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• *ESOPHAGEAL CANCER* •

Inhibitory effects of extracellular adenosine triphosphate on growth of esophageal carcinoma cells

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Abstract

AIM: To study the growth inhibitory effects of ATP on TE-13 human squamous esophageal carcinoma cells *in vitro*.

METHODS: MTT assay was used to determine the inhibition of proliferation of ATP or adenosine (ADO) on TE-13 cell line. The morphological changes of TE-13 cells induced by ATP or ADO were observed under fluorescence light microscope by acridine orange (AO)/ethidium bromide (EB) double stained cells. The internucleosomal fragmentation of genomic DNA was detected by agarose gel electrophoresis. The apoptotic rate and cell cycle after treatment with ATP or ADO were determined by flow cytometry.

RESULTS: ATP and ADO produced inhibitory effects on TE-13 cells at the concentration between 0.01 and 1.0 mmol/L. The IC_{50} of TE-13 cells exposed to ATP or ADO for 48 and 72 h was 0.71 or 1.05, and 0.21 or 0.19 mmol/L, respectively. The distribution of cell cycle phase and proliferation index (PI) value of TE-13 cells changed, when being exposed to ATP or ADO at the concentrations of 0.01, 0.1, and 1 mmol/L for 48 h. ATP and ADO inhibited the cell proliferation by changing the distribution of cell cycle phase via either G_0/G_1 phase (ATP or ADO, 1 mmol/L) or S phase (ATP, 0.1 mmol/L) arrest. Under light microscope, the tumor cells exposed to 0.3 mmol/L ATP or ADO displayed morphological changes of apoptosis. A ladder-like pattern of DNA fragmentation was obtained from TE-13 cells treated with 0.1-1 mmol/L ATP or ADO in agarose gel electrophoresis. ATP and ADO induced apoptosis of TE-13 cells in a dose-dependent manner at the concentration between 0.03 and 1 mmol/L. The maximum apoptotic rate of TE-13 cells exposed to ATP or ADO for 48 h was 16.63% or 16.9%, respectively.

CONCLUSION: ATP and ADO inhibit cell proliferation, arrest cell cycle, and induce apoptosis of TE-13 cell line.

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Key words: Extracellular adenosine triphosphate; Esophageal carcinoma cells; Apoptosis; Growth inhibition

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INTRODUCTION

Extracellular ATP and adenosine (ADO) are important signaling molecules in both intracellular and extracellular microenvironments of cells. Though the regulatory control is exerted by ectonucleotidases, which maintain its low physiologic concentrations, extracellular ATP may reach high concentrations when released exocytotically from various cell types such as neurons, platelets, basophils, and mast, or when released nonexocytotically from damaged cells[1]. Since the pioneering work of Rapaport and Fontaine[2,3] showing the anticancer activities of extracellular adenine nucleotides on tumor, inhibitory effects of extracellular ATP have been described in the majority of cells and tissues studied so far, including human histiocytic leukemia cell line U-937[4], macrophages[5], mouse neuroblastoma cell line N1E-115[6], pancreatic cancer cells[7], endothelial cells[8], pulmonary artery endothelial cells[9], colorectal carcinoma cells[10], prostate carcinoma cells[11], murine dendritic cells, etc.[12-16]. But in other cell lines, such as human ovarian tumor cells and breast tumor cells, ATP shows opposite effects[17,18]. Recently, Maaser *et al*.[19], reported that extracellular nucleotides inhibit growth of moderately differentiated human esophageal cancer cells. However, the effects of ATP on poorly differentiated esophageal cancer cells have not been reported. In this study, we observed the growth inhibitory and apoptotic effects of ATP and its final metabolite, ADO, on poorly-differentiated human TE-13 esophageal cancer cells.

MATERIALS AND METHODS

Drugs and reagents

ATP, ADO, acridine orange (AO), ethidium bromide (EB), 3-(4,5-dimethyiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma. RNase, SDS, proteinase K, trypsin and agarose were from Sino-American Biotec Co., RPMI 1640 medium was from GIBCO, and fetal bovine serum (FBS) was from Hangzhou Sijiqing Biotec Co. ATP and ADO were dissolved in sterile PBS, and stored at -20 ¡æ

Cell culture

Human esophageal carcinoma TE-13 cells, obtained from Japanese Cancer Cell Database, were cultured in RPMI 1640 medium supplemented with 100 mL/L FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ¡æin a humidified CO_2-controlled (50mL/L) incubator.

