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Extracellular ATP Induces Apoptotic Signaling in Human Monocyte Leukemic Cells, HL-60 and F-36P

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Extracellular adenosine 5'-triphosphate (ATP) affects the function of many tissues and cells. To confirm the biological activity of ATP on human myeloid leukemic cells, F-36P and HL-60, cells were treated with a variety of concentrations of ATP. The stimulation with extracellular ATP induced the arrest of cell proliferation and cell death from the analysis of Annexin-V staining and caspase activity by flow cytometry. The Annexin-V positive cells in both cell lines were dramatically increased following ATP stimulation. The expression of P2 purinergic receptor genes was confirmed, such as P2X1, P2X4, P2X5, P2X7 and P2Y1, P2Y2, P2Y4, P2Y5, P2Y6, P2Y11 in both leukemic cell lines. Interestingly, ATP induced intracellular calcium flux in HL-60 cells but not in F-36P cells, as determined by Fluo-3 AM staining. Cell cycle analysis revealed that ATP treatment arrested both F-36P and HL-60 cells at G1/G0. Taken together, these data showed that extracellular ATP *via* P2 receptor genes was involved in the cell proliferation and survival in human myeloid leukemic cells, HL-60 and F-36P cells by the induction of apoptosis and control of cell cycle. Our data suggest that treatment with extracellular nucleotides may be a novel and powerful therapeutic avenue for myeloid leukemic disease.

Key words: Acute myeloid leukemia, MDS, ATP, Apoptosis, Calcium signaling, P2 receptor

INTRODUCTION

Adenosine 5'-triphosphate (ATP) is known to be an important molecule in both the intracellular and extracellular microenvironments of the cells. Several studies on the biologic roles of extracellular ATP in different cell types have revealed its involvement in cell proliferation, differentiation, chemotaxis, cytokine secretion, release of lysosomal consitituents, generation of reactive oxygen intermediates (ROI) and cell death effects (Di Virgilio *et al.*, 2001). The effects of extracellular ATP are mediated by specific plasma membrane receptors, which are grouped of two families: the G-protein coupled P2Y receptors (P2YRs), and the ligand-gated ion channels P2X receptors (P2XRs).

Typically, P2YRs are G-protein-coupled receptors containing seven transmembrane domains. Eleven P2Y receptors have been identified to date (P2Y1-11) and eight have been cloned so far. They have been shown to be involved in signal transduction via the activation of phospholipase C or stimulation/inhibition of adenylate cyclase. On the other hand, P2X receptors are ion-gated channels, which lead to the influx of monovalent and divalent cations and which change the permeability of plasma membrane. Seven P2XRs have been identified and cloned so far (Burnstock *et al.*, 1985; Burnstock, 1997; Ralevic *et al.*, 1998; Von kugelgen *et al.*, 2000).

The expression of two P2 receptor family members has been detected in human peripheral blood leukocytes. P2Y1 receptor was identified in peripheral leukocytes, endothelial cells, HL-60 cells, K562 cells and Dami cells, but not in U937 cells. P2Y2 receptor mRNA was detected in peripheral blood leukocytes, endothelial cells, U937 cells, HL-60 cells, but not in K562 cells (Jin *et al.*, 1998; Di Virgilio *et al.*, 2001). Extracellular effects of nucleotides such as ATP were initially recognized in the contexts of smooth muscle contraction, neurotransmission, regulation of cardiac function, and platelet aggregation (Holton *et al.*, 1959; Burnstock *et al.*, 1976; Furchgott *et al.*, 1980; De Mey *et al.*, 1981). However, over the last ten years, the intercellular mediator role of these molecules has also been investigated in hematopoietic cells such as thymocytes,

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peripheral T lymphocytes, mast cells, monocytes, macrophages and phagocytic cells of the thymic reticulum. There are two major sources of extracellular ATP in the bloodstream, deriving from vascular injury and from the degranulation of platelets, both of which release stored ATP and ADP. These extracellular nucleotides can act on a number of blood cells to trigger physiological responses. In human neutrophils and macrophages, ATP activates phospholipase C and eventually increases intracellular calcium in monocytes. Intracellular calcium mobilization by ATP was demonstrated in T-leukemic cells, and was shown to be mediated by P2Y receptor. Furthermore, vascular endothelial cells are regulated by nucleotides released from platelets, neurons and damaged cells, resulting in enhanced binding of neutrophils (Wilkinson *et al.*, 1993; Kunapuli *et al.*, 1998). Macrophages express receptors specific for ATP, which inhibit Fc receptor-mediated phagocytosis (Steinberg *et al.*, 1987; Greenberg *et al.*, 1988). Moreover, in human and murine dendritic cells, P2X7 mediates cytokine release and might also participate in antigen presentation (Mutini *et al.*, 1999; Ferrari *et al.*, 2000). Leukemia results from defects in cell cycle control, resulting in aggressive cell proliferation. Most treatments for this type of cancer seek to remove leukemic cells via cytotoxic drugs or radiation therapy.

Purines including ATP are known to have cytotoxic properties. Current evidence suggests that purine nucleotides induce cell death both apoptosis or necrosis in the thymocytes, promyelocytic leukaemia HL-60 cells, embryonic neurons, and endothelial cells (Kizaki *et al.*, 1990; Tanaka *et al.*, 1994; Wakade *et al.*,1995; Dawicki *et al.*, 1997).

