. Recent evidence suggests that apoptosis of cells is closely related to the occurrence, progress and metastasis of tumors[1–3]. Study of the mechanisms of apoptosis in tumor cells is an important field of tumor therapy and cancer molecular biology[4–7]. Adenosine has been shown to induce apoptosis in various systems[8,9] and in the clinical setting, adenosine has also been used to treat some diseases, including arrhythmia. ATP, a precursor of adenosine, has also been used in the treatment of advanced lung cancer in a clinical phase II study [10]. However, there are very limited reports about adenosine-mediated apoptosis in hepatocytes and hepatoma cells.

Adenosine is thought to arise from the sequential dephosphorylation of extracellular ATP by ecto-ATP diphosphohydrolase and 5'-nucleotidase, and also from diverse cells such as fibroblasts, endothelial cells, epithelial cells, cardiac myocytes, muscle cells and platelets at micromolar

concentrations. This concentration of adenosine has been documented in the interstitial fluid of carcinomas growing in laboratory animals[11,12]. It is transported in and out of cells through specific nucleoside transporter proteins or specific cell surface receptors. These receptors, all of which are G protein-coupled, are classified into 4 categories (A1, A2a, A2b, and A3) on the basis of their molecular structures, their distinct pharmacological profiles, and tissue distribution[13]. The availability of agonists and antagonists specific for the 4 adenosine receptor subtypes has allowed investigators to explore the roles of adenosine and these receptors[2,3].

Up to now, several mechanisms of adenosine-mediated cell apoptosis have been described. In bovine endothelial cells[14], U-937 human histiocytic leukemia cells[15], and human leukemia HL-60 cells[16], adenosine induces apoptosis after being transported into the cells, but in human epidermoid carcinoma (A431 cells)[17], human arterial smooth muscle cells[18], and astrocytes[19], adenosine exerts its effect extracellularly, mediated by the adenosine receptors.

Evidence has indicated that caspases play an important role in the apoptotic response in some cell lines. In the unstimulated state, caspases are present in the form of inactive proenzymes in cells. Upon treatment with anticancer drugs or stimulation with other factors, cellular caspases are activated. There are at least 2 regulatory pathways of the caspase activation cascades. The first extra-mitochondrial pathway (extrinsic pathway) is mediated by death receptors such as Fas and caspase-8. The other intra-mitochondrial pathway (intrinsic pathway) is mediated by cytochrome c and caspase-9. Caspase-8 and caspase-9 activate effector caspase-3, which cleaves target proteins, causes DNA degradation, and eventually leads to programmed cell death[19].

In the present study, we explore whether adenosine induces apoptosis in human HepG2 cells, and examine the molecular mechanisms underlying adenosine-mediated cytotoxicity. To investigate the possibility, the effects of extracellular adenosine on cell proliferation, apoptosis induction, mitochondrial membrane potential and caspase activity were studied.

Materials and methods

Materials Dulbecco's modified Eagle's medium (DMEM), penicillin G, streptomycin, glutamine, trypsin/ethylenediamine tetraacetic acid (EDTA), phosphate-buffered saline (PBS), theophylline, and fetal bovine serum (FBS) were purchased from Equitech-Bio (USA). An *in situ* cell death detection kit was purchased from Boehringer Mannheim Biochemicals (Indianapolis, USA); a Hoechst 33342 staining-Vybrant Apoptosis Assay Kit was purchased from Eugene Biochemical Co (Oregon, USA); a caspase-3, -8, -9 colorimetric protease assay kit (R&D Systems, Minneapolis, USA); adenosine, 3,7-dimethyl-1-propargylxanthine (DMPX), 8-cyclopenthyl-1,3-dioropylxanthine (8-cpt), and 3-ehtyl-*S*-benzyl-2-methyl-4-phenylethnyl-6-phenyl-1,4(±)-dihydrophyridime-3,5-dicarboxylate (MRS1191) were purchased from Biotrend (Germany); and 3-(4,5-dimehthyl-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Dojindo (Japan).

Cell lines and culture conditions HepG2 cells derived from a human hepatocellular carcinoma line (HB 8065, American Type Culture Collection) were grown in DMEM supplemented with 10% heat-inactivated FBS, antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin) and 2 mmol/L glutamine at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide. The media were changed every 3 d, and the cells were separated by trypsinization using trypsin/edetic acid when they reached 90% confluence.

MTT assay Cell proliferation was determined by using the MTT method[20]. Approximately 5000 cells/well were seeded in 96-well plates (Coster) and incubated for 24 h before treatment with 0.1–5 mmol/L adenosine. The initial number of viable cells at the time of treatment, termed t=0, was then determined to correct for differences in starting cell number between experiments and to monitor changes in cell number over time. At the indicated times, MTT tetrazolium salt with phenazine methosulfate were added directly to the culture media and the cells were allowed to incubate for 2–3 h. Mitochondrial dehydrogenases of viable cells convert MTT into a color-dense formazan. The media from each well were collected in microfuge tubes, and detached cells were recovered by centrifugation. Dimethylsulfoxide (Me_2SO) was added to the cell pellets in the tubes as well as the attached cells remaining in the wells to dissolve the insoluble formazan. The Me_2SO solutions were combined, and absorbance was measured at 570 nm in a microplate reader (SpectraMax Plus 384; Molecular Devices, USA) to determine the number of viable cells at 12, 24, 36, and 48 h after adenosine treatment. All data presented in the present report were obtained from 6 independent experiments.

