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Extracellular ATP and adenosine induce cell apoptosis of human hepatoma Li-7A cells *via* the A3 adenosine receptor

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1 Extracellular ATP is a potent signaling molecule that modulates a myriad of cellular functions through the activation of P2 purinergic receptors and is cytotoxic to a variety of cells at higher concentrations. The mechanism of ATP-elicited cytotoxicity is not fully understood. In this study, we investigated the effect of extracellular ATP on the human hepatoma Li-7A cells.

2 We observed a time- and dose-dependent growth inhibition of Li-7A cells by ATP, which is accompanied by an increase in the active form of caspase-3 as well as increased cleavage of its substrate, poly (ADP-ribose) polymerase. The cytotoxic effect of extracellular ATP was not mediated by the $P2X_7$ receptor, since (1) the effect was not abolished by the $P2X_7$ receptor antagonists oxidized ATP and KN-62, and (2) extracellular ADP, AMP, and adenosine were also cytotoxic.

3 We found that ATP and ADP were degraded to adenosine by Li-7A cells and that treatment of Li-7A cells by adenosine resulted in growth inhibition and caspase-3 activation, indicating that adenosine is the apoptotic agent. Using adenosine receptor agonists and antagonists, as well as inhibitors of adenosine transport and deamination, we showed that the cytotoxic effect of adenosine is specifically mediated by the A3 receptor even though transcripts of A1, $A2_A$, $A2_B$, and a splice variant of the $P2X_7$ receptors were detected in Li-7A cells by RT–PCR.

4 Cytotoxicity caused by exogenous ATP and adenosine was completely abolished by the caspase-3 inhibitor Z-DEVD-FMK, demonstrating the central role of caspase-3 in apoptosis of Li-7A cells.
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Keywords: ATP; adenosine; $P2X_7$ receptor; A3 receptor and human hepatoma Li-7A cells

Abbreviations: DAB, diaminobenzidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KN-62, l-(*N*, *O*-bis[5-isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine; Li-7A cells, human hepatoma cell line; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; oxATP, periodate oxidized ATP; PARP, poly (ADP-ribose) polymerase; RT–PCR, reverse transcriptase–polymerase chain reaction; TUNEL, Tdt-mediated dUTP nick end labeling

Introduction

Extracellular adenosine and ATP elicit a variety of physiological responses in different cells. These effects are mediated by specific purinergic receptors for extracellular nucleosides or nucleotides. It is increasingly clear that purinergic signaling plays important roles in various pathological conditions such as cancer, ischemia-associated injury, inflammation, wound healing, drug toxicity, and pain. For the development of therapeutic agents, it is important to define the involvement of specific receptor subtypes in different tissues.

The effect of extracellular purines on transformed cells was first reported in the 1970s. The observation that nucleosides and phosphate esters traffic through the membranes of ATP-treated cells suggested that relatively large pores are formed in the cell membranes (Rozengurt *et al*., 1977; Heppel *et al*., 1985). Subsequently, it was shown that ATP-induced ion fluxes and membrane permeabilization also occur in normal cells, especially in cells of the immune system (Cockcroft & Gomperts, 1980; Steinberg & Silverstein, 1987; Steinberg & Di Virgilio, 1991). Interestingly, the effects of extracellular ATP on cytolytic T lymphocytes bear all the hallmarks of apoptosis, that is, surface blebbing and nuclear DNA fragmentation, in addition to dye uptake (Zheng *et al*., 1991), thus identifying extracellular ATP as an apoptotic agent.

It has now been established that the effects of extracellular ATP are mediated by members of the P2 purinergic receptor family. Of the two P2 receptor subfamilies, P2X receptors are ligand-gated ion channels, whereas P2Y receptors are G protein-coupled heptahelical receptors (see Ralevic & Burnstock (1998) for review). Subtypes of each of the subfamily have been characterized and cloned. Accumulating evidence indicated that the $P2X_7$ receptor (Surprenant *et al*., 1996) mediates the apoptotic responses of a variety of cells (see Chow *et al*. (1997) for a review). ATP-induced cell death in transformed cells has been exploited in inhibiting tumor cell growth (Rapaport, 1983; Chahwala & Cantley, 1984; Fang *et al*., 1992; Spungin & Friedberg, 1993; Vandewalle *et al*., 1994). However, the molecular mechanisms of ATP-induced apoptosis are only beginning to be investigated (Ferrari *et al*., 1999; Humphreys *et al*., 2000; Wen *et al*., 2003).

Owing to the ubiquitous presence of a variety of ectonucleotidases (see Zimmermann (2001) for review), extracellular

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ATP can be degraded to adenosine, a ligand of the adenosine receptors. Adenosine has also been implicated in the induction of apoptosis in several cell types (for a review, see Chow *et al*., 1997). Extracellular adenosine acts mainly through the activation of the adenosine receptors, of which four subtypes have been cloned from various tissues and species (reviewed in Ralevic & Burnstock, 1998). These receptors, all of which are G protein-coupled, are classified into four categories, A1, $A2_A$, $A2_B$, and A3, on the basis of their molecular structures, their distinct pharmacological profiles, and tissue distributions. Agonists selective for these receptors are known. Among these, only the A3 receptor agonists, such as IB-MECA and Cl-IB-MECA, have been shown to induce apoptosis in leukemia cells and cardiac myocytes (Kohno *et al*., 1996a, b; Shneyvays *et al*., 2000).

In the present study, we have investigated the effect of extracellular nucleotides in a human hepatoma cell line, Li-7A, in an attempt to understand the mechanism of extracellular nucleotide-induced cell death of cancer cells. Our results suggest that extracellular ATP induces apoptosis of Li-7A cells after being degraded to adenosine. We further show that adenosine-induced apoptosis in Li-7A cells is an A3 receptor-dependent process and that activation of the caspase-3 cascade is required.

Methods

Cell culture

Human hepatoma Li-7A cells (Knowles, 1988a) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics (50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin). Human embryonic kidney cells (HEK293) stably transfected with the human $P2X_7$ receptor (HEK-$P2X_7$ cells) (kindly provided by Dr G.R. Dubyak, Case Western Reserve University) were grown in DMEM containing 5% new born calf serum and 5% fetal calf serum, streptomycin, penicillin, and 25 μg ml^{-1} hygromycin. Cells were grown in 24-well or 10-cm culture plates, in a humidified atmosphere of 5% CO_2 at 37°C.

Cytotoxicity assays

The effect of nucleotides and nucleosides on the proliferation of Li-7A cells was evaluated by the MTT bioassay (Cory *et al*., 1991). After Li-7A cells were grown to confluency in 24-well plates, the cells were washed twice with serum-free DMEM and then cultured in the absence or presence of the ligands of the purinergic receptors. At the indicated times, MTT tetrazolium salt with phenazine methosulfate was added directly to the culture media and allowed to incubate with the cells for 2–3 h. Mitochondrial dehydrogenases of viable cells will convert MTT into a color-dense formazan. The media from each well were collected in microfuge tubes and detached cells were recovered by centrifugation. DMSO was added to the cell pellets in the tubes as well as attached cells remaining in the wells to dissolve the insoluble formazan. The DMSO solutions were combined and the absorbance was measured at 550 nm with a reference wavelength of 630 nm after appropriate dilution. At least three independent experiments were performed. Data presented were selected from representative experiments.