In this report, in order to determine the effects of extracellular ATP on the myeloid leukemic cell lines F-36P and HL-60, we have investigated its effects on cell proliferation activity, cell cycle, and apoptosis. Both cell lines, HL-60 and F-36P, which were used for functional assay of ATP, have different origin and characteristics. HL-60 cell was derived from acute myeloid leukemia (AML, FAB M2) patient, but F-36P cell was established from myelodysplastic syndrome (MDS) patient showing acute myeloid leukemia (AML, FAB M6) and cytokine dependency, such as GM-CSF or IL-3 for cell growth and survival (Gallagher *et al.*, 1979; Chiba *et al.*, 1991). We observed that extracellular ATP caused the arrest of proliferation in both cell lines, resulting from severe apoptotic signaling via intracellular caspase activation and also via cell cycle arrest at G0/G1 stage. Interestingly, stimulation with extracellular ATP had distinct effects on the two cell lines: intracellular calcium was induced in the HL-60 cell line, but not in the F-36P cell line. Our results indicate that the activation of P2X and P2Y receptors by extracellular ATP stimulation causes the regulation of myeloid leukemic cells through the retardation of cell proliferation by cell cycle arrest and apoptosis. We suggest that these findings may be applied to the clinical management of myeloid leukemia.

MATERIALS AND METHODS

Cells and cell culture

F-36P cells, a human IL-3-dependent myeloid leukemia cell line established from a patient with myelodysplastic syndrome, were obtained from Riken Cell Bank (Tsukuba, Japan). F-36P cells were cultured in RPMI (GIBCO/BRL, NY, USA) supplemented with 10% fetal bovine serum (GIBCO/BRL, NY, U.S.A.) in the presence of 5 ng/mL rhIL-3. HL-60 cells, a promyelocytic leukemia cell line, were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (GIBCO/BRL, NY, U.S.A.) containing 10% fetal bovine serum. (GIBCO/BRL, NY, U.S.A.). Both lines were maintained at 37°C in humidified 5% CO_2 atmosphere. Cytokines were purchased from R&D systems (Minneapolis, U.S.A.).

Cell proliferation assay

The MTT assay is a colorimetric method using metabolic competence as an indicator of cell viability. This method assesses the ability of the cell to convert 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Roche, Germany) to formazan. 2×10^4 cells per well were plated in 96-well plates in a final volume of 100 μL culture medium per well and were treated with ATP (Sigma, St .Louis, MO, U.S.A.) at the concentrations of 0 mM, 0,1 mM, 1 mM, 10 mM, 50 mM. After treatment with ATP, the cells were cultured for up to 4 days and 10 μL of the MTT labeling reagent (final concentration; 0.5 mg/mL) was added to each well on the indicated time points. The plates were incubated for 4 h at 37°C and then 100 μL of solubilization solution was added to each well for over 12 h. The absorbance was measured with immunoreader at 595 nm.

Apoptotic cell death assay

Cells (1×10^5) were plated in 24-well plates in a final volume of 500 μL culture medium per well. Cells were treated with ATP at concentrations ranging from 0-50 mM. After treatment of ATP, the cells were cultured for up to 4 days and were harvested at different time points. Harvested cells were washed twice with PBS and the cells were resuspended in binding buffer to a final concentration of 1×10^6 cells/mL. Annexin-V-PE (BD Pharmingen, CA, U.S.A.), binding to phosphatidyl serine (PS) exposed to external side of plasma membrane during the early stage of apoptosis, and PI (Sigma, St. Louis, MO), which is used

to distinguish apoptotic cells (Annexin V positive and PI negative) from necrotic cells (Annexin V negative and PI positive), were added and the cells were kept in dark at room temperature for 15 minutes. The samples were analyzed by flow cytometry. We also detected intracellular caspase activity by FITC-conjugated ApoStat reagent (R&D systems, U.S.A.), which is pan-caspase inhibitor of caspase activation by coupling caspase-specific peptides to certain aldehyde, nitrile or kitone compounds. It allows for intracellular detection upon binding to active caspase enzymes (Garcia-Calvo *et al.*, 1998). F-36P cells cultured under the same conditions as for the annexin and PI apoptosis assay were collected, washed, resuspended with PBS, and then stained with ApoStat at 37°C for 15 minutes. Cells samples were washed once with PBS to remove unbound reagent and analyzed by FACSvantageSE flow cytometry (Becton Dickinson, San Jose, CA, U.S.A.).

Measurement of intracellular Ca^{2+}

Changes in the intracellular free Ca^{2+} concentration were measured with the fluorescent indicator Fluo-3 AM (Sigma, St. Louis, MO, U.S.A.) by flow cytometry. F-36P cells and HL-60 cells were harvested and washed three times with HBSS and resuspended with HBSS (GIBCO/BRL, NY, U.S.A.) containing 4 mM Fluo-3 AM and 0.01% pluronic F-127 (Sigma, St. Louis, MO, U.S.A.) in a final concentration of 5×10^6 cells/mL. Incubation was performed at 37°C for 30 minutes. Cells were then washed three times with saline solution and incubated for 30 minutes at 37°C for deestification of dye in cells. Ca^{2+} changes were measured after stimulation with 0 mM, 1 mM and 10 mM ATP by FACSvantage flow cytometry. Emission was detected at 525 nm.