Identification of apoptosis by Hoechst 33342 staining To observe cells undergoing apoptosis, Hoechst 33342 staining was performed as described previously[6]. Briefly, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then washed once with PBS. Hoechst 33342 (50 ng/mg) was added to the fixed cells, incubated for 30 min at room temperature, and then washed with PBS. Cells were counted and examined by fluorescence microscopy at 480

nm (Eclipse TE300; Nikon, Japan). Apoptotic cells were identified by their characteristic nuclei condensation and fragmentation, whereas nuclei from normal cells demonstrated a normal uniform chromatin pattern. The percentage of apoptotic cells was calculated from the ratio of apoptotic cells to total cells counted. At minimum, 500 cells were counted from more than 5 random microscopic fields by two observers.

Identification of apoptosis by flow cytometry and TUNEL assay To determine the effects of adenosine on cell apoptosis, DNA fragmentation was determined using a Tdt-mediated dUTP-FIFC nick-end labeling (TUNEL) assay as described previously[3] according to the manufacturer's instructions. Briefly, HepG2 cells (1.5×10^6 cells/mL) were incubated for 48 h in the presence or absence of adenosine, then these cells were washed with PBS, and cell suspensions were prepared by trypsinization. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and labeled with 0.3 nmol FIFC-12-dUTP, 3 nmol dATP, 2.5 mmol/L $Cocl_2$, 12.5 U Tdt, and 5 μL 10×Tdt buffer in a total volume of 50 μL at 37 °C for 1 h. Samples were analyzed on a FACScan flow cytometer using lysis-11 software (Becton Dickinson, Germany). At least 10 000 cells were analyzed per sample.

Use of adenosine receptor antagonists or adenosine transport inhibitors Extracellular adenosine interacts with cells mainly through two mechanisms: (1) cellular uptake by transmembrane transporter proteins[1]; or (2) specific activation of adenosine receptors[2]. To investigate the mechanisms responsible for the adenosine-mediated cytotoxic effects, adenosine receptor antagonists or an inhibitor of transport protein were used. Cells were seeded into 96-well plates (5000 cells/well) and cultured for 24 h. Then, the medium was removed and replaced with fresh medium, containing various concentrations of the compounds to be tested, for another 48 h: adenosine alone, adenosine plus its A1 receptor antagonist 8-cpt, A2a receptor antagonist DMPX, A3 receptor antagonist MRS1191, nonspecific receptor antagonist theophylline, or the inhibitor of the nucleoside transmembrane carrier, dipyridamole[18]. Cell viability was determined by using the MTT assay.

Assessment of mitochondrial membrane potential To determine whether the mitochondria-mediated apoptotic pathway is involved in adenosine-induced apoptosis, mitochondrial membrane potential was analyzed using the lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (DePsipher, Molecular Probes)[22]. DePsipher is a fluorescent compound that exists as a monomer at low concentrations and fluoresces green. At higher concentrations, DePsipher forms aggregates and fluoresces red. Under physiologic conditions, DePsipher aggregates upon membrane polarization and forms an orange-red fluorescent compound. However, de-energized mitochondria cannot concentrate DePsipher and fluoresce green. If the potential is disturbed, the dye has no access to the transmembrane space and remains in or reverts to its green monomeric form. Induction of mitochondrial damage is a key step for apoptosis in many experimental systems, so changes in mitochondrial membrane potential are considered to be indicators of mitochondrial damage[21]. DePsipher fluoresces red in its multimeric form in healthy mitochondria and is the active reagent in the DePsipher Mitochondrial Potential Assay Kit (Trevigen). HepG2 cells were seeded onto 96-well plates and incubated with or without adenosine as described earlier. Approximately 25 μg/mL of DePsipher reagent was added at 48 h after treatment with adenosine and incubated for a further 20 min. The aggregate red form was observed by fluorescence microscopy (Eclipse TE300; Nikon) after excitation at 518 nm and emission at 605 nm. Quantification of the fluorescent signal was determined using Metamorph imaging software.

Assay of caspase activation After treatment with 3 mmol/L adenosine for 48 h, cells were washed with PBS and lysed in lysis solution. Activity of caspase-3, -8, or -9 was detected by using Fluorometric Assay Kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA)[22]. In brief, control or treated cells were lysed in 50 μL of cold lysis buffer, kept on ice for 10 min and centrifuged for 10 min at 15 000×*g*, after which the supernatant was collected. Cell lysate was added to 50 μL of reaction buffer and 5 μL of fluorogenic report substrates specific for caspase-3 (DEVD-ρNA), caspase-8 (IETD-ρNA) or caspase-9 (LEHD-ρNA). After incubation at 37 °C for 4 h, the fluorescence was detected by a fluorescence microplate reader (SpectraMax Plus 384, Molecular Devices, USA) with excitation at 400 nm and emission at 505 nm. Comparison of the absorbance of ñNA in cells with and without adenosine allows the determination of relative caspase activity (expressed as a percentage of control).

