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Research Article

Growth Inhibition of Lung Cancer Cells by Adenosine 5′-Triphosphate

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Strategy, Management and Health Policy				
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ABSTRACT Preliminary clinical data suggest that adenosine 5′-triphosphate (ATP) may inhibit lung tumor growth. Because studies of ATP on lung cancer cells are lacking, the aim of the present study was to explore effects of extracellular ATP on the growth and morphology of human lung tumor cells. Five human lung tumor cell lines derived from tumors with different cellular characteristics, i.e., a small cell carcinoma (GLC4), a large cell carcinoma (H460), a squamous cell carcinoma (H520), a mesothelioma (MERO82), and a papillary adenocarcinoma (H441), were exposed to 0, 0.5, 1, 2, and 3 mM ATP. Total cell numbers and dead or damaged cells were measured on days 1, 2, and 3. ATP induced a significant, dose-dependent growth inhibition in GLC4, H460, H520, and MERO82 cells. In contrast, H441 cells showed already maximal inhibition at 0.5 mM. Compared to untreated control cell lines, a significant growth inhibition (mean ± SEM) of 65 ± 5% (GLC4), 59 ± 5% (H460), 45 ± 5% (H520), 38 ± 2% (MERO82), and 55 ± 8% (H441) was shown after 3 days incubation with 3 mM ATP. ATP also induced changes in morphology and attachment to the substratum. Although not demonstrated by the Trypan Blue exclusion test, on photographs it seems that ATP induces death of GLC4 and H460 cells at higher concentrations. In conclusion, in four out of five explored lung tumor cell lines, ATP induces a dose-dependent growth inhibition. Lung adenocarcinoma cells show already maximal inhibition at the lowest tested ATP dose. There is a relationship between growth inhibition and morphology changes. Drug Dev. Res. 60:196–203, 2003.

Key words: adenosine 5′-triphosphate; tumor cells; growth inhibition; cell death

INTRODUCTION

Adenosine 5′-triphosphate (ATP) is a naturally occurring nucleotide that is present in every cell of the human body. In addition to its well-established intracellular energy-transferring role, extracellular ATP is involved in biological processes including neurotransmission, muscle contraction, cardiac function, platelet function, vasodilatation, and liver glycogen metabolism [Burnstock, 1990]. The physiological concentration of ATP in plasma is low, i.e., 0.02–10 μM [Forrester, 1972; Harkness et al., 1983]. Extracellular ATP exerts its effects through cell surface P2 receptors, classified in G protein-coupled P2Y, and ionotropic P2X receptors [Fredholm et al., 1997].

In vitro studies have shown that extracellular ATP can modulate the growth of neoplastic cells. In transformed cells, ATP induced mitogenesis at a

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concentration below 50 μM [Huang et al., 1991; Wang et al., 1992], whereas higher ATP concentrations inhibited growth [Belzer and Friedberg, 1989; Chahwala and Cantley, 1984; Fang et al., 1992; Hatta et al., 1994; Rapaport, 1983; Rapaport et al., 1983; Seetulsingh-Goorah and Stewart, 1998; Spungin and Friedberg, 1993; Vandewalle et al., 1994]. These effects were not observed in untransformed control cells [Hatta et al., 1993, 1994; Kitagawa et al., 1988; Mure et al., 1992; Rapaport, 1983]. In human tumor cell lines, ATP inhibited the growth of pancreatic adenocarcinoma cells [Rapaport, 1983], colon adenocarcinoma cells [Rapaport, 1983], melanoma cells [Kitagawa et al., 1988; Rapaport, 1983], androgenindependent prostate carcinoma cells [Fang et al., 1992], breast cancer cells [Abraham et al., 1996; Spungin and Friedberg, 1993; Vandewalle et al., 1994], myeloid and monocytic leukemia cells [Hatta et al., 1994], and multidrug resistant colon carcinoma cells [Correale et al., 1995]. In rats and mice, ATP was found to inhibit the growth of lymphomas [Nayak et al., 1990], colon carcinomas [Rapaport and Fontaine, 1989], fibrosarcomas [Froio et al., 1995], Ehrlich ascites tumors [Lasso de la Vega et al., 1994], and breast tumors [Abraham et al., 1996].

Recently, 15 patients with advanced non-small-cell lung cancer were treated with courses of intravenous ATP. Stable disease was found in 10 of these patients suggesting an inhibitory effect of ATP on tumor growth [Haskell et al., 1998]. So far, studies on growth inhibitory effects of ATP on lung cancer cell lines are lacking. Therefore, the aim of this study was to investigate the effects of ATP on the growth and morphology of different human lung cancer cell lines.

MATERIALS AND METHODS

Chemicals

Adenosine 5′-triphosphate (ATP-$Na_2 \cdot 3H_2O$; MW = 605) of >98% purity was obtained from Merck (Darmstadt, Germany). Two stock solutions of ATP were prepared to a final concentration of 10.5 mM (stock A) and 21 mM (stock B). ATP was dissolved in RPMI-1640 medium, adjusted to pH 7.4 with NaOH, and stored at −20°C until use. ATP stock solutions were sterilized by filtration through 0.2-μm syringe filters. High-performance liquid chromatograph (HPLC) testing showed that under these conditions ATP remained stable in time (data not shown).