Cell cycle analysis

Cells (5×10^5) were treated with various concentration of ATP and cultured for a day. Cells were harvested, washed, and resuspended with cold PBS. The cells were fixed in 100% ethanol at 4°C for 1 h, washed and resuspended in PBS with RNAse (50 ug/mL). Cellular DNA was stained with PI (50 ug/mL) for 30 minutes and cell cycle data were acquired using FACSvantage flow cytometry and subsequent cell cycle analysis was performed using ModFit software.

Expression of P2X and P2Y receptor family members by RT-PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen, California, U.S.A.) according to the manufacture's instructions and five micrograms of RNA were

Table I. P2Y receptor families specific primers used

Primer	Strand	Sequence	Correspond to nt
ACTIN	S AS	5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3' 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3'	294bp
P2X1	S AS	5'-CTG TGA AGA CGT GTG AGA TCT TTG G-3' 5'-TTG AAG AGG TGA CGG TAG TTG GTC-3'	463bp
P2X4	S AS	5'-GAG ATT CCA GAT GCG ACC-3' 5'-GAC TTG AGG TAA GTAGTG G-3'	296bp
P2X5	S AS	5'-TCG ACT ACA AGA CCG AGA AG-3' 5'-CTT GAC GTC CAT CAC ATT G-3'	596bp
P2X7	S AS	5'-AAC ATC ACT TGT ACC TTC C-3' 5'-TGT GAA GTC CAT CGC AGG-3'	675bp
P2Y1	S AS	5'-CTA CAT CTT GGT ATT CAT CAT CGG-3' 5'-GAG ACT TGC TAG ACC TCT TGT CAC C-3'	772bp
P2Y2	S AS	5'-CTC TAC TTT GTC ACC ACC AGC GCG-3' 5'-TTC TGC TCC TAC AGC CGA ATG TCC-3'	637bp
P2Y4	S AS	5'-CCA CCT GGC ATT GTC AGA CAC C-3' 5'-GAG TGA CCA GGC AGG GCA CGC-3'	425bp
P2Y5	S AS	5'-TGG TTA ACT GTG ATC GGA GG-3' 5'-AGT CAC TTC TCC TGA CAG ACC-3'	520bp
P2Y6	S AS	5'-CGC TTC CTC TTC TAT GCC AAC C-3' 5'-CCA TCC TGG CGG CAC AGG CGG C-3'	365bp
P2Y10	S AS	5'-CAT CAG TCT TCA AAG GTG C-3' 5'-GGA CAA CTG CTA ATG ATG G-3'	456bp
P2Y11	S AS	5'-CAG CGT CAT CTT CAT CAC C-3' 5'-GCT ATA CGC TCT GTA GGC-3'	273bp

reverse transcribed to cDNA using SuperScript™ (Life technologies). The preparation of cDNA was performed for 1 h at 42°C and was stopped by heating for 5 minutes. As a control for cDNA synthesis, actin-PCR was performed. PCR reactions were performed in 25 μL final volumes containing 100 ng cDNA, 10 pM of each specific primer (Table I), 0.2 mM dNTP and Taq DNA polymerase. PCR cycling conditions were as follows: for P2X1 and P2Y1 receptor 45s at 94°C, 45s at 64°C, 1 min 30s at 72°C; for P2X4, P2X7 and P2Y5 receptors: 45s at 94°C, 45s at 54°C, 1 min at 72°C; for P2X5 receptor: 45s at 94°C, 45s at 57°C, 45s 30s at 72°C; for P2Y2 receptor: 45s at 94°C, 45s at 67°C, 1 min 30s at 72°C; for P2Y4 and P2Y6 receptor 45s at 94°C, 45s at 56.5°C,1 min at 72°C; for P2Y10 receptor: 45s at 94°C, 45s at 52°C, 1 min at 72°C; for P2Y11 receptor: 45s at 94°C, 45s at 57°C, 1 min at 72°C; for b-actin: 45s at 94°C, 45s at 67°C, 1 min 30s at 72°C. All PCR were carried out for 35 cycles (except 25 cycles for human β-actin) and included an initial 3 minute denaturation step at 94°C and a final 10 minute extension at 72°C (Berchtold *et al.*, 1999).

RESULTS

Effect of extracellular ATP on cell proliferation of human leukemic cells

Extracellular ATP affects the function of many tissues and cell types and has also been shown to exert an immunomodulatory function in B and T cells. In this study, to examine the biological effects of extracellular ATP in human myeloid cells, we have used a human myeloid leukemic cell line, F-36P, derived from a myelodysplastic syndrome patient, which was depends on IL-3 or GM-CSF for survival, as well as a human promyelocytic leukemia cell line, HL-60. The effect on cell proliferation of treatment with a range of doses of extracellular ATP for up to four days in these cell lines was determined. Both cell lines showed a significant decrease in proliferation in the presence of high concentrations of ATP, especially at 10 mM and 50 mM (Fig. 1). As shown in Fig. 1 A, there are no significant differences in effects at 0.1 mM or 1 mM compared to the control cells in F-36 P cells. However, the HL-60 cell line appears to be more sensitive to ATP; an effect is seen at 1 mM. The data above demonstrate that extracellular ATP negatively regulates cell survival and/or cell proliferation in F-36P and HL-60 human myeloid leukemic cell lines.