Statistical analysis All data are presented as mean±SD. The statistical significance of differences was determined using one-way ANOVA with SPSS 11.5 software (SPSS, Chicago, IL, USA). $P<0.05$ was considered statistically significant.

Results

Adenosine reduces HepG2 cell number As seen in Figure 1A, cell growth inhibition was observed with expo-

sure to adenosine at different concentrations. Adenosine time- and dose-dependently increased cell death with 12–48 h treatment. The effective adenosine concentration for 50% inhibition (EC_{50}) of HepG2 cell growth after 48 h was 3 mmol/L. So we choose 3 mmol/L for the subsequent experiments. The effect of adenosine on cell proliferation over time is shown in Figure 1B; treatment with 3 mmol/L adenosine for 12 h caused a reduction in viable cell number to 90.7%±6.3% as compared with 120.3%±10.8% in controls, representing a 24.6±1.5% inhibition of cell growth. After 24 h, 36 h, and 48 h of treatment, the percentages of viable cells were 79.6%±5.5%, 46.2%±3.8%, and 33.3%±2.1%, respectively, in adenosine-treated cells; and 135.1%±12.1%, 211.7%±16.8%, and 248.1%±21.6%, respectively, in normal controls, representing decreases of 41.1%±3.3%, 78.2%±5.1%, and 86.6%± 6.8%, respectively. There was a significant difference after

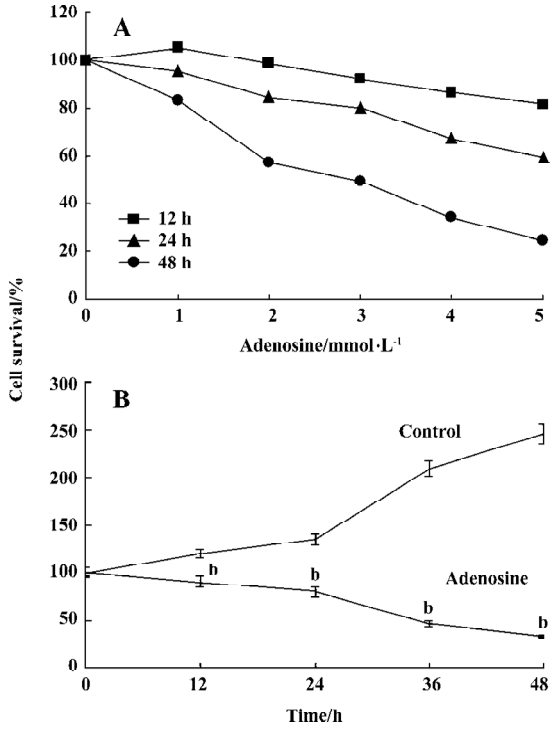

Figure 1. Adenosine inhibited HepG2 cell proliferation. (A) Time- and dose- dependent cytotoxic effects of extracellular adenosine on HepG2 cells. Cells (5000 cells/well) were treated with different concentrations of adenosine (0.1–5 mmol/L) for 12, 24, or 48 h. Cell viability was determined by using the MTT assay. Results are expressed as percentages of cell growth relative to initial number of viable cells in controls. (B) Effects of 3 mmol/L adenosine on cell proliferation over time. Equal numbers of HepG2 cells were seeded in 96-well culture plates in DMEM and allowed to adhere for 24 h. DMEM and 3 mmol/L adenosine was then added, and the number of viable cells was determined at 12, 24, 36, and 48 h by using the MTT assay. Mean±SD. n=6. ^bP<0.05 *vs* control.

12 h, 24 h, 36 h, and 48 h of treatment between the control and adenosine-treated cells (all P<0.05).

Adenosine induces apoptotic HepG2 cell death To explore the potential mechanisms by which adenosine inhibited HepG2 cell proliferation, we further studied whether adenosine induced apoptosis in HepG2 cells by using Hoechst 33342 staining and TUNEL techniques. In experiment 1, cells were treated with 3 mmol/L adenosine for 48 h, and apoptotic cell death was analyzed by Hoechst 33342 staining and quantified by using fluorescence microscopy. In the control, most cells contained intact genomic DNA (Figure 2A); however, in adenosine-treated cells, many cells had condensed chromatin (Figure 2B). Approximately 27.3%±2.3% of adenosine-treated cells showed DNA changes, but in the controls, only 2.1%±0.2% of cells were apoptotic. There was a significant difference between the control and adenosine-treated cells (Figure 4; P<0.01). In experiment 2, cells were treated with 3 mmol/L adenosine, and DNA fragmentation was determined 24 h later by using the TUNEL and FACScan flow cytometer assays. As shown in Figure 3A, cells without adenosine formed a very single population in which only 1.1%±0.1% cells exhibited DNA fragmentation (low dUTP-FITC fluorescence with a mean channel fluorescence intensity of 3). Following exposure to 3 mmol/L adenosine for 48 h, a second population of cells (27.5%±2.1%) appeared that exhibited DNA fragmentation (increased dUTP-FITC fluorescence with a mean channel fluorescence intensity of 136; Figure 3B). There was a significant difference in the relative cell number between the controls and adenosine-treated cells (P<0.01). The same results were obtained by using 2 different experimental methods, which indicates