Cell Lines, Media, and Culture Conditions

Five cell lines were studied. Human papillary lung adenocarcinoma (H441), human large cell lung carcinoma (H460), and human squamous cell lung carcinoma (H520) cell lines were obtained from the American Type Culture Collection (Mananas, VA, USA). Human small cell lung carcinoma (GLC4), and human mesothelioma cell lines (MERO82) were kindly given by Dr. T. Boersma (Department of Oncology, Erasmus University Medical Center Rotterdam, The Netherlands), and Dr. M. Versnel (Department of Immunology, Erasmus University Medical Center Rotterdam, The Netherlands), respectively.

Cells were cultured on RPMI-1640 culture medium (BioWhittaker Europe, Vervier, Belgium) containing L-Glutamine (2 mM), supplemented with 2% $NaHCO_3$ (BioWhittaker Europe), 100 U/ml penicillin (BioWhittaker Europe), 100 μg/ml streptomycin (BioWhittaker Europe), and supplemented with 10% heat inactivated fetal calf serum (GIBCO BRL, Life Technologies, Breda, The Netherlands). Cells were detached from their substratum by 0.5 g Trypsin/0.2 g EDTA (GIBCO BRL, Life Technologies).

The different cell lines were cultured in 75 cm^2 flasks (Costar, Cambridge, MA) on RPMI-1640 culture medium at 37°C in a 5% CO_2 humidified incubator. Heat-inactivated serum was prepared by heating FCS in a water bath at 60°C for 30 min. The cells were routinely passaged at approximately 90% confluency with medium changes once per week.

Experimental Protocol

On day-1 of the experiment, the different cell lines were seeded in a number of 4.75×10^4 cells per well on 6-well culture plates (Costar) and suspended in 5 ml RPMI-1640 medium per well. On day 0, cells were incubated in triplicate with ATP to final concentrations of 0, 0.5, 1, 2, and 3 mM. On days 1, 2, and 3, cell numbers and viability were determined. Cells were detached from their substrate by incubation with 1.2 ml Trypsin/EDTA per well for 10 min at 37°C. The wells were rinsed once with 1 ml phosphate buffered saline (PBS). The solution (cells, Trypsin/EDTA and PBS) was centrifuged at 1,000 RPM for 5 min at 22°C and the supernatant was removed. Subsequently, the cells were resuspended in PBS and counted.

Determination of Morphological Changes Due to ATP

Cells were inoculated and incubated as described. Photographs were taken at 1, 2, and 3 days after incubation with ATP, using a Sony RBG CCD video camera attached to a Zeiss Axiovert 100 microscope and stored on a videotape by use of a Panasonic time-lapse video recorder.

Cell Growth Determination

Cell numbers and viability were determined microscopically by Trypan Blue dye exclusion, and each sample was scored in triplicate.

Statistical Analysis

Growth data are expressed as cell numbers per well, and as percentage growth inhibition compared to control cell cultures. Cell death or damage is expressed as percentage of the total cells counted. Each value is the mean of 27 measurements: i.e., all experiments were performed three times, every incubation was performed in triplicate, and cell numbers were determined in triplicate. Values are expressed as mean ± SEM. Statistical significance of differences was appraised using Student's *t*-test. *P* values below 0.05 were considered statistically significant.

RESULTS

Growth Inhibition

Figure 1A–E shows the growth curves of five human lung tumor cells exposed to ATP at concentrations