Expression of P2X and P2Y receptors in F-36P and HL-60 cells

Since extracellular ATP was found to affect cell growth and proliferation in myeloid leukemia cell lines, we next tried to determine through which receptors might be acting in this case. RT-PCR was employed to assess whether the following purinergic receptor genes were expressed in these lines: P2X1, P2X4, P2X5, P2X7, P2Y1, P2Y2, P2Y4, P2Y5, P2Y6, P2Y10 and P2Y11. Of the P2Y family members, P2Y1, P2Y4, P2Y5, P2Y6 and P2Y11 were expressed at a similar level in both cell lines. P2Y2 receptor was detected only in the HL-60 cell line, and neither line expressed P2Y10. Of the P2X receptor genes tested, P2X4 and P2X5 were detected in both cell lines, but only HL-60 cells expressed P2X7. In addition, P2X1 gene was expressed at a very low level in both cell lines (Fig. 2). These expression data demonstrate that several purinergic receptor family genes are expressed in human myeloid leukemic cell lines and may mediate the effects of extracellular ATP stimulation on cell proliferation and survival.

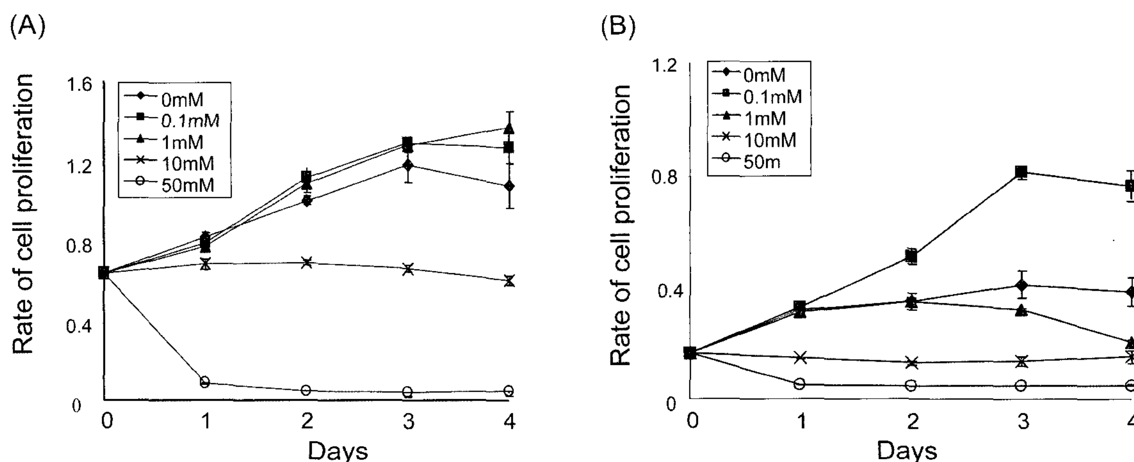

Fig. 1. The effect of ATP on survival in F-36P cells (A) and HL-60 cells (B). Cytotoxicity induced by ATP. F-36P and HL-60 cells were incubated for 0, 1, 2, 3, or 4 days in media containing ATP at the concentrations of 0 mM (◆), 0.1 mM (■), 1 mM (▲), 10 mM (x), and 50 mM (○). Cell viability was determined by the MTT assay. The results represent the mean (±S.D) of triplicate determinations.

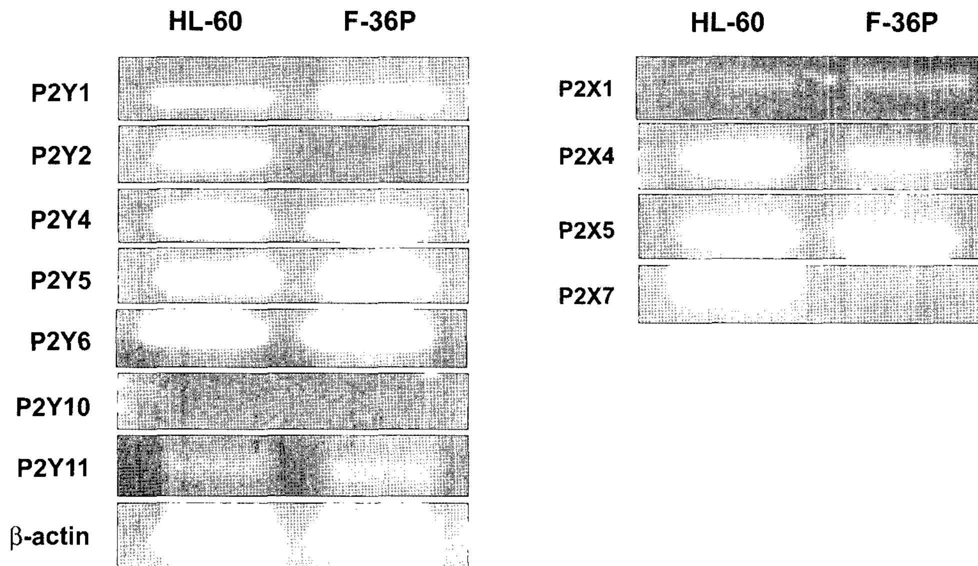

Fig. 2. Expression of P2Y and P2X receptor genes. Messenger RNA from HL-60 and F-36P cells was used for RT-PCR analysis which was performed using a pair of gene specific PCR primers for indicated P2X and P2Y genes.