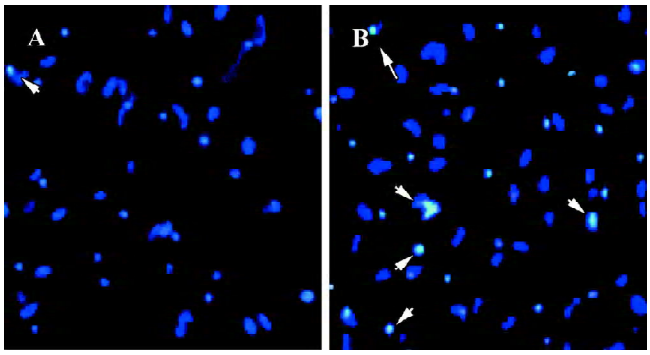

Figure 2. Morphological changes in the nuclei of HepG2 cells. (A) In controls, the majority of cells had uniformly stained nuclei after staining with the membrane-permeable DNA-binding dye Hoechst 33342. (B) A total of 48 h exposure to 3 mmol/L adenosine induced morphological changes typical of apoptosis (ie nuclei fragmentation with condensed chromatin), as detected by fluorescent microscopy. ×400.

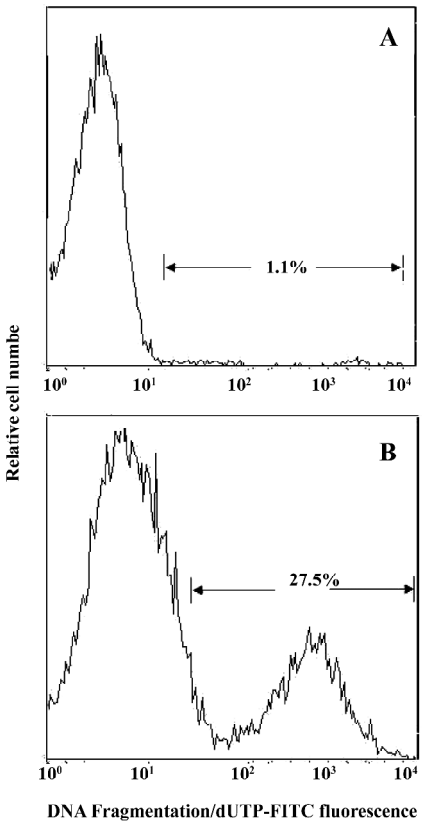

Figure 3. Effect of adenosine on DNA fragmentation. HepG2 cells were incubated with or without 3 mmol/L adenosine for 48 h. DNA fragmentation was determined by using the TUNEL assay. Data shown are DNA fragmentation (as dUTP-FITC fluorescence detected by flow cytometry). (A) Control cells form a very single population in which only 1.1%±0.1% of cells exhibit DNA fragmentation. (B) After exposure to 3 mmol/L adenosine for 48 h, 27.5%±2.1% cells exhibit DNA fragmentation. n=6. P<0.01 *vs* control.

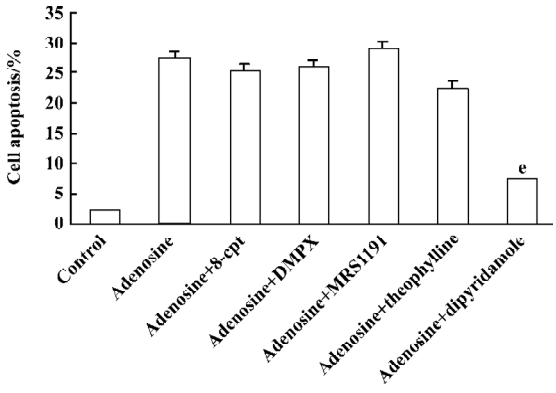

Figure 4. Effects of adenosine receptor antagonists and dipyridamole (inhibitor of the adenosine transporter) on adenosine-induced apoptosis. Cells were incubated for 24 h, then the cells were pretreated with one of 4 adenosine receptor antagonists or dipyridamole for 30 min. The antagonists used were 8-cpt (A1), DMPX (A2a), MRS1191 (A3), and theophylline, a nonspecific adenosine receptor antagonist, each at a concentration of 100 μmol/L. Dipyridamole was used at a concentration of 10 μmol/L. Cells were treated with 3 mmol/L adenosine and the above compounds for 48 h. The percentages of apoptotic cells were determined by using fluorescence microscopy. n=6. Mean±SD. ^bP<0.05 *vs* control. ^eP<0.05 *vs* adenosine-treated cells.

that adenosine mediates apoptotic cell death in HepG2 cells.