Tdt-mediated dUTP nick end labeling (TUNEL) assay

TUNEL staining was performed using an *in situ* cell detection kit, BrdUTP-FragEL™, according to the manufacturer's instruction (Oncogene Research Products, San Diego, CA, U.S.A.). The cells were plated on Lab-Tek chamber slides, grown overnight and then treated with or without ATP. Cells were washed, fixed with 4% paraformaldehyde in PBS solution, and incubated with hydrogen peroxide to block endogenous peroxidase. After rinsing, the cells were incubated with the TUNEL reaction mixture. Thereafter, the cells were washed and incubated with biotinylated monoclonal anti-BrdU and a streptavidin-horseradish peroxidase conjugate, followed by incubation with diaminobenzidine (DAB)-substrate solution, and then counterstained with methyl green.

cDNA microarray analysis

Total cellular RNA was prepared from Li-7A cells cultured in the absence or presence of 6 mM ATP for 24 h. A measure of 10 μg of the total RNA was used as template for [^{32}P] cDNA probe synthesis. The RNA was first annealed with a primer mix specific for apoptosis-related genes and the cDNA probes were synthesized according to the protocols provided by the manufacturer. The cDNA probes were denatured and hybridized with the apoptosis-related cDNA microarrays (Super Array Inc., Bethesda, MD, U.S.A.). Hybridization was carried out at 68°C overnight. The membrane was washed twice with wash solution (2 × SSC, 1% SDS) for 20 min at 68°C. The membrane was wrapped in a plastic wrap and exposed to an X-ray film with an intensifying screen at −70°C, developed or scanned by a phosphorimager. Each array is composed of 23 marker genes in duplicates, with two positive controls, β-actin and (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), and a negative control, bacterial plasmid pUC18. In addition, it contained cDNA gene fragments of the Bcl-2 gene family, caspases, caspase-regulatory molecules, as well as Fas, E2F, TRAIL, p21, pig, p53, and NF-κB. Relative amounts of mRNA transcripts were quantified using a Computing Densitometer™ scanner and ImageQuant™ V3.3 (Molecular Dynamics), and compared with the signals derived from GAPDH and β-actin.

Immunoblotting analysis

Cells were lysed and protein extraction was performed. Protein concentrations of the cell lysates were determined by the *Dc* protein assay (Bio-Rad, Hercules, CA, U.S.A.). Cell proteins (25 μg) were separated in 10 or 13% sodium dodecyl sulfate–polyacrylamide gel, and electrophoretically transferred to a PVDF membrane (NEN Life Science, Boston, MA, U.S.A.). The membranes were blocked with 5% nonfat milk, washed, and subsequently incubated with a mouse monoclonal antibody against poly (ADP-ribose) polymerase (PARP) (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.) at 1 : 1000 dilution, or a rabbit polyclonal antibody against caspase-3 (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.) at 1 : 1000 dilution. After washing, the membrane was incubated with horseradish peroxidase-conjugated sheep anti-mouse antibody or donkey anti-rabbit antibody (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), and then visualized by enhanced chemiluminescence (ECL) according to the manufacturer's

recommendations (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

HPLC separation of adenosine and adenine nucleotides

Nucleotides were separated by HPLC (Waters, 'Millennium' system) on a DNApac™ PA-100 anion exchange column ($4 \times 250\,mm^2$, Dionex), using a 45-min linear gradient developed from 20 mM sodium phosphate (pH 7.0). to 20 mM sodium phosphate with 1 M sodium chloride (pH 7.0). The method allowed clear separation of ATP, ADP, AMP, and adenosine. Peaks in the eluates were identified by comparison to known standards for their characteristic retention times.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

The presence of the transcripts of human $P2X_7$, Al, $A2_A$, $A2_B$, and A3 receptors in Li-7A cells were confirmed using RT–PCR. Total cellular RNA from Li-7A and HEK-$P2X_7$ cells was prepared using the RNAzol method (Teltest, Firendwood, TX, U.S.A.), according to the manufacturer's instruction. First-strand cDNA was synthesized from 1 μg of total cellular RNA, using an oligo$(dT)_{18}$ primer and Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD, U.S.A.) at 42°C for 1 h. PCRs were performed in a 50 μl reaction mixture containing 0.5 μl *Taq* DNA polymerase (5 U μl^{-1}), 1 μl cDNA from the cDNA synthesis reaction mixture, 0.2 mM dNTP, and 50 nM of various primer pairs specific for the human $P2X_7$ receptor (accession number Y09561), and the human Al, $A2_A$, $A2_B$ (accession number BC025722), and A3 receptors. For the human P2X7 receptors, two forward primers directed to bases 1–23 (5′-GCAGGGAGG-GAGGCTGTCACCAT-3′), 85–106 (5′-CCAGAGCATGA-ATTATGGCACC-3′) and two reverse primers directed to bases 1321–1440 (5′-TATCCCTGGATCTAGGAGTC-3′), 1860–1885 (5′-GCCTGGCTTCAGTAAGGACTCTTGAA-3′) were used. Forward primer 5′-CAACATTGGGCCACA-GACCT-3′ and reverse primer 5′-TAGGTAAG-GATGCTGGGCTT-3′ (Suzuki *et al*., 1998) were used for the Al receptor, giving a 606 bp product. Forward primer 5′-AGATGGAGAGCCAGCCTCTGC-3′, and reverse primer 5′-GCTAAGGAGCTCCACGTCTGG-3′ (Suzuki *et al*., 1998) were used for the $A2_A$ receptor, giving a 535 bp product. Forward primer 5′-CAAGTCACTGGCCATGA-TTG-3′ (785–805) and reverse primer 5′-CCACCA-TAAACAAGG-CAGAC -3′ (1362–1381) were designed and used for the $A2_B$ receptor, giving a 596 bp product. Forward primer 5′-ACCCCCATGTTTGGCTG-3′ and reverse primer 5′-GCA-CAAGCTGTGGTACCTCA-3′ (Kohno *et al*., 1996a) were used for the A3 receptor, giving a 361 bp product. The PCR products were analyzed after electrophoresis on 1.5% agarose gel and staining by ethidium bromide. The PCR products were also cloned into the pcDNA3.1 vector (TA cloning kit, Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's instructions, and sequenced completely using an automated DNA sequencer.

Statistical analysis

Statistical comparison was carried out by the Student's *t*-test. Data are expressed as means ± s.d. *P*-values less than 0.05 were considered as significant.

Materials

DMEM, fetal and new born calf sera were purchased from GIBCO Life Technologies (Gaithersburg, MD, U.S.A.). All nucleotides and nucleosides, dipyridamole, N^6-cyclohexyladenosine (CHA), 5-(*N*-cyclopropyl) carboxamidoadenosine (CPCA), 8-cyclopentyl-l,3-dipropylxanthine (DPCPX), alloxazine, l-(*N*, *O*-bis[5-isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62), and periodate-oxidized ATP (oxATP) were obtained from Sigma (St Louis, MO, U.S.A.). Adenosine receptor agonists and antagonists, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylurinamide(2-C1-IB-MECA), ZM241385, MRS 1220, and MRS 1523 were from Tocris (Ellisville, MO, U.S.A.). Protease inhibitors, Z-DEVD.fmk, Z-FA.fmk, and *N*-acetyl-leucyl-leucyl-norleucinal (ALLN), and adenosine deaminase inhibitors, coformycin and erythro-9-(2-hydroxy-3-nonyl)adenine, HC1(EHNA), were from Calbiochem (La Jolla, CA, U.S.A.).