Effects of extracellular ATP on intracellular Ca^{2+} flux

Most P2Y receptors couple to G proteins and activate phospolipase C, which lead to the cleavage of phosphatidyl inositol 4, 5 bisphosphate (PIP_2) into inositol 1.4-5 triphospate (IP_3), which releases Ca^{2+} from intracellular stores, thereby activating protein kinase C (PKC). Several purinergic receptor genes were found to be expressed in both myeloid leukemic cell lines, F-36P and HL-60. Therefore, we assessed whether extracellular ATP treatment would affect intracellular Ca^{2+} release, using fluorescence indicator Fluo-3AM. As shown Fig. 3, HL-60 cells responded to ATP stimulation by fluxing calcium. However, in F-36P cells, no calcium flux was detected. These data indicate that signaling initiated by stimulation with extracellular ATP has divergent effects of calcium release in these two cell lines. Their differences in response could be due to differences in purinergic receptor gene expression between the two cell lines.

Induction of apoptosis by extracellular ATP

The decrease in metabolic activity in response to ATP treatment detected by the MTT assay could be due either to growth arrest or to apoptosis. To determine whether apoptosis was a contributing factor, both human myeloid cell lines were exposed to 0, 1, 10, or 50 mM ATP for 3

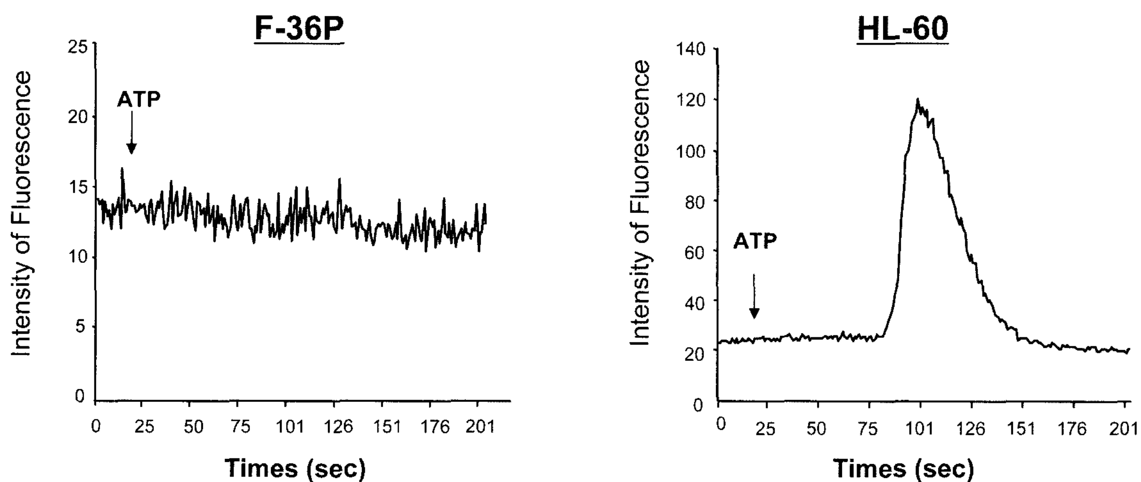

Fig. 3. Effects of extracellular ATP on cytoplasmic Ca^{2+} concentration in F-36P cells (left) and HL-60 cells (right). Cells were loaded with the Ca^{2+} indicator, Fluo-3AM, as detailed in "Material and Methods" and then stimulated with 10mM ATP. Changes in fluorescence intensity reflecting levels of free intracellular Ca^{2+} over the time are shown.

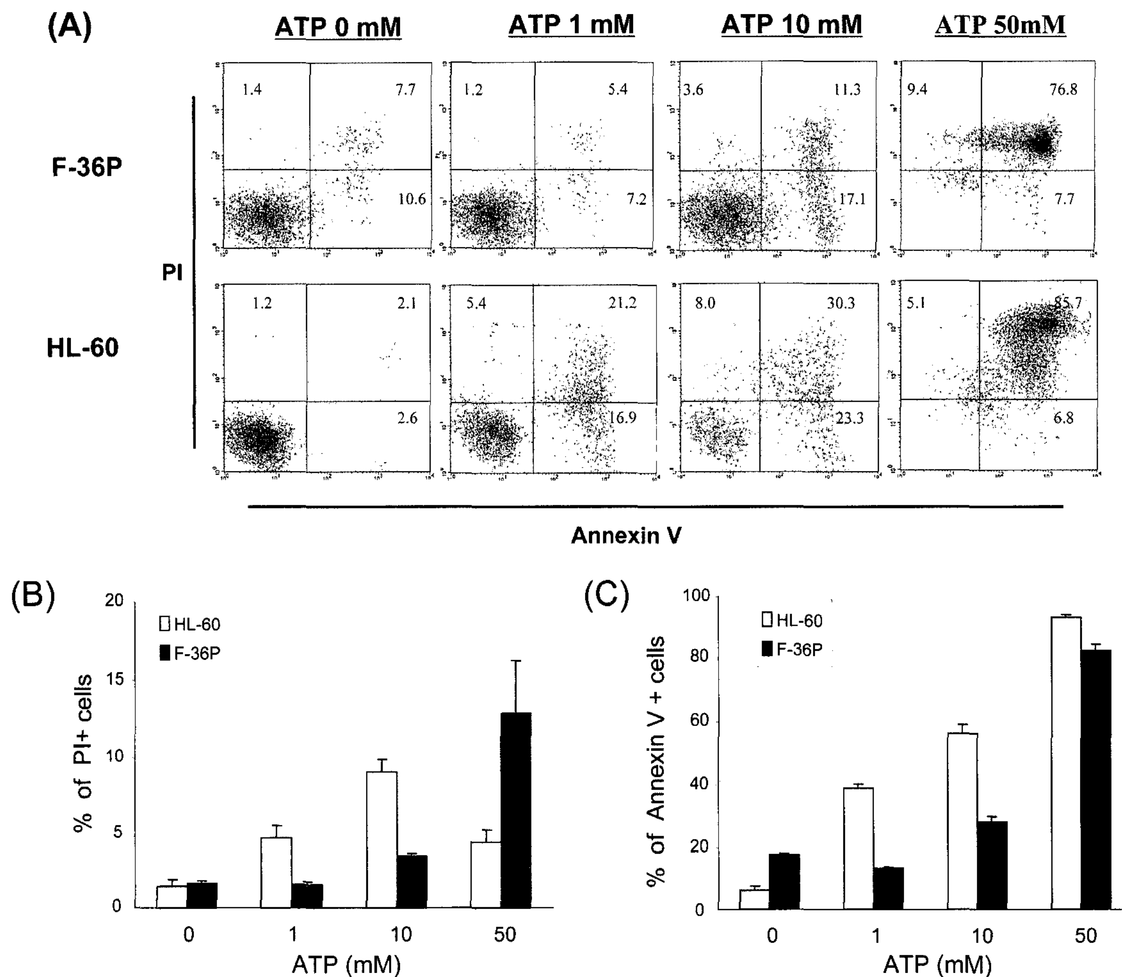

Fig. 4. FACS analyses of annexin-V-PE and PI staining cells. F-36P (upper panel) and HL-60 (lower panel), treated with ATP at the indicated concentrations, were cultured for 3 days. PS externalization and PI-permeable cells were assessed by each staining with annexin-V-PE and PI. A representative profile of flow cytometry is shown (A). The mean of percentage of apoptotic cells, PI+ and Annexin V+, in both cells was demonstrated (B, C).

days, and the percent apoptotic cells was assessed by staining with Annexin V and propidium iodide and performing flow cytometric analysis. As shown at Fig. 4, the percent of Annexin-V positive cells after 3 days was 17.6±0.7, 13.0±0.5, 28.0±1.9, 82.9±2.0% in F-36P cells, respectively. In HL-60 cells, the percentage of Annexin-V positive cells after 3 days was 6.0±1.4, 39.2±1.1, 56.3±2.9, 93.5±0.8% respectively (Fig. 4A-C).

A caspase assay using ApoStat, which can determine intracellular caspase activities by extracellular ATP stimulation *via* purinergic receptors, was performed to confirm the induction of apoptosis in the F-36P cell line. As shown in Fig. 5, in F-36P cells, the activity of caspase enzymes was increased in proportion of the concentrations of ATP. These data indicate that extracellular ATP activates intracellular caspases, resulting in severe cell apoptosis in both human myeloid leukemic cell lines.

Cell cycle arrest by extracellular ATP

Since extracellular ATP was shown to induce apoptosis in both cell lines, we further assessed whether it also exerted an effect on cell cycle. Propidium iodide was used for cell cycle analysis. In the case of cells treated with 0.1 mM and 1 mM ATP, the results were not significantly different from those of cells untreated with ATP (data not shown). However, cells exposed to 10 mM of ATP showed arrest at the G_0/G_1 cell stage (0 mM: 55.6±0.7, 10 mM: 77.6±0.9 in F-36P; 0 mM: 59.0±2.4, 10 mM: 65.0±1.1 in HL-60) in both lines, resulting in lower percentage in S phase of the cell cycle (17.32±0.5 in F-36P, 30.6±1.7 in HL-60), compared with control cells (36.47±0.7 in F-36P, 37.1±1.5 in HL-60) (Fig. 6). These data demonstrate that the extracellular ATP signaling in both human myeloid leukemic cells, in addition to inducing apoptosis, also negatively regulates cell cycle.

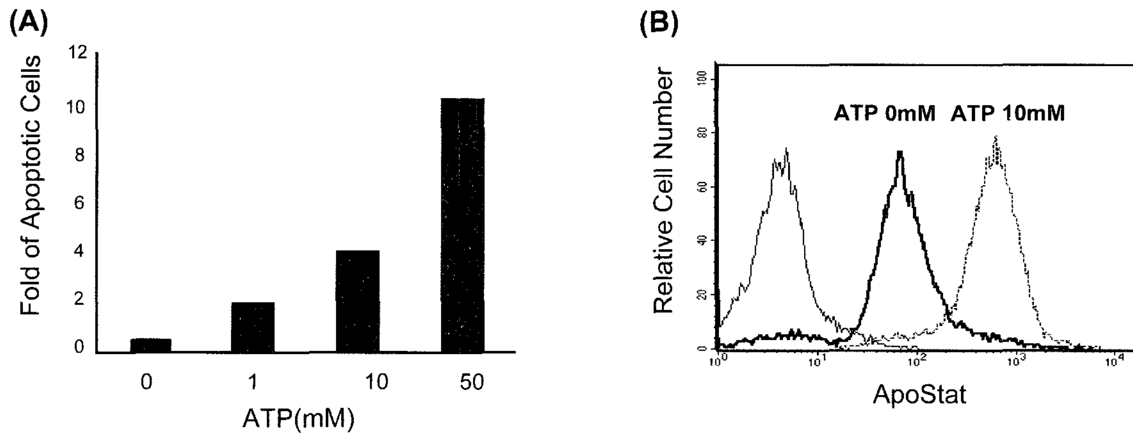

Fig. 5. Induction of intracellular caspase activation by ATP. F-36P cells were used to determination intracellular caspase activation effects resulting from stimulation with extracellular ATP. Cells were cultured at the indicated concentrations of ATP for 3 days. Cells were stained with Apostat and detected by flow cytometry. The results shown are values compared to those prior to ATP treatment (A). Flow cytometry results depicted as histograms (0 mM and 50 mM) (B).

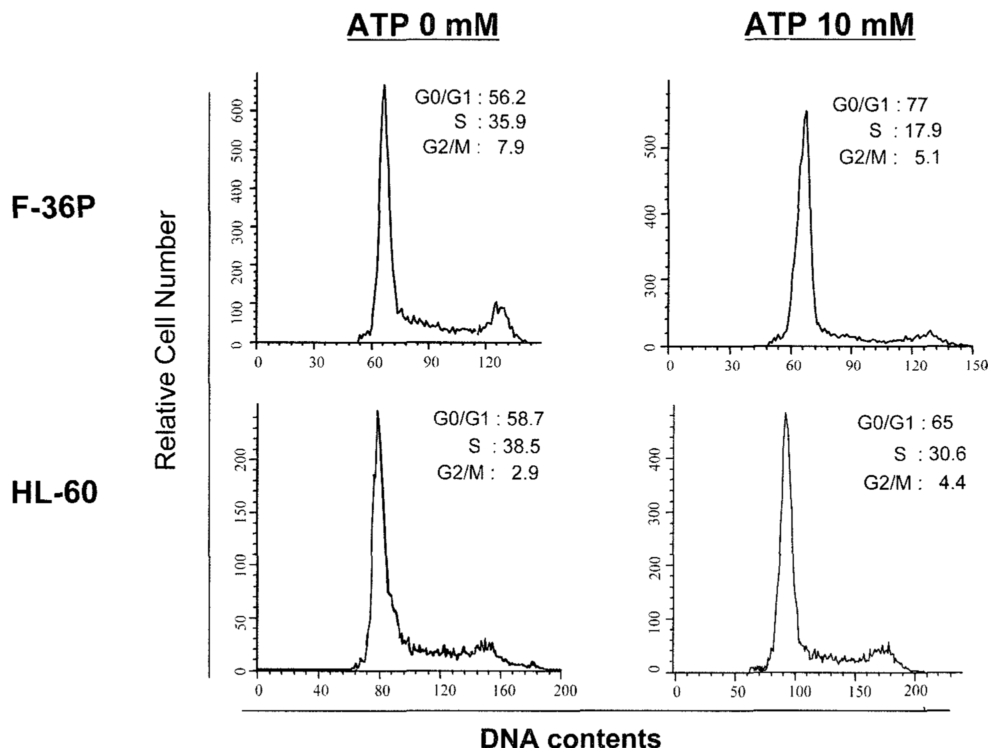

Fig. 6. Effects of extracellular ATP on cell cycles in F-36P and HL-60 cells. Cells were cultured with the indicated concentrations of ATP. After one day, the cells were stained with PI to quantitatively assess DNA content and were analyzed by flow cytometry.

DISCUSSION

Extracellular nucleotides exert a wide range of biological effects such those on platelet aggregation, neurotransmission, inflammation and muscle contraction in various cell types (Holton, 1959; Burnstock, 1976; Furchgott and Zawadzki, 1980; De Mey and Vanhoutte, 1981). Effects as diverse as proliferation, differentiation, chemotaxis, release of cytokines or lysosomal constituents, and generation of reactive oxygen or nitrogen species are elicited upon stimulation of blood cells with extracellular adenosine triphosphate (ATP). These various effects of ATP are mediated by plasma membrane P2 purinergic receptors. P2 receptors are divided into two main classes, P2X and P2Y receptors (Abbracchio and Burnstock, 1994; Barnard *et al.*, 1994). P2X receptors are ligand-

gated ion channels which mediate rapid and selective permeability to cautions such as Na^+, K^+ and Ca^{2+} (Bean, 1992). In contrast, P2Y receptors are G-protein coupled receptors. Signaling *via* P2Y receptors results in hydrolysis of PIP_2 by phospholipase C to produce diacylglycerol and inositol-1,4,5-triphosphate (IP_3), stimulating mobilization of Ca^{2+} (Harden *et al.*, 1995).

Extracellular ATP inhibits the growth of a variety of cells including human PC-3 prostate cancer cells, human CAPAN-1 pancreatic adenocarcinoma cells, human HT29 colon adenocarcinoma cells, and mouse 3T6 fibroblasts (Rapaport, 1988; Weisman *et al.*, 1988; Fang *et al.*, 1992). Human monocyte-derived dendritic cells express several P2X and P2Y receptor genes. In a biological survey of dendritic cells, ATP was found to induce the expression of the DC surface markers, CD80, CD83 and CD86, indicating a maturation promoting effect (Berchtold *et al.*, 1999). In contrast, in human monocytes, ATP is a powerful stimulus not only for caspase-1 activation but also for the externalization of mature caspase-1 subunits. Various mechanisms for effects have been proposed including activation of P2 receptors (Fang *et al.*, 1992) and pyrimidine starvation induced by adenosine derived from the extracellular breakdown of adenine nucleotides (Weisman *et al.*, 1988; Chow *et al.*, 1997).

Recently, several reports have revealed that the response elicited by extracellular ATP depends on the P2 receptor subtype expressed by the responding cell and on the intensity of stimulation. For example, all murine macrophage lines expressed P2Y receptors coupled to release of Ca^{2+} from intracellular stores and to IP_3 generation, but the individual subtypes have not been investigated in detail. While some reports were mentioned the expression of some of P2Y receptor subtype genes in human leukemic cell lines, Jurkat, LB223, U937, K562 and Dami cells (Jianguo *et al.*, 1998).

In this study, we assessed the expression of P2X genes, such as P2X1, P2X4, P2X5 and P2X7, and P2Y genes, such as P2Y1, P2Y2, P2Y4, P2Y5, P2Y6, P2Y10 and P2Y11 receptor genes in both myeloid cell lines, F-36P and HL-60. From RT-PCR analysis, P2X or P2Y receptors expression profiles on F-36P and HL-60 cells were shown very similar expression pattern. This may be explained by the fact that both cells derive from the related type of human myeloid progenitor cells derived from the development of hematopoietic stem cells into mature myeloid lineage. However, P2Y2, and P2X7 receptors genes were not expressed in F-36P cell lines compared from that of expression on HL-60 cells. This data may be suggested that P2 receptors genes during myeloid development from hematopoietic stem cells will be related with the differential functional involvement through the different expressed pattern, such as HL-60 and F-36P cells (Adrian *et al.*, 2000).

In this report, we confirmed that several different P2X and P2Y receptor genes were expressed in both cell lines with relatively different expression pattern by RT-PCR analysis. Interestingly, P2X7 receptor is known as a cytotoxic inducer in monocytes following the stimulation with ATP (Humphreys *et al.*, 2000). Recent evidence suggests that induction of lytic pore formation in cells by extracellular ATP results in cell death independently of Ca^{2+} whereas its action as a selective ligand-gated ion channel result in apoptosis due to excessive Ca^{2+} influx after prolonged or repeated activation of P2X7R. Stimulation of the receptor leads to the depletion of the intracellular K^+, which has been suggested to activate interleukin-1β-converting (ICE) enzyme and the maturation of IL-1β (Perregaux and Gabal, 1994).

We observed that P2X7 was expressed at a higher level in HL-60 cells compared to F-36P cells. When we assessed the effects of extracellular ATP on apoptosis, HL-60 cells showed relatively high apoptosis induction efficiency at a low dose of ATP, compared to F-36P cells. Furthermore, in our analysis of intracellular calcium release induced by ATP, HL-60 cells was showed a normal secretion pattern, but F-36P cell did not respond at even high dose of ATP stimulation. Those results, the high expression and high susceptibility against from Ca^{2+} signaling result in 10 fold increased Annexin + and PI+ cells even low dose of ATP stimulation in HL-60 cells compared with F-36P cells. These result implicated that these two different developmental stages of cells had slightly different cellular biological mechanism in respect to calcium release against the stimulation of ATP, because HL-60 cell was M2 stage of myeloid leukemic cell, but F-36P cells was M6 developmental stage cells from myelodysplatic syndrome (MDS) patient.

We also examined the cytotoxic effects of extracellular ATP in the human myeloid cell lines F-36P and HL-60. The type of death pathway of these tumor cells was evaluated with the aim of determining whether cell death resulted from apoptosis or necrosis mechanism. The two cell lines showed similar sensitivity rate in respect of the rate of apoptosis against the dose of extracellular ATP applied. In presence of low dose of ATP, such as 10 nM to 100 uM had no significant apoptotic effect on F-36P and HL-60 cells (data not shown). However, both tumor cells treated with even 1 mM ATP started the distinguishably high cell death induction on both cell lines. UTP showed the similar results as that of ATP (data not shown). The dose response range of ATP stimulations was roughly consistent with the known physiologic level of cytosolic ATP (5-10 mM) in mammalian cells.

Since both cell lines express P2Y and P2X receptors, which mediate cell signaling and lead to physiological

effects in the cells, a change in concentration of intracellular Ca^{2+} was expected after ATP treatment. Interestingly, we observed that the different response of intracellular Ca^{2+} efflux from the stimulation of ATP in both cells. In HL-60 cells, calcium flux was detected in response to ATP treatment, but F-36P cell was not shown the calcium flux even under the stimulation with 1 mM and 10 mM ATP. We also discovered that the rate of intracellular calcium flux from high dose of stimulation of ATP was shown faster peak level in HL-60 cell lines. This phenomenon may results from homologous feedback regulation of PKC and PKA activation (Lee *et al.*, 1997). We considered that ATP signaling pathway and strength of signaling via P2X and P2Y receptor expressing on both cell lines had differences between HL-60 and F-36P, due to different expression pattern.

In this study, we investigated the biological effect by stimulation of extracellular ATP in human acute myeloid leukemic cells, HL-60 and F-36P. We discovered ATP induces cell apoptosis through the induction of intracellular caspase activation in both leukemic cells, resulting in dramatically reduced cell proliferation activity. This result directly showed the evidence that the extracellular ATP regulates the growth and proliferation of human myeloid leukemic cells by cell death induction.

Taken together, these data demonstrate that the extracellular ATP might be applied as novel therapeutic agent for human myeloid leukemia patient in the future.

Abbreviations: ATP, adenosine 5'- triphosphate; MDS, myelodysplastic syndrome; FACS, fluorescence-activated cell sorter.

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