Adenosine induces HepG2 cell death via an intracellular pathway HepG2 cells were pretreated with 4 receptor antagonists and then treated with 3 mmol/L adenosine for 48 h, after which apoptotic cell death was quantified by analysis of nuclear chromatin morphology as previously described. Neither 8-cpt (A1 receptor antagonist), DMPX (A2a receptor antagonist), MRS1191 (A3 receptor antagonist), nor theophylline (nonspecific adenosine receptor antagonist) affected apoptosis induced by adenosine. The percentage of apoptotic cells in cultures with adenosine plus 8-cpt, adenosine plus DMPX, adenosine plus MRS911, and adenosine plus theophylline was 25.1%±2.0%, 25.9%±2.3%, 28.8%±2.2%, and 22.5%±1.7%, respectively; there was no significant difference between adenosine alone and adenosine plus any of these antagonists (all P>0.05; Figure 4). The results indicate that the cytotoxic effects of adenosine on HepG2 cells were not mediated by adenosine receptors. To further investigate whether adenosine-induced apoptosis is caused by transmembrane transporter proteins, dipyridamole, an inhibitor of facilitated intracellular transport of adenosine, was tested. HepG2 cells were pretreated with dipyridamole, then treated with 3 mmol/L adenosine for 48 h, after which the apoptotic effect was evaluated. Control cells were not treated with dipyridamole. The percentage of apoptotic cells in the adenosine plus dipyridamole group (7.1%±0.5%) was significantly lower than that in cells treated with adenosine alone (27.3%±2.3%; P<0.05), suggesting that the cytotoxic effects of adenosine on HepG2 cells are mediated by an intracellular pathway.

Effect of adenosine on mitochondrial membrane potential The mitochondria of both controls and adenosine-treated cells exhibited bright red fluorescence signals and no accumulation of green fluorescence signals (Figure 5). Average DePsipher fluorescence was quantified using Metamorph imaging software. The fluorescence of adenosine-treated cells and controls was 94% and 100%, respec-tively; there was no significant difference between the two groups (P>0.05), indicating that adenosine does not perturb mitochondrial function.

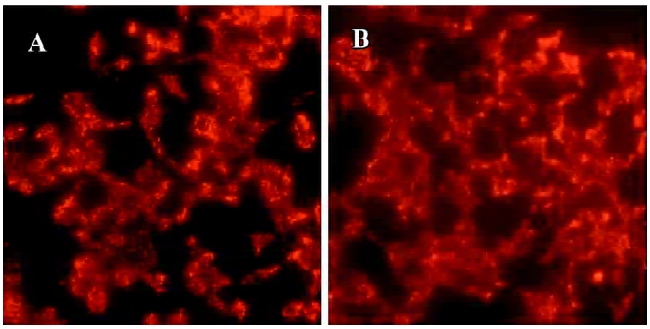

Figure 5. Mitochondrial membrane potential assay. Cells were incubated with or without 3 mmol/L adenosine (control) for 48 h, and then adherent cells were incubated with medium containing DePsipher (25 μg/mL) for 30 min at 37 °C. Red fluorescence represents mitochondria with intact membrane potential. Representative fluorescent images of DePsipher fluorescence in control (A) and adenosine-treated cells (B) are shown. Photographs were taken with a Nikon Eclipse E800 microscope. ×400.

Effects of adenosine on caspase-3, -8, and -9 activity

When HepG2 cells were incubated with 3 mmol/L adenosine, caspase-3 was significantly activated (Figure 6). Caspase-3 activity was increased 3.5-fold (P<0.01). However the activities of caspase-8 and caspase-9 were unchanged. Dipyridamole significantly decreased caspase-3 activation 1.6-fold (P<0.05) but did not affect caspase-8 and caspase-9 activity (P>0.05). These observations indicate that caspase-3 activation plays a role in adenosine-induced apoptosis in HepG2 cells.

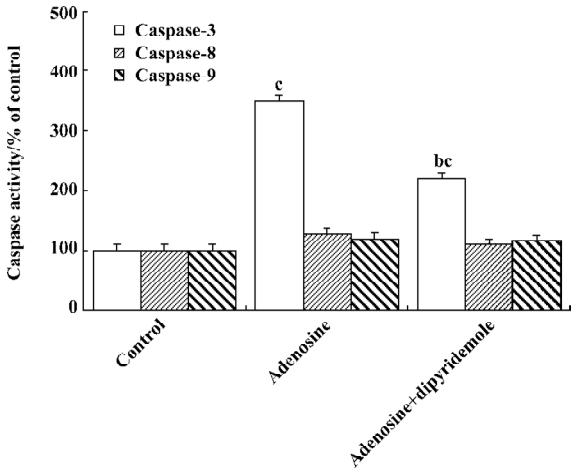

Figure 6. Detection of caspase activity. After adherence, cells were cultured in 10% fetal calf serum medium and incubated with or without 3 mmol/L adenosine for 48 h. Caspase activity in lysates was measured using a caspase-3 substrate (DEVD-ρNA), a caspase-8 substrate (IETD-ρNA) and a caspase-9 substrate (LEHD-ρNA). After incubation at 37 °C for 1 h, fluorescence was detected by using a fluorescence microplate reader. Comparison of the absorbance of ρNA from a treatment sample with that of the controls allowed the determination of the relative caspase activities. n=6. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* control. ^eP<0.01 *vs* Adenosine.