MTT assays

The cell viability was determined by MTT assay[20]. TE-13 cells in exponential phase of growth were harvested and seeded in 96-well plates (Costar, USA) at a density of 10 000 cells/well, and cultured for 24 h. ATP or ADO (0.01, 0.03, 0.1, 0.3, and 1 mmol/L), and control (PBS) were added into the wells and incubated continuously for 48 or 72 h at 37 ¡æwith 50 mL/L CO_2. The drugs were added daily and replaced with fresh medium every 2 d. A 20-μL sample of MTT solution (5 g/L dissolved in PBS) was added to each well and the plates were incubated at 37 ¡æfor 4 h. The supernatant was discarded and 150 μL dimethylsulfoxide was added to dissolve the blue insoluble MTT formazan produced by mitochondrial succinate dehydrogenase. The absorbance was measured at 492 nm in a spectrophotometer (Zhengzhou Bosai Biotech Co., ht2010), and the negative control well contained only the medium. The percentage of viable cells was calculated as the relative ratio of their absorbances to the control. All determinations were performed in quadruplicate and each experiment was repeated at least thrice.

Morphological assessment of apoptotic cells induced by ATP or ADO

Morphological assessment of apoptotic cells was performed using the AO/EB double staining method[21]. TE-13 cells in exponential phase of growth were harvested and seeded in a 25-mL cultured flask. The cells were incubated for 24 h at 37 ¡æwith 50 mL/L CO_2, and then treated with 0.3 mmol/L ATP or ADO for 48 h. Freshly isolated TE-13 cells (1×10^6) were harvested in an Ependorf centrifuge tube, centrifuged for 5 min at 1 000 r/min and suspended in PBS containing fluorescence dye AO/EB (both AO and EB were at the concentration of 100 mg/L in PBS). The cells were prepared, and dropped on slides. The morphology of the cells was observed under fluorescence light microscope (UFX-II; Nikon, Japan) and photographed.

Agarose gel electrophoresis of DNA[22]

After treatment with ATP or ADO (0.1, 0.3, and 1 mmol/L) for 72 h, TE-13 cells (1×10^6) were harvested in an Ependorf centrifuge tube and washed twice with PBS. The cells were resuspended in a cell lysis buffer (50 mmol/L Tris-HCl buffer, 20 mmol/L EDTA, pH 8.0, 1% SDS) and then mixed by vortexing briefly. After the cells stood for 30 min on ice, proteinase K was added at a final concentration of 0.25 g/L. The cell lysates were incubated at 37 ¡æovernight in a water bath, and RNase was added at a final concentration of 0.5 g/L and incubated at 37 ¡æfor 1 h. The lysates were mixed with an equal volume of Tris-saturated phenol–chloroform (1:1, v/v) and mildly shaken for 30 min, and the mixture was centrifuged at 3 000 r/min for 10 min at room temperature to separate the aqueous phase from the organic phase. Extraction of each aqueous phase was repeated using the Tris-saturated phenol-chloroform-isopropanol (25:24:1, v/v/v) mixture, and the aqueous phase was further extracted with an equal volume of chloroform. Mixing with 2 volumes of ice-cold ethanol and 0.1 volume of 3 mol/L NaAc precipitated DNA in the final aqueous phase. At this point, the mixture could be stored overnight. DNA was recovered by centrifugation at 13 000 *g* for 20 min in an Ependorf centrifuge tube. The supernatant was discarded, the DNA pellet was washed once with 70% ethanol, air-dried, and then redissolved in an appropriate volume of deionized distilled water and electrophoresed for 3 h at a constant voltage of 60 mV on an 1.8% agarose gel containing 0.5 mg/L EB, using an electrophoresis buffer (40 mmol/L Tris/acetate buffer, 1 mmol/L EDTA, pH 8. 0). Each DNA sample contained bromophenol blue as a front-running dye. Ladder formation of oligonucleosomal DNA was made visible by an ultraviolet transillumination and photographed using a gel imaging system (PE Co., USA).

Determination of apoptosis by flow cytometric analysis

After the cells were incubated with different concentrations of ATP or ADO for 48 h, they were harvested by centrifugation, washed with ice-cold PBS once and fixed in 70% ethanol at 4 ¡æovernight. The cells were then washed once with ice-cold PBS and resuspended in PBS (pH 7.4) containing 0.5% pepsin, 5 mg/L EB and RNase at room temperature for 30 min. Finally, cells were analyzed by flow cytometry on a FACS420 (Becton Dickinson Corporation, USA), equipped with an argon ion laser (488 nm), using the HP-300 Consort 30 software to determine percentage of the apoptotic cells and the proportion of cells in G_0/G_1, S, G_2/M phases of cell cycle. The proliferation index (PI) of cells was calculated by the following formula:

$$PI(\%) = ([S+G_2/M]/[G_0/G_1+S+G_2/M])\times100\%.$$

Statistical analysis

The data shown were mean values of at least three independent experiments and expressed as mean±SD. Statistical comparisons were made by ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

Inhibitory effects of ATP or ADO on TE-13 cell proliferation

The proliferation of TE-13 cells was significantly inhibited in a dose- and time-dependent manner, by 0.01-1.0 mmol/L of ATP or 0.3-1.0 mmol/L of ADO for 48 h of incubation, as well as 0.01-1.0 mmol/L of ADO for 72 h of incubation. The inhibitory fraction of TE-13 cells exposed to ATP and ADO for 48 and 72 h was 59.6% and 46.5%, and 80.5% and 74%, respectively (Figures 1A and B). The IC_{50} of TE-13 cells exposed to ATP or ADO for 48 and 72 h was 0.71 or 1.05 mmol/L, and 0.21 or 0.19 mmol/L, respectively.

Effects of ATP or ADO on cell cycle and proliferation index (PI) of TE-13 cells

The cell cycle phase and PI value of TE-13 cells changed when exposed to ATP or ADO at the concentrations of 0.01, 0.1, 1 mmol/L for 48 h. The proportion of cells in

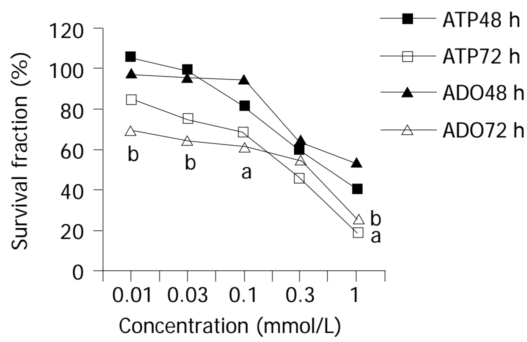

Figure 1 Effects of various concentrations of ATP or ADO on survival fraction of TE-13 cells. [a]*P*<0.05, [b]*P*<0.01 *vs* 48 h groups.

the S phase of cell cycle significantly increased, and that of the G_0/G_1, G_2/M phases and PI value did not alter after sustained incubation of TE-13 cells with ATP (0.1 mmol/L). In contrast, when exposed to ATP or ADO at the concentration of 1 mmol/L, the proportion of cells in the G_0/G_1 phase of cell cycle significantly increased, while that in the S phase of cell cycle and PI value significantly decreased. In accordance with cell proliferation results, neither the cell cycle phase nor PI value changed when exposed to ATP or ADO at the concentration of 0.01 mmol/L. The proportion of cells in G_2/M phase when exposed to ATP at various concentrations did not alter, but that significantly decreased when exposed to ADO at the concentration of 1 mmol/L. These results showed that ATP and ADO inhibited the cell proliferation by changing the distribution of cell cycle phase via either S phase delay (ATP, 0.1 mmol/L) or G_0/G_1 phase delay (ATP and ADO, 1 mmol/L, Table 1 and Figures 2A and B).

Table 1 Effects of ATP or ADO on PI value of TE-13 cells (*n* = 3, mean±SD)

Concentration (mmol/L)		PI (%)
ATP	0	37.57±2.02
	0.01	39.17±3.65
	0.1	31.24±1.04[a]
	1.0	28.69±1.33[a]
ADO	0	36.85±1.15
	0.01	29.91±1.78
	0.1	32.34±3.39
	1.0	21.76±5.20[b]

[a]*P*<0.05, [b]*P*<0.01 *vs* control group.

Morphological changes of TE-13 cells induced by ATP or ADO

Under fluorescence light microscope, the tumor cells exposed to 0.3 mmol/L ATP or ADO displayed morphological changes of apoptosis by AO/EB double staining, such as cell shrinkage, chromatin condensation, cell nuclear fragmentation, cell nucleolus disappearance, increased nuclei fluorescence or labeled orange or red-orange color (Figures 3A-C).

Agarose gel electrophoresis results of TE-13 cells induced by ATP or ADO

By agarose gel electrophoresis, a ladder-like pattern of DNA fragmentation was obtained from TE-13 cells treated with 0.1-1 mmol/L ATP or ADO, indicating that ATP or ADO induced apoptosis of TE-13 tumor cells (Figures 4A and B).

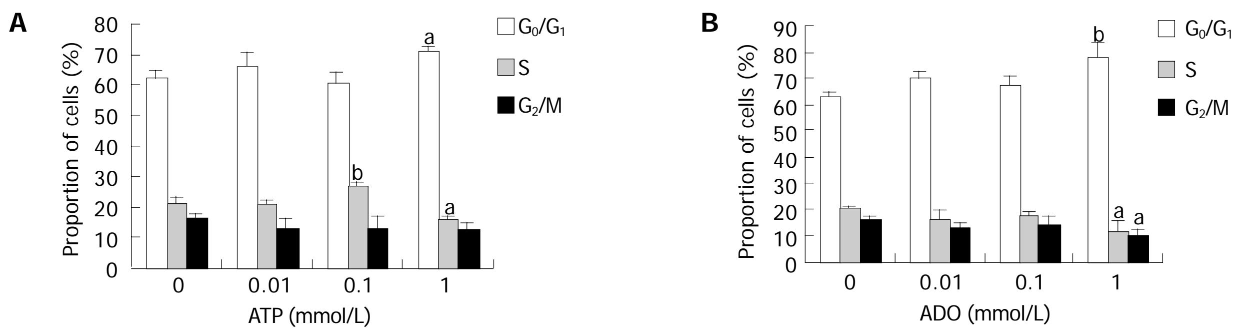

Figure 2 Effects of ATP (**A**) and ADO (**B**) on cell cycle of TE-13 cells (*n* = 3). [a]*P*<0.05, [b]*P*<0.01 *vs* 0 mmol/L.

Figure 3 Fluorescence micrographs of TE-13 cells incubated for 48 h without treatment (**A**) and treated with ATP (**B**) or ADO (**C**) (×400).

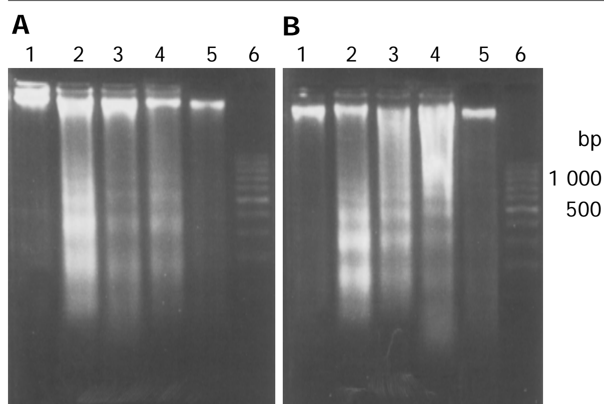

Figure 4 Agarose gel electrophoresis of DNA extracted from apoptotic TE-13 cells treated with ATP (**A**) or ADO (**B**) for 72 h. Lane 1: control; lanes 2-5: 1, 0.3, 0.1, and 0.03 mmol/L, respectively; lane 6: marker.

Apoptotic rate of TE-13 cells induced by ATP or ADO

ATP or ADO induced apoptosis of TE-13 cells in a dose-dependent manner at the concentration between 0.03 and 1 mmol/L for 48 h. The apoptotic rate of TE-13 cells treated with ATP or ADO was markedly higher than that of the control. The maximum apoptotic rate of TE-13 cells exposed to ATP or ADO (1 mmol/L) for 48 h was (16.6±1.1)% or (16.9±1.2)%, respectively (Table 2 and Figures 5A-C).

Table 2 Apoptosis of TE-13 cells induced by extracellular ATP or ADO (n = 3, mean±SD)

Concentration (mmol/L)		Apoptotic rate (%)
ATP	0	1.35±0.07
	0.03	3.89±0.29
	0.1	7.73±0.57[a]
	0.3	12.40±0.61[b]
	1.0	16.60±1.10[a]
ADO	0	2.43±0.85
	0.03	6.75±0.49[a]
	0.1	9.73±1.70[d]
	0.3	13.10±0.53[d]
	1.0	16.90±1.20[d]

[a]P<0.05, [b]P<0.01, and [d]P<0.001 *vs* control (0 mmol/L).

DISCUSSION

ATP and related compounds are widespread transmitters for extracellular communication in many cell types. By coupling to specific purinergic receptors, ATP is involved in a large variety of cellular functions. Receptors for purines and pyrimidines (P receptor) are divided into two major classes termed as ADO or P1 receptors at which ADO is the principal natural ligand, and P2 receptors at which ATP, ADP, UTP, and UDP are the principal natural ligands. To date four P1 receptor subtypes have been identified (A1, A2a, A2b, and A3), all of them are coupled to G proteins with distinct tissue distribution and pharmacological properties. The P2 receptors are divided into two families: the ligand-gated ion channels (P2X) and the G protein-coupled receptors (P2Y)[23-25]. ATP can inhibit cancer growth, induce apoptosis in various tumor models[26-30]. Both growth inhibition and programmed cell death are mediated by ionotropic P2-receptors and metabotropic P2-receptors[10,19]. Here we provide evidence that extracellular ATP induces apoptosis and causes cell cycle arrest in poorly differentiated human squamous cancer cells of the esophagus, and ADO plays an important role in them.

Recently, Maaser *et al*.[19], studied the effects of ATP and ADO on moderately differentiated esophageal cancer Kyse-140 cells, and found that ATP (100-500 μmol/L) inhibits cell growth, causes a delay in the S phase of cell cycle, and induces apoptosis. However, ADO has no contribution to the antiproliferative and apoptotic action of Kyse-140 cells. In our study, both ATP (0.1-1 mmol/L) and ADO (0.03-1 mmol/L) inhibited growth of TE-13 cells, caused cell cycle arrest in S phase (ATP, 0.1 mmol/L) or in G_0/G_1 phase (ATP or ADO, 1 mmol/L). The reasons why our results did not accord with those of Maaser *et al*.[19], may be due to the different kinds of esophageal cancer cell line and different concentrations of ATP used in our study. Additionally, a positive correlation between S-phase fraction and the response to anticancer agents has recently been documented[31]. Hence, in addition to the antiproliferative action of ATP on its own, possible synergistic effects of ATP and anticancer drugs should be investigated.

Besides its cell cycle interfering effects, ATP or ADO was shown to induce apoptosis in esophageal cancer TE-13 cells as assessed simultaneously by morphological study, agarose gel electrophoresis, and flow cytometry analysis. After being exposed to various concentrations of ATP or ADO for 48 or 72 h, TE-13 cells displayed a series of apoptotic event, such as chromatin condensation, fragment nuclei, apoptotic body, apoptotic peak in the flow cytometry imaging

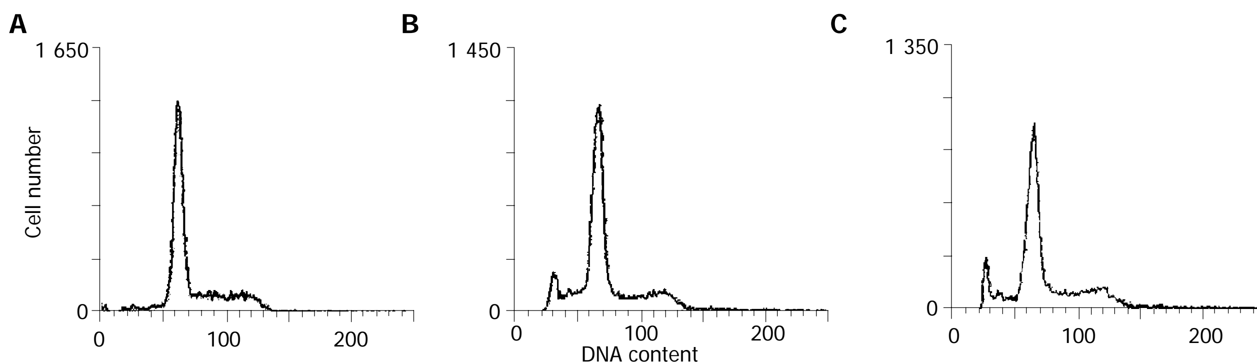

Figure 5 ATP- or ADO-induced apoptosis of TE-13 cells detected by flow cytometry in control (**A**) and treated with ADO (**B**) and ATP (**C**) for 48 h, respectively.

as well as DNA ladder. ADO is the final metabolite of ATP, and the result of ADO contributing to the growth inhibition and apoptosis suggests that the above effects of ATP might be partially related to its metabolite, ADO.

In conclusion, extracellular ATP inhibits cell growth, causes cell cycle arrest, and induces apoptosis. These actions might be partially related to its metabolite, ADO. Although the average concentration of nucleosides in plasma and other extracellular fluids is generally in the range of 0.4-6 μmol/L, these values can increase at sites of vascular inflammation and platelet degranulation[32]. Taken together, to further investigate the effects of ATP on tumor cells may provide an innovative treatment strategy for esophageal cancer.

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