Results

Extracellular ATP induces cytotoxicity in Li-7A cells

To investigate if ATP exerts cytotoxic effect on Li-7A cells, we treated Li-7A cells with 0–10 mM ATP at various time intervals, and used the MTT colorimetric assay to quantify the effect of ATP on Li-7A cell proliferation. As seen in Figure 1, ATP caused a time- and dose-dependent inhibition of the proliferation of Li-7A cells. Treatment of Li-7A cells by ATP for only 7 h had no effect even at 10 mM ATP. Cell growth inhibition was observed when exposure to extracellular ATP was prolonged. The effective ATP concentration for 50% inhibition (EC_{50}) of Li-7A cell growth after 39 h is 5 mM. In the following experiments, 6 mM (higher than EC_{50}) of ATP was used as the standard concentration.

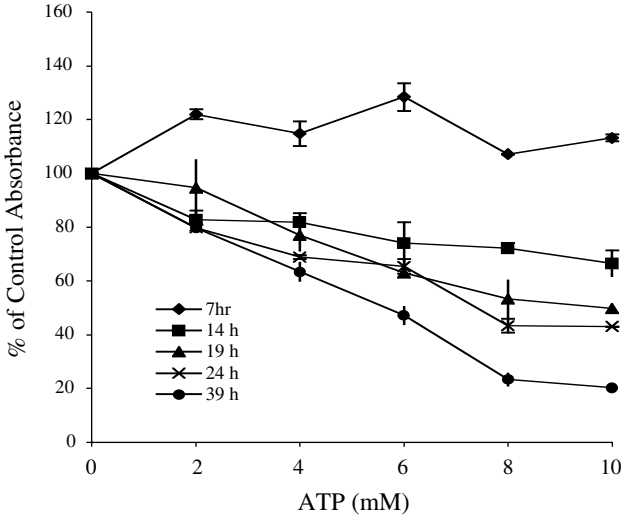

Figure 1 Cytotoxic effect of extracellular ATP on Li-7A cells. Cells were treated with different concentrations of ATP for 7, 14, 19, 24, and 39 h. Cell viability was determined by the MTT assay, as described in 'Methods'. Results are expressed as percentages of cell growth relative to untreated controls. Data are averages ± s.d. of triplicate determinations.

We then verified that ATP-induced cell death in Li-7A cells was the result of apoptosis. Apoptosis is characterized by internucleosomal degradation of genomic DNA. DNA cleavage and free 3′-OH groups generated by cellular endonucleases in apoptotic cells can be determined using *in situ* TUNEL assay (Gavriell *et al*., 1992). A dark brown DAB signal indicates positive staining, while shades of blue-green to greenish tan indicate a nonreactive cell. Figure 2 shows the result of a representative experiment demonstrating that the number of TUNEL-positive cells increased with increasing ATP concentrations. These results were confirmed by FACS analysis (data not shown), where cells were stained with propidium iodide and FITC-conjugated annexin V antibodies, which only bind to apoptotic cells. We conclude that extracellular ATP induces apoptosis in Li-7A cells.

Extracellular ATP induces caspase-3 gene expression

To define the molecular basis of the cytotoxic action of ATP, we examined the effect of ATP on the expression of apoptosis-related genes using cDNA microarray analysis. Through side-by-side hybridization with cDNA probes prepared from RNAs of untreated and ATP-treated Li-7A cells, the expression profiles of apoptosis-related genes were compared. The results in Figure 3 show that the bacterial plasmid pUC18 was not expressed in either control or ATP-treated Li-7A cells (spots 1G and 2G) and served as negative control. ATP treatment of Li-7A caused no changes in the expression of the housekeeping genes, β-actin (spots 3G and 4G) and GAPDH (spots 5G, 6G, 7G, 8G, 8E, and 8F). Importantly, ATP treatment caused a significant upregulation of caspase-3 gene expression (spots 3C and 3D) in Li-7A cells. The expression of other cDNAs showed either slight or no changes after ATP treatment.

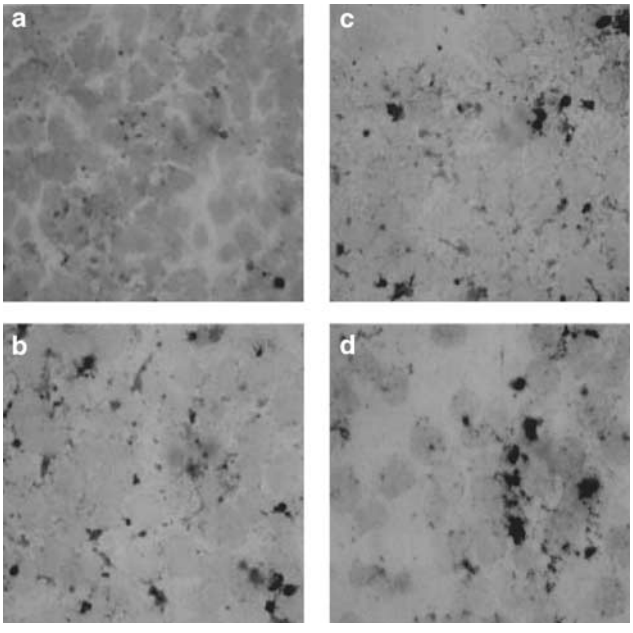

Figure 2 Extracellular ATP induces apoptosis in Li-7A cells as determined by *in situ* TUNEL assays. Li-7A cells were treated by various concentrations of ATP for 24 h, and then used for *in situ* TUNEL assays. Diaminobenzidine reacts with the labeled cells to generate a brown product at the site of DNA fragmentation. Brown staining, therefore, indicates apoptotic cells. Cells were counter-stained with methyl green to aid in the morphological evaluation of normal and apoptotic cells. (a) no ATP; (b) 2 mM ATP; (c) 4 mM ATP; (d) 6 mM ATP. After TUNEL assays, cells were examined by light microscopy. Magnification, $\times 40$; bar $= 25\,\mu$m.

Activation of caspase cascade in ATP-induced cell death of Li-7A cells

Caspases have been implicated as the principal mediators of apoptosis. Increased caspase 3/CPP32 activity results in increased cleavage of its substrate PARP (He *et al*., 1998). Caspase-3, like the other caspases, is synthesized as an inactive zymogen. Upon activation, it is converted to a tetrameric complex composed of two heterodimeric subunits of 17 kDa. Using Western blot analysis, we demonstrated the appearance of the 17 kDa caspase-3 subunits in Li-7A cells after 39 h of treatment with 6 mM ATP (Figure 4a). As observed for other cells undergoing apoptosis, significant amount of the 32 kDa zymogen remained (He *et al*., 1998; Wen *et al*., 2003). Nevertheless, the active caspase-3 cleaved its substrate, the 117 kDa PARP, into its characteristic 89 kDa fragment (Figure 4a). Figure 4b shows that the 17 kDa form of caspase-3 as well as the 89 kDa cleavage product of PARP were detected in Li-7A cells after being treated with 2–8 mM ATP for 36 h.