Discussion

Apoptosis is a morphologically distinct form of programmed cell death that plays a major role during development, homeostasis, and in many diseases, including cancer, acquired immunodeficiency syndrome, and neurodegenerative disorders. Diverse signals originating from either within or outside a cell (eg tumor suppressor protein, cyclin death kinase inhibitor, cell surface receptor, and cysteine proteases) can modulate this cell-intrinsic suicide program and lead to cell apoptosis. Induction of apoptosis can be recognized by characteristic biochemical and morphological alterations of the plasma membrane, mitochondria, and nucleus, including cell shrinkage, membrane blebbing, chromatin condensation, and formation of a DNA ladder with multiple fragments[22]. The present study demonstrates that HepG2 cells undergo cell death when exposed to adenosine, because adenosine causes antiproliferation in a dose- and time-dependent manner. In the present study, to confirm that cell death was apoptosis, we used two established methods to detect apoptosis. The coincident results were obtained by observing chromatin condensation using fluorescence microscopy (Figure 2B) and DNA fragmentation (TUNEL) in a flow cytometry assay (Figure 3B), which demonstrates that the extensive cell death induced by adenosine in the HepG2 cell line is due mainly to apoptosis.

Adenosine has been shown to inhibit cell growth and induce apoptosis of several types of cells via at least 2 independent pathways[15,19]. Adenosine can exert its effects extracellulary, mediated by the adenosine receptors, resulting in an increase of the activation of adenylate cyclase, phospholipase C, protein kinase C and intercellular Ca^{2+} [23] as shown for human epidermoid carcinoma (A431 cells)[17], human arterial smooth muscle cells[18], and astrocytes[19]. Alternatively, adenosine causes cell growth inhibition and apoptosis induction after being transported into the cells, via intracellulary or non-receptor-mediated pathways. Our results show that none of the adenosine receptor antagonists tested effectively inhibited apoptosis of the cells, but dipyridamole significantly reduced the percentage of adenosine-induced apoptotic cells from 27.3%±2.3% to 7.1%±0.5% (P<0.05; Figure 4). These findings are consistent with those of previous studies using bovine endothelial cells[14], human breast cancer cells[1], U-937 human histiocytic leukemia cells[15], and human leukemia HL-60 cells [16]. These findings demonstrate that the cytotoxicity of adenosine in HepG2

cells is mediated by an intracellular mechanism rather than by the adenosine receptors.

There are several different metabolic routes for intracellular adenosine[14]: it can be phosphorylated to AMP by adenosine kinase or deaminated by adenosine deaminase to form inosine, which is then phosphorylized to hypoxanthine, and is also related to homocystine metabolism by S-adenosyl-*L*-homocysteine hydrolase. These mechanisms involving active adenosine catabolic products and key enzymes in the salvage pathways have been further investigated in our study.

Although extracellular stimuli-induced apoptosis may involve multiple mechanisms, accumulated data suggest that the mitochondria-initiated death pathway plays an important role in triggering apoptosis in response to those stimuli. In the mitochondria-initiated death pathway, mitochondria undergoing permeability transition release apoptogenic proteins such as cytochrome c or apoptosis-inducing factor from the mitochondrial intermembrane space into the cytosol. Released cytochrome c can activate caspase-9, and activated caspase-9 in turn cleaves and activates executioner caspase-3. After caspase-3 activation, some specific substrates for caspase-3 such as poly(ADP-ribose)polymerase (PARP) are cleaved, and eventually lead to apoptosis[24]. However, mitochondria are not always involved in the process of apoptosis[25,26]. In the present study, the mitochondrial membrane potential was maintained in adenosine-induced HepG2 cell apoptosis (Figure 5). The fluorescence of adenosine-treated cells and controls was 94% and 100%, respectively (P>0.05), indicating that adenosine does not perturb mitochondrial function, and that other pathways might be involved in the apoptotic process in HepG2 cells. Similar findings have been reported for human gastric cancer cells (GT3-TKB cells)[26], but these results are contradictory to those of another study using HL-60 cells[27]. HL-60 cells were exposed to extracellular adenosine, and apoptosis was associated with adenosine active transport. These findings may indicate that adenosine uses a number of different pathways to exert its action and that it acts via different mechanisms in different cell types.