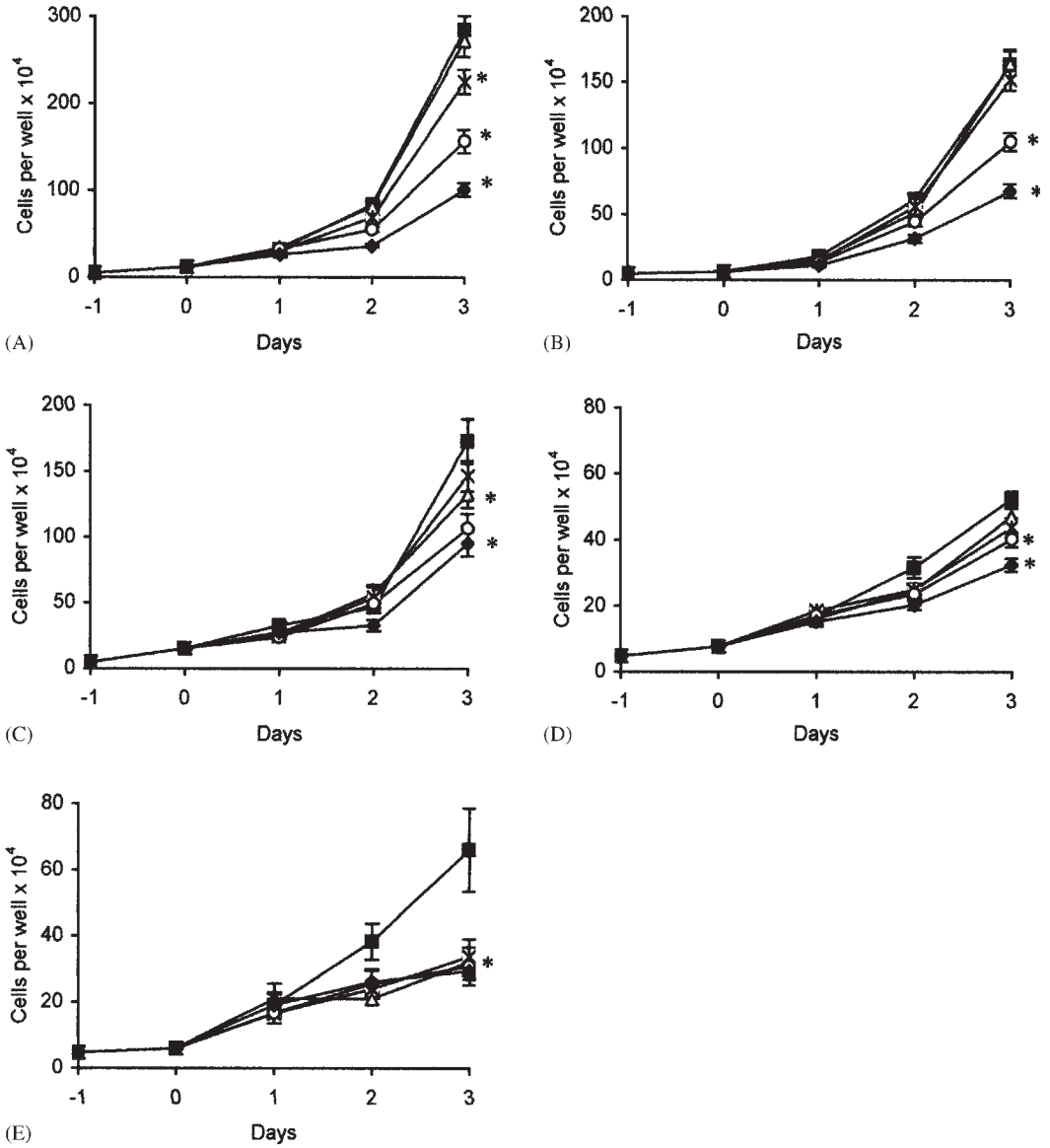

Fig. 1. Growth of (**A**) small cell carcinoma (GLC4), (**B**) large cell carcinoma (H460), (**C**) squamous cell carcinoma (H520), (**D**) mesothelioma (MERO82), and (**E**) papillary adenocarcinoma (H441) cell lines after administration of 0 (black square), 0.5 (open triangle), 1 (cross), 2 (open circle), and 3 (black diamond) mM adenosine 5′-triphosphate (ATP). Graphs represent mean values and error bars SEM. *Significantly different growth inhibition compared to the control cell line (0 mM ATP) at 3 days.

of 0 (= control), 0.5, 1, 2, and 3 mM ATP for a period of 3 days. As shown in Figure 1 and in Table 1, ATP induced a significant, dose-dependent growth inhibition in GLC4, H460, H520, and MERO82 cells. Three days after exposure to ATP, the growth inhibition of GLC4 cells was maximal at 3 mM ATP, less at 2 mM, minimal at 1 mM ATP, and absent at 0.5 mM ATP. At the same time point, the growth of H460, H520, and MERO82 cells was maximally inhibited at 3 mM, less at 2 mM, and not at either 1 and 0.5 mM ATP. In contrast, H441 cells showed already maximal growth inhibition at 0.5 mM ATP, with no additional growth inhibition at ATP concentrations of 1, 2, and 3 mM.

TABLE 1. Percentage Growth Inhibition of Lung Cancer Cells after 3 Days of Exposure to Different Concentrations of ATP as Compared to Control Cell Lines[†]

	mM ATP			
	0.5	1	2	3
GLC4	5±0	21±1*	45±4*	65±5*
H460	0±0	7±0	36±2*	59±5*
H520	23±2	15±1	38±4*	45±5*
MERO82	10±1	16±1	23±1*	38±2*
H441	51±7*	49±8*	53±7*	55±8*

[†]Values are mean of 27 measurements. Results are expressed as mean±SEM.
*Significant difference compared to control.

Morphological Changes

Besides growth inhibitory effects, ATP had effect on the attachment of the lung tumor cells to the substratum and on their morphology. At higher ATP concentrations, the cells became spheroidal, and detached from the substratum (Fig. 2). As shown in Figure 3, higher concentrations of ATP induced more pronounced changes in morphology of GLC4, H460, H520, and MERO82 cells than lower ATP concentrations. In contrast, H441 cells showed similar changes in morphology at both low and high ATP concentrations. Comparison of Table 1 and Figure 3 shows a relationship between growth inhibition and changes in morphology of lung cancer cells.

Cell Death

As tested by the Trypan Blue exclusion test ATP at several concentrations did not have