Effect of other nucleotides and adenosine on Li-7A cell proliferation

To determine if the cytotoxic effect of ATP on Li-7A cells is mediated by the $P2X_7$ receptor, we determined the effects of two $P2X_7$ receptor antagonists, oxATP (Murgia *et al*., 1993) and KN62 (Humphreys *et al*., 1998), on ATP-induced cell death in Li-7A cells. In previous studies using HEK cells stably transfected with the human $P2X_7$ receptor, we showed that

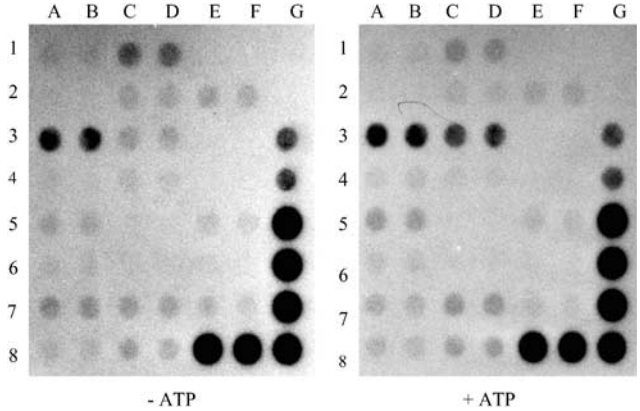

Figure 3 ATP modulates caspase-3 expression in Li-7A cells. Total RNA was isolated from untreated Li-7A cells (a) and Li-7A cells treated with 6 mM ATP (b) for 24 h. The RNAs were reverse transcribed to cDNAs, as described in 'Methods'. The ^{32}P-labeled cDNAs were hybridized to apoptotic pathway-specific DNA microarray. Each gene is present in duplicate. The expression of positive controls β-actin (3G, 4G), GAPDH (5G, 6G, 7G, 8G, 8E, and 8F) remained constant in the presence and absence of ATP. The negative control bacterial plasmid pUC18 (1G, 2G) was not expressed in either. Caspase-3 (3C, 3D) expression is upregulated in ATP-treated cells. A more than two-fold difference was detected between the signals obtained from untreated and ATP-treated Li-7A cells.

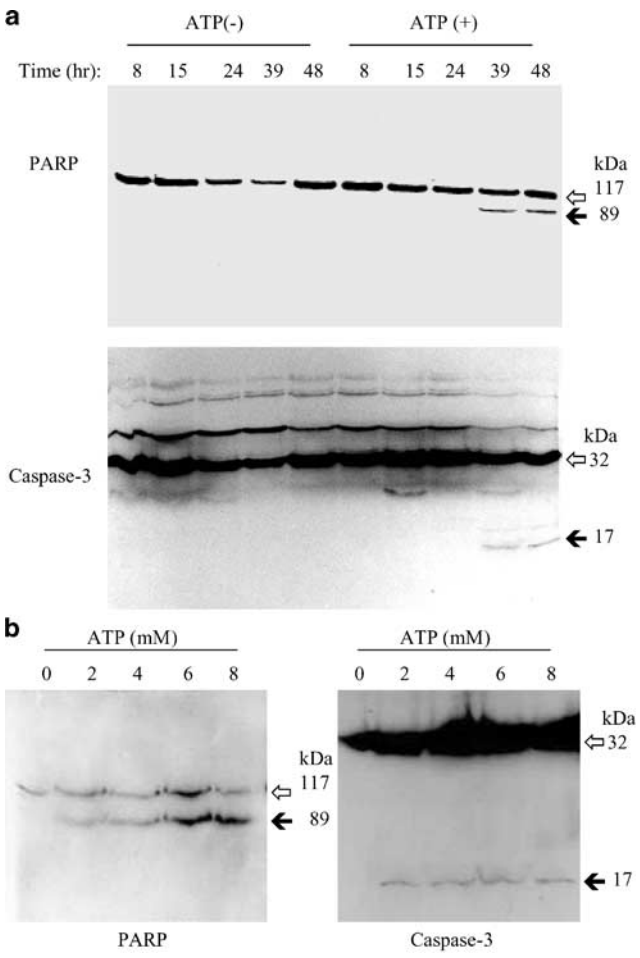

Figure 4 ATP treatment causes cleavage of PARP and caspase-3 in Li-7A cells. Cell lysates were obtained from untreated and ATP-treated Li-7A. After SDS–PAGE of the cell lysates (25 μg), Western blot analysis was carried out with antibodies of capase-3 and the caspase substrate PARP. Open arrowheads indicate the full-length and closed arrowheads, the cleaved forms of the proteins. (a) Protein samples were obtained from cells cultured without and with 6 mM ATP for the indicated times, (b) Protein samples were obtained from Li-7A cells treated with different concentrations of ATP for 36 h.

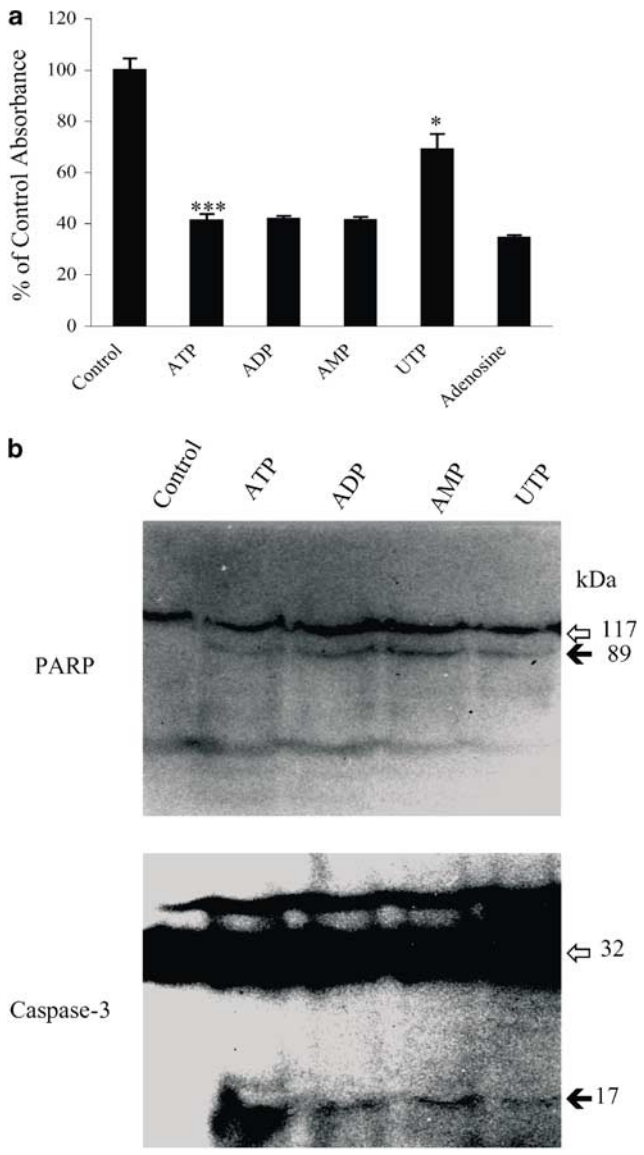

Figure 5 Effect of other nucleotides on cell growth and caspase-3 activation in Li-7A cells. (a) Cells were either left untreated (control) or treated with 6 mM ATP, ADP, AMP, UTP, or adenosine for 36 h. Cell viability was determined by the MTT assay. Data are presented as mean percentage of basal $\pm$ s.d. of four replicates; $^{*}P<0.05$; $^{***}P<0.001$ (compared with appropriate control values). (b) Cells were treated with 6 mM ATP, ADP, AMP, or UTP, as described above. Cellular lysates were prepared and subjected to immunoblotting using antibodies against the caspase substrate PARP and caspase-3.

ATP-induced apoptosis was blocked by 1 mM oxATP and 3.5 μM KN62 (Wen *et al*., 2003). We found that oxATP itself inhibited the growth of Li-7A cells at 0.25 and 1 mM. Inhibition of growth by oxATP did not involve the activation of caspase-3. ATP-induced caspase-3 activation was also not diminished by oxATP (data not shown). Furthermore, the more potent $P2X_7$ receptor antagonist, KN-62, was ineffective in blocking ATP-induced cell death of Li-7A cells (data not shown). These results indicate that ATP-induced cell death in Li-7A cells is not mediated by the $P2X_7$ receptor.

We then investigated the effects of other purinergic receptor ligands on Li-7A cell proliferation. Figure 5a shows that 6 mM adenosine, AMP, and ADP inhibited cell growth to the same extent as ATP, whereas inhibition by 6 mM UTP was less. Similar to the effect of ATP, cytotoxicity induced by ADP, AMP, and UTP also involved activation of caspase-3, as indicated by the appearance of the 17 kDa caspase subunit and the 89 kDa PARP cleavage product (Figure 5b).

Effect of adenosine on Li-7A cell proliferation

The observation that extracellular AMP, which is not a ligand of P2 receptor, was effective in inducing the cell death of Li-7A cells, suggests that adenosine, which can be produced from AMP by the action of 5′-nucleotidase, is most likely the apoptotic agent. The effects of ATP and ADP could also be explained by the generation of adenosine from these nucleotides by the action of ectonucleotidases in these cells (Knowles, 1988b). Figure 6 shows that adenosine was indeed produced from ATP and ADP added to Li-7A cells. HPLC analysis of

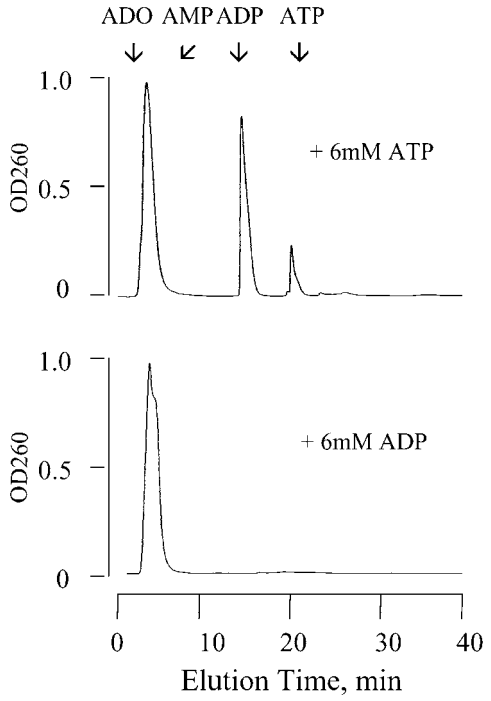

Figure 6 HPLC analysis of metabolic products of ATP and ADP. Li-7A cells were incubated for 24 h with 6 mM ATP and ADP. Adenine metabolites were separated by HPLC, as detailed in 'Methods'. The retention times for various standards of adenine nucleosides and nucleotides are indicated with arrows. The results are representative of two independent experiments with duplicate samples.

adenine metabolites in the culture media showed that ~50% of the added ATP and all of the added ADP were converted to adenosine after 24 h.

Figure 7a shows that adenosine was as effective as ATP in inducing cell cytotoxicity in Li-7A cells. While treatment for 24 h by 4 mM ATP caused an ~30% inhibition of cell growth (Figure 1), 4 mM adenosine inhibited cell growth by ~70% (Figure 7a). Similar to the effect of ATP, treatment of Li-7A cells by 6 and 8 mM adenosine for 15 h also resulted in the cleavage of PARP into the characteristic 89 kDa fragment and the conversion of the inactive caspase-3 precursor to the 17 kDa subunit (Figure 7b). The disappearance of the 32 kDa caspase zymogen was more pronounced in adenosine-treated than in ATP-treated Li-7A cells. These results support the conclusion that it is the metabolite of ATP, adenosine, rather than ATP *per se* that causes the cell death of Li-7A cells.

To further establish that adenosine-induced cell death in Li-7A cells is caspase-3-dependent, the effect of the caspase-3 inhibitor Z-DEVD.fmk was tested. Figure 8 shows that 200 μM Z-DEVD.fmk effectively inhibited both ATP- and adenosine-induced cell death by 84%. On the other hand, 200 μM Z-FA.fmk, a structural analog of Z-DEVD.fmk in which the amino-acid sequence DEVD has been replaced, and ALLN, a proteasome inhibitor, were ineffective. Taken together, these data are consistent with the conclusion that caspase-3 activation is required for adenosine-mediated apoptosis of Li-7A cells.

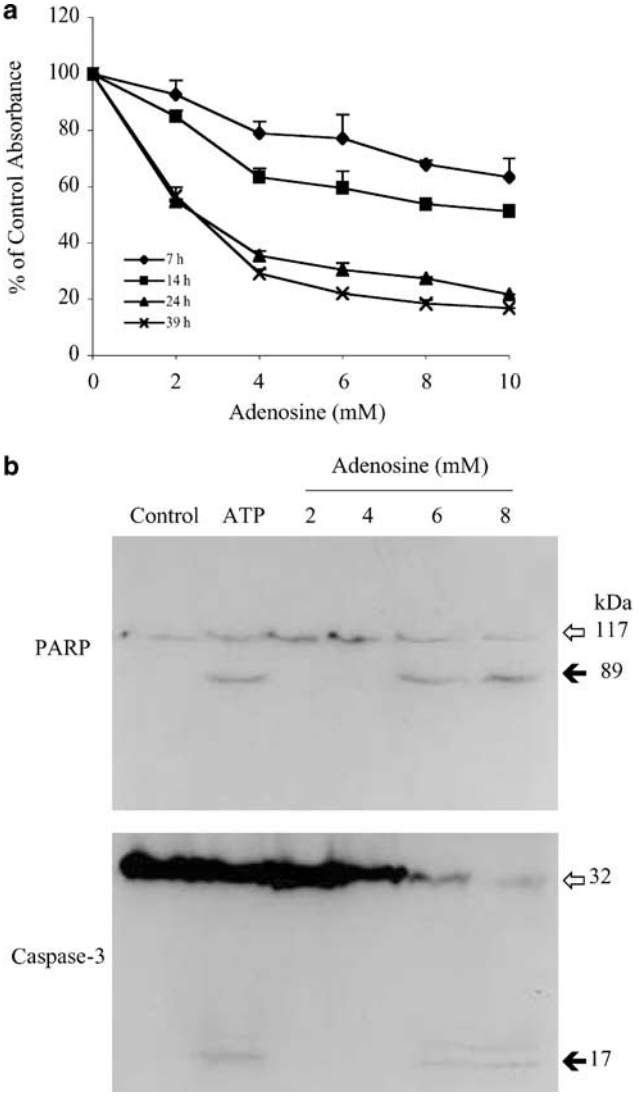

Figure 7 Extracellular adenosine inhibits cell growth and activates caspase-3 in Li-7A cells. (a) Cells were treated with different concentrations of adenosine for 7, 14, 24, and 39 h. Cell viability was determined by the MTT assay. Results are expressed as percentages of cell growth relative to untreated controls. Data are averages ± s.d. of triplicate determinations. (b) Li-7A cells were incubated for 15 h in the absence and presence of the indicated concentrations of adenosine and 6 mM ATP. Following SDS–PAGE of cellular lysates, the amounts of full-length and cleaved PARP and caspase-3 protein levels were determined by Western blot analysis.

Effect of adenosine deaminase and nucleoside transport inhibitors on ATP- and adenosine-induced cell death

To demonstrate that apoptosis of Li-7A cells is induced specifically by extracellular adenosine, we investigated the effects of inhibitors of adenosine deaminase and adenosine uptake on adenosine-induced cytotoxicity. Li-7A cells were incubated with ATP or adenosine in the absence or presence of adenosine deaminase inhibitors EHNA and coformycin. Figure 9 shows that 300 μM EHNA had no effect on cytotoxicity induced by either ATP or adenosine, whereas equal concentration of coformycin, a more potent adenosine deaminase inhibitor, significantly enhanced the cytotoxic

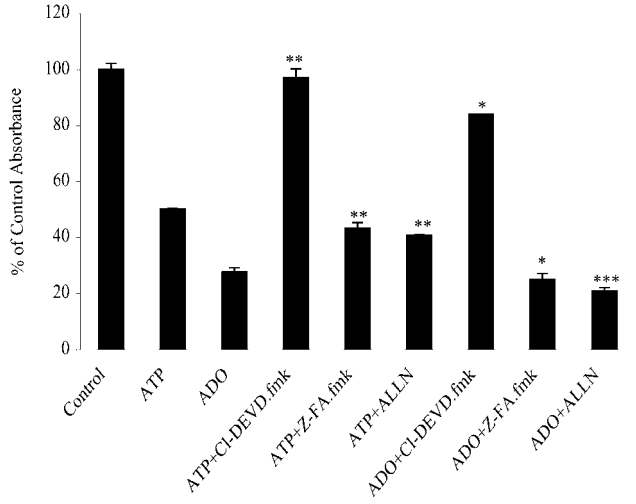

Figure 8 ATP- and adenosine-induced cytotoxicity is inhibited by caspase-3 inhibitor. Li-7A cells were incubated at 37°C in the absence or presence of 350 μM caspase-3 inhibitor Cl-DEVD.fmk, the inactive analog Z-FA.fmk, and a proteasome inhibitor, ALLN. After 2 h, 6 mM ATP or adenosine were added and incubation was carried out for an additional 22 h. Cell viability was determined by the MTT assay. Data are presented as mean percentage of basal ± s.d. of four replicates; $*P<0.05$; $**P<0.01$; $***P<0.001$ (compared with appropriate control values).

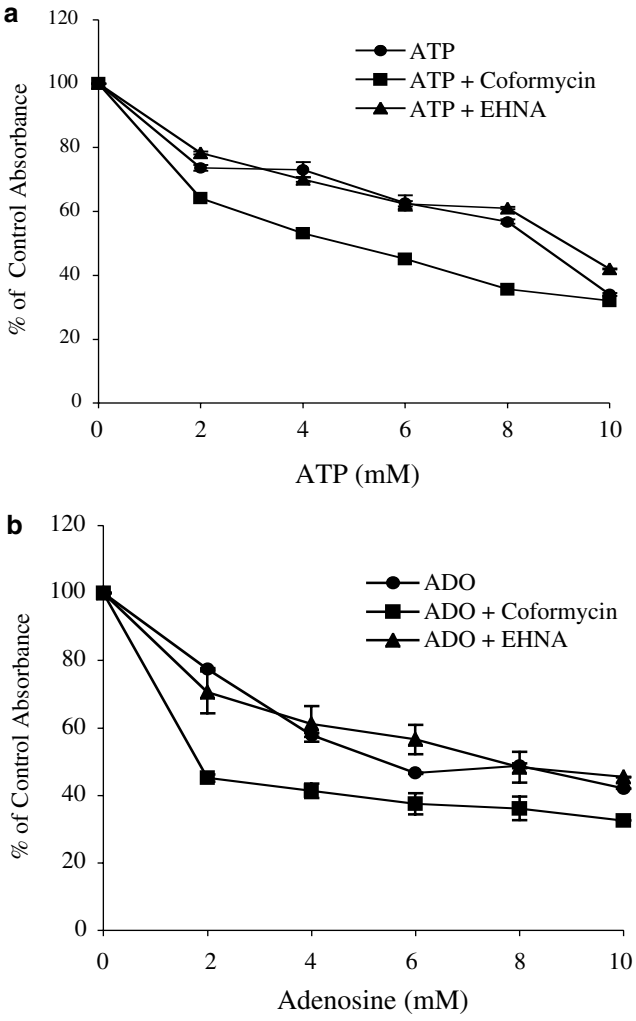

Figure 9 Effect of adenosine deaminase inhibitors on ATP- and adenosine-induced cytotoxicity in Li-7A cells. Li-7A cells were incubated with 6 M different concenteratins of ATP (a) or adenosine (b) in the absence or presence of 300 μM coformycin or EHNA. After 48 h, cell viability was determined by the MTT assay. Results are expressed as percentages of cell growth relative to untreated controls. Data are averages ± s.d. of triplicate determinations.

effects of ATP and adenosine. This observation indicates that the cytotoxic effects of ATP and adenosine on Li-7A cells were mediated by adenosine, and not its deaminated metabolite inosine.

It has been shown that adenosine can exert its cytotoxic effect intracellularly after it is transported into cells (Chow *et al*., 1997; Schrier *et al*, 2001; 2002). If this is the case, then adenosine-induced cytotoxicity in Li-7A cells will be abolished by the nucleoside transport inhibitor dipyridamole, or another substrate of the transporter, uridine. We found that neither dipyridamole nor uridine had any effect on adenosine-induced cytotoxicity in Li-7A cells (data not shown), supporting the conclusion that the cytotoxic effect is exerted by extracellular adenosine.

Effect of adenosine receptor agonists and antagonists on ATP- and adenosine-induced cell death of Li-7A cells

To determine whether the apoptotic effect of adenosine was mediated by a specific adenosine receptor, we tested several adenosine receptor agonists. N6-cyclohexyladenosine (CHA), CPCA, and 2-C1-IB-MECA are selective agonists for the Al, A2, and A3 receptors, respectively. At concentrations of 300 μM, CHA and CPCA did not induce cell death. However, in the concentration range of 100–300 μM, the adenosine A3 receptor agonist 2-C1-IB-MECA induced cell death (Figure 10a). To further confirm that adenosine-induced cell death in Li-7A cells is mediated by the A3 receptor, various adenosine receptor antagonists were tested. DPCPX, ZM241385, alloxazine, and MRS1220 are selective antagonists for the Al, $A2_A$, $A2_B$, and A3 receptors, respectively. Figure 10b shows that DPCPX, ZM241385, and alloxazine did not block adenosine-induced cell death at 200 μM. Only MRS 1220, a human A3 receptor-selective antagonist, partially inhibited cell death induced by either adenosine or the A3 receptor agonist Cl-IB-MECA. We conclude that the cytotoxic effect of adenosine on Li-7A cells is mediated by the A3 receptor.

RT–PCR of human adenosine receptors and $P2X_7$ receptor of Li-7A cells

The presence of the A3 receptor in Li-7A cells can also be demonstrated by RT–PCR. However, transcripts of the Al, $A2_A$, and $A2_B$ receptors are present as well (Figure 11a). Interestingly, we also detected the presence of the $P2X_7$ receptor transcript in Li-7A cells by RT–PCR. A 1.8 kb PCR product was obtained using the forward primer directed to bases 85–106 and reverse primer to bases 1860–1885 (Figure 11b, lane 4), and a 1.35 kb product was obtained using forward primer directed to bases 85–106 and reverse primers directed to bases 1321–1440 (Figure 11b, lane 3). The amplified partial cDNAs were subcloned and their sequences were found to be identical to that reported for the same region

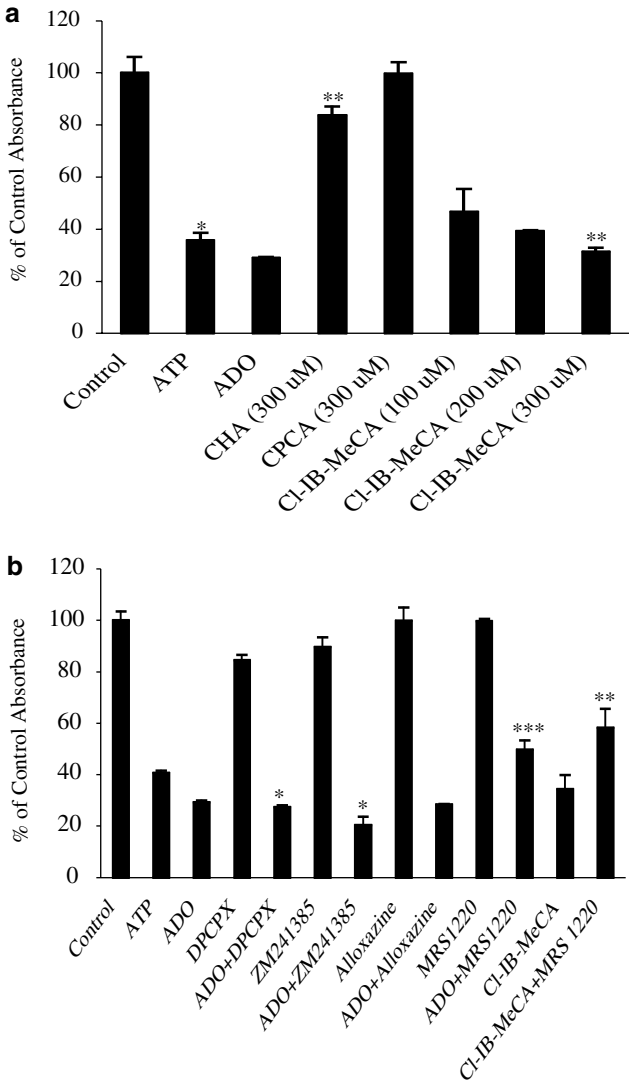

Figure 10 Effect of adenosine receptor agonists and antagonists on the growth of Li-7A cells. (a) Effect of adenosine receptor agonists on growth of Li-7A cells. ATP and adenosine were added at 6 mM. The concentrations of Al agonists (CHA) or the A2 agonist (CPCA) were 300 μM. The concentrations of A3 agonist (Cl-IB-MECA) used were 100, 200, and 300 μM. (b) Effect of adenosine antagonists on adenosine- and Cl-IB-MECA-induced cytotoxicity in Li-7A cells. Adenosine was added at 6 mM and Cl-IB-MECA was added at 100 μM. The antagonists used were DPCPX (A1), ZM241385 ($A2_A$), alloxazine ($A2_B$), and MRS 1220 (A3), each at a concentration of 100 μM. After 39 h, cell viability was determined by the MTT assay. Data are presented as mean percentage of basal $\pm$ s.d. of four replicates, $*P<0.05$; $**P<0.01$; $***P<0.001$ (compared with appropriate control values).

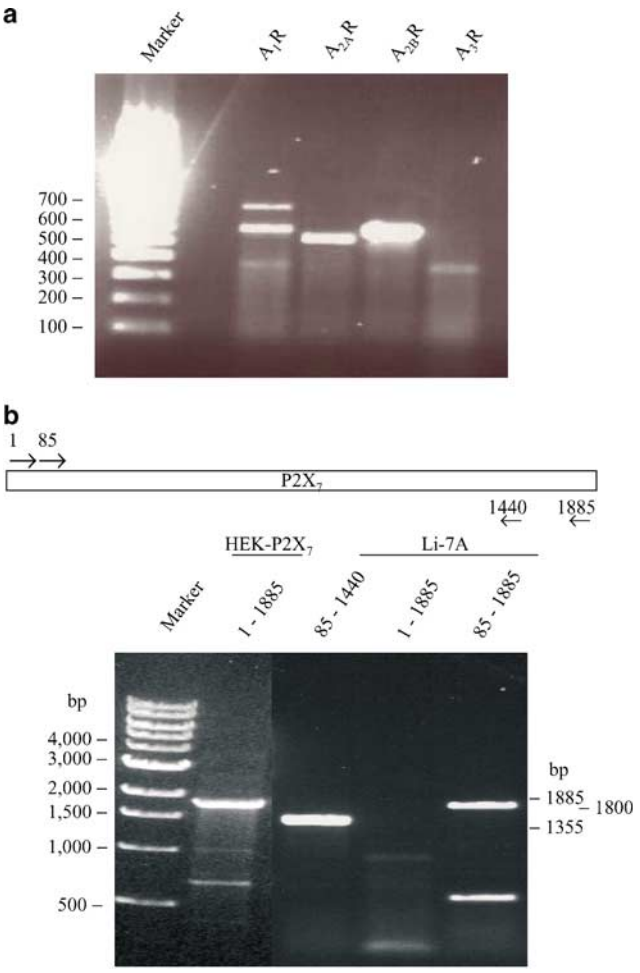

Figure 11 Expression of adenosine and $P2X_7$ receptors in Li-7A cells. Total RNA was isolated from Li-7A cells and reverse transcribed. Equal amounts of cDNA were used as templates in PCR reactions with primers specific for human adenosine and $P2X_7$ receptors. The PCR products were analyzed by agarose gel electrophoresis. Veracity of the amplified products was confirmed by cloning the PCR products into pcDNA3.1, followed by DNA sequencing. (a) Primers used were specific for the human adenosine receptors. Lengths of the different PCR products are: 606 bp for Al receptor; 535 bp for $A2_A$ receptor; 596 bp for $A2_B$ receptor; 361 and bp for A3 receptor. (b) Primers used were specific for the human $P2X_7$ receptor. Primer pairs used in the PCR reactions are indicated in the scheme. The full-length $P2X_7$ cDNA was obtained from HEK cells stably transfected by the P2X7 receptor cDNA (lane 2), but not from Li-7A cells (lane 4).

of the human $P2X_7$ receptor (Rassendren *et al*., 1997). On the other hand, we failed to obtain a product corresponding to the full-length cDNA in RT–PCR reaction using the 5′-terminal forward primer directed to bases 1–23, designed from the same sequence (Figure 9b, lane 2), while an $\sim$1.9 kb PCR product could be obtained using cDNA prepared from HEK cells stably transfected with $P2X_7$ cDNA (HEK-$P2X_7$ cells) (Figure 9b, lane 1). These results suggest that a splice variant of the $P2X_7$ receptor is present in Li-7A cells. Its functionality remains to be determined.

Discussion

In this report, we examined the effect of extracellular ATP on the human hepatoma cells Li-7A. Rozengurt *et al*. (1977) first reported that exogenous ATP caused permeability changes in the membranes of transformed cultured cells. Subsequently, it was shown that this is not a general property of all transformed cells (Heppel *et al*., 1985). Nevertheless, ATP-induced changes in membrane permeability are most likely related to its cytotoxic effects on cultured tumor cells (Rapaport, 1983; Chahwala & Cantley, 1984; Fang *et al*., 1992; Spungin & Friedberg, 1993; Vandewalle *et al*., 1994), and has been exploited in the treatment of tumor-bearing murine hosts (Rapaport, 1988).

Based on our current understanding of signal transduction of extracellular ATP, plasma membrane permeability changes are mediated by the P2X receptors, which are ligand-gated ion channels. Two subtypes of the P2X receptors, $P2X_7$ and $P2X_1$, have been implicated in apoptosis (Chvatchko *et al*., 1996; Chow *et al*., 1997; Wen *et al*., 2003). $P2X_7$ receptor is unique among the P2X receptors in that its activation results in large pores in the membrane that permit the fluxes of molecules of up to 900 Da. This receptor is most certainly involved in dye uptake of ATP-treated cells (Surprenant *et al*., 1996; Rassendren *et al*., 1997; Kim *et al*., 2001). Recently, we demonstrated the involvement of the $P2X_7$ receptor in ATP-induced apoptosis in HEK293 cells stably transfected with the human $P2X_7$ receptor cDNA (Wen *et al*., 2003). We showed that the apoptotic response of $P2X_7$-HEK cells was only induced by ATP, while ADP and AMP were ineffective. We further showed that ATP-induced cell death could be prevented by two $P2X_7$ receptor antagonists, that is, oxATP and KN62. In contrast to the HEK-$P2X_7$ cells, the present study showed that cell growth inhibition of Li-7A cells was induced not only by extracellular ATP, but also extracellular ADP and AMP (Figure 5). Furthermore, the cytotoxic effect of extracellular ATP was not diminished by the $P2X_7$ receptor antagonists. Since extracellular adenine nucleotides could be degraded to adenosine by the combined actions of several ectonucleotidases, these results indicated that adenosine might be the cytotoxic agent of Li-7A cells. HPLC analysis revealed that indeed the majority of the ATP and ADP added to the media were degraded to adenosine by the cells (Figure 6). The time required for adenosine production from ATP hydrolysis may explain the length of time (24–39 h) needed for the manifestation of apoptosis.

Adenosine has been shown to inhibit the growth of several types of cells (Chow *et al*., 1997; Schrier *et al*., 2001; 2002). There are two different underlying mechanisms of the growth-inhibitory actions of adenosine. Adenosine can exert its effect extracellularly, which is mediated by the adenosine receptors, as shown for HL-60 leukemia cells (Kohno *et al*., 1996a). Alternatively, adenosine causes cell growth inhibition after being transported into the cells, bringing about pyrimidine starvation (Lee & Jarvis, 1988; Pajor & Wright, 1992). To distinguish between these two possibilities, we conducted experiments in the presence of the nucleoside transporter inhibitor dipyridamole. Dipyridamole is expected to abolish the cytotoxic effect of adenosine on Li-7A cells if the nucleoside affects intracellular nucleotide metabolism. We found that dipyridamole is ineffective in blocking growth inhibition by adenosine, indicating that its effect is mediated by one of the adenosine receptors. This conclusion was supported by the finding that the A3 adenosine receptor agonist Cl-IB-MECA was effective in inhibiting Li-7A cell growth at concentrations (100 μM) significantly lower than that required for adenosine or the adenine nucleotides (2–6 mM) (Figure 10a, b). Furthermore, the cytotoxic effect of Cl-IB-MECA was partially blocked by the specific A3 receptor antagonist MRS 1220 (Figure 10b).

It is interesting that the A3 receptor is specifically involved in the apoptotic response of Li-7A cells to adenosine or ATP, since RT–PCR demonstrated the presence of all the four subtypes of the adenosine receptor, as well as a splice variant of the $P2X_7$ receptor in these cells. The A3 receptor has also been shown to be involved in the apoptosis of astrocytes (Iorio *et al*., 2002), cardiac myocytes (Shneyvays *et al*., 1998), and HL-60 leukemia cells (Kohno *et al*., 1996a). As for the truncated $P2X_7$ receptor, reported here for the first time, it remains to be determined if it codes for a functional receptor protein.

While $P2X_7$ receptor mediates the apoptotic action of extracellular ATP in HEK-$P2X_7$ cells and A3 receptor mediates the apoptotic effect in Li-7A cells, their effects on the intracellular mediators of apoptosis, that is, caspase and PARP, are similar. In both cases, there is substantial increase in caspase-3 activation, as evidenced by proteolysis of procaspase-3 and the caspase substrate PARP. Similar findings have been reported for adenosine-induced cell death of human HL-60 leukemia cells, human U-937 lymphoma cells, and mouse neuroblastoma cells (Schrier *et al*., 2001; 2002). In Li-7A cells, the crucial roles of caspase-3 activation and PARP cleavage in adenosine-induced cell death were supported by evidence that the caspase-3 inhibitor Cl-DEVD.fmk completely abolished adenosine-induced Li-7A cell death.

In summary, we demonstrated that the cytotoxic effect of extracellular ATP and other adenine nucleotides on the human Li-7A hepatoma cells is mostly due to the generation of adenosine by ectonucleotidases. The cytotoxic effect of adenosine is mediated by the A3 adenosine receptor. Ligand binding to the receptor activates caspase-3, leading to apoptosis.

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