Recently, many investigators have suggested that caspases play an important role in the apoptotic response in some cell lines[6,28,29]. In particular, caspase-3 is a key executioner of apoptosis, whose activation is mediated by the inhibitor caspases such as caspase-8 and caspase-9. Corbiere *et al* reported that diosgenin-induced apoptosis in different human cancer cells is caspase-3-dependent, and is concomitant with a fall of mitochondrial membrane potential[30]. Shieh *et al* investigated the role of caspase-3 in emodin-induced apoptosis in HepG2/C3A cells. They found that the activity of caspase-3 was not significantly increased at the early stage, that it reached a maximal activity value after 48 h emodin treatment, and that the time point was in accordance with the appearance of DNA fragmentation and upregulation of p53 and p21 proteins[31]. In the present study, we observed that adenosine resulted in a time- and dose-dependent increase of apoptotic cell number, and that 3 mmol/L adenosine increased caspase-3 activity 3.5-fold after 48 h treatment (Figure 6). Dipyridamole markedly decreased caspase-3 activity 1.6-fold (Figure 6) and attenuated apoptotic cell numbers (Figure 4), supporting the earlier findings that adenosine is a potent apoptogen and that caspase-3 participates in adenosine-induced apoptotic pathways in human hepatoma cells[20,31]. Okamura *et al* also reported similar results for cisplatin-induced cell apoptosis in human hepatoma and oral squamous cell carcinoma cell lines[22]. However, we noticed that there were no significant changes in the activities of caspase-8 and -9, or in mitochondrial membrane potential after adenosine treatment; the inhibitor of transporter protein had no effect on caspase-8 or -9 activity, which suggests that adenosine mediates apoptosis in part in a caspase-dependent manner, but that an additional, as yet unidentified, apoptotic pathway underlies cell death[32,33]. To address this question, we are currently carrying out further experiments.

In summary, the present study shows that adenosine can inhibit HepG2 cell proliferation via induction of apoptosis. An intracellular pathway is involved, not activation of adenosine receptors and mitochondria dysfunction. The nucleoside transporter activates caspase-3 and causes cell apoptosis. This result indicates that adenosine could be a useful chemotherapeutic agent for the treatment of hepatocellular carcinoma. The primary anticancer effect of adenosine *in vitro* is reported here, and the potential effects, including elucidation of its active catabolic components and evaluation of its anticancer activity *in vivo* need further investigation.

References

1 Hashemi M, Karami-Tehrani F, Ghavami S, Maddika S, Los M. Adenosine and deoxyadenosine induces apoptosis in oestrogen receptor-positive and -negative human breast cancer cells via the intrinsic pathway. Cell Prolif 2005; 38: 269–85.

2 Duann P, Ho TY, Desai BD, Kapoian T, Cowen DS, Lianos EA. Mesangial cell apoptosis induced by stimulation of the adenosine A3 receptor: signaling and apoptotic events. J Investig Med 2005; 53: 37–43.

3 Gorczyca W, Bigman K, Mittelman A, Ahmed T, Gong J, Melamed MR, *et al*. Induction of DNA strand breaks associated with apoptosis during treatment of leukemia. Leukemia 1993; 7:

659–70
4 Notarbartolo M, Lo Cicero S, Meli M, Poma P, Labbozzetta M, Cervello M, *et al*. Induction of apoptosis by the adenosine derivative IB-MECA in parental or multidrug-resistant HL-60 leukemia cells: possible relationship to the effects on inhibitor of apoptosis protein levels. Chemotherapy 2005; 51: 272–9.
5 Ghias K, Ma C, Gandhi V, Platanias LC, Krett NL, Rosen ST. 8-Amino-adenosine induces loss of phosphorylation of p38 mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2, and Akt kinase: role in induction of apoptosis in multiple myeloma. Mol Cancer Ther 2005; 4: 569–77.
6 Zhang YX, Yu SB, Ou-Yang JP, Xia D, Wang M, Li JR. Effect of protein kinase C alpha, caspase-3, and survivin on apoptosis of oral cancer cells induced by staurosporine. Acta Pharmacol Sin 2005; 26: 1365–72.
7 Yang FG, Zhang ZW, Xin DQ, Shi CJ, Wu JP, Guo YL, *et al*. Peroxisome proliferator-activated receptor g ligands induce cell cycle arrest and apoptosis in human renal carcinoma cell lines. Acta Pharmacol Sin 2005; 26: 753–761.
8 Lee EJ, Min HY, Chung HJ, Park EJ, Shin DH, Jeong LS, *et al*. A novel adenosine analog, thio-Cl-IB-MECA, induces G0/G1 cell cycle arrest and apoptosis in human promyelocytic leukemia HL-60 cells. Biochem Pharmacol 2005; 70: 918–24.
9 Lee HT, Gallos G, Nasr SH, Emala CW. A1 adenosine receptor activation inhibits inflammation, necrosis, and apoptosis after renal ischemia-reperfusion injury in mice. J Am Soc Nephrol 2004; 15: 102–11.
10 Haskell CM, Mendoza E, Pisters KM, Fossella FY, Figlin RA. Phase II study of intravenous adenosine 5'-triphosphate in patients with previously untreated stage IIIB and stage IV non-small lung cancer. Invest New Drugs 1998; 16: 81–5.
11 Grierson JP and Meldolesi J. Shear stress-induced $[Ca^{2+}]_i$ transient and oscillations in mouse fibroblasts are mediated by endogenously released ATP. J Bio Chem 1995; 270: 4451–56.
12 Ferguson DR, Kennedy I ,Burton TJ. ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes: a possible sensory mechanism? J Physiol 1997; 505: 503–11.
13 Ralevic V, Burnstock G. Receptors for purinces and pyrimidines. Pharmacol Rev 1998; 50: 413–92.
14 Dawicki DD, Chatterjee D, Wyche J, Rounds S. Extracellular ATP and adenosine cause apoptosis of pulmonary artery endothelial cells. Am J Physiol 1997; 273: L485–94.
15 Schneider C, Wiendl H, and Ogilive A. Biophasic cytotoxic mechanism of extracellulat ATP on U-937 human histiocytic leukemia cells: involvement of adenosine generation. Biochim Biophys Acta 2001; 1538: 190–205.
16 Tanaka Y, Yoshihara K, Tsuyuki M, Kamiya T. Apoptosis induced by adenosine in human leukemia HL-60 cells. Exp Cell Res 1994; 213: 242–52.
17 Atey HB, Tan CH and Khoo HE. Modulation of DNA synthesis via adenosine receptors in human epidermoid carcinoma (A431) cells. Biofactors 1994; 4: 161–5.
18 Peyot ML, Gadeau AP, Dandre F, Belloc I, Dupuch F, Desgrane C. Extracellular adenosine induces apoptosis in human arterial smooth muscle cells via A2b-purioceptor. Circ Res 2000; 86: 76–85.
19 Iorio PD, Kleywegt S, Ciccarelli R, Traversa U, Andrew CM, Crockkr CE, *et al*. Mechanisms of apoptosis induced by purine nucleosides in astrocytes. Glia 2002; 38: 179–90.
20 Wen LT, Knowles AF. Extracellular ATP and adenosine induce cell apoptosis of human hepatoma Li-7A via the A3 adenosine receptor. Br J Pharmacol 2003; 140: 1009–18.
21 Wedgwood S, Black SM. Molecular mechanisms of nitric oxide-induced growth arrest and apoptosis in fetal pulmonary arterial smooth muscle cells. Nitric Oxide 2003; 9: 201–10.
22 Okamura M, Hashimoto K, Shimada J, Sakagami H. Apoptosis-inducing activity of cisplatin (CDDP) against human hepatoma and oral aquamous cell carcinoma cell lines. Anticancer Res 2004; 24: 655–62.
23 Kim KT, Yeo EJ, Choi H, Park SC. The effect of pyrimidine nucleosides on adenosine-induced apoptosis in HL-60 cells. Cancer Res Clin Oncol 1998; 124: 471–7.
24 Hou DE, Uto T, Tong X, Takeshita T, Tanigawa S, Imamura I, *et al*. Involvement of reactive oxygen species-independent mitochondrial pathway in gossypol-induced apoptosis. Arch Biochem Biophy 2004; 428: 179–87.
25 Siu KPY, Chan JYW, Fung KP. Effect of arsenic trioxide on human hepatocellular carcinoma HepG2 cells: inhibition of proliferation and induction of apoptosis. Life Sci 2002; 71: 275–85.
26 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.
27 Shin S, Sung BJ, Cho YS, Kim HJ, Ha NC, Hwang JI, *et al*. An antiapoptotic protein human survivin is a direct inhibitor of caspase-3 and -7. Biochemistry 2001; 40: 1117–23.
28 Kang CM, Sun Y, Jang IS, Park SC. Thymidine-dependent attenuation of the mitochondrial apoptotic pathway in adenosine-induced apoptosis of HL-60 cell. J Cancer Res Clin Oncol 2001; 12: 570–6.
29 Tai CJ, Chang SJ, Chien LY, Leung PC, Tzeng CR. Adenosine triphosphate induces activation of caspase-3 in apoptosis of human granulosa-luteal cells. Endocr J 2005; 52: 327–35.
30 Corbiere C, Liagre B, Terro F, Beneytout JL. Induction of antiproliferative effect by diosgenin through activation of p53, release of apoptosis-inducing factor (AIF) and modulation of caspase-3 activity in different human cancer cells. Cell Res 2004; 14: 188–96.
31 Shieh DE, Chen YY, Yen MH, Chiang LC, Lin CC. Emodin-induced apoptosis through p53-dependent pathway in human hepatoma cells. Life Sci 2004; 74: 2279–90.
32 Sun W, Khoo HE, Tan CH. Adenosine induced apoptosis in BHK cells via P1 receptors and equilibrative nucleoside transporters. J Biochem Mol Biol 2005; 38: 314–9.
33 Wang XJ, Kong KM, Qi WL, Ye WI, Song PS. Interleukin-1 beta induction of neuron apoptosis depends on p38 mitogen-activated protein kinase activity after spinal cord injury. Acta Pharmacol Sin 2005; 26: 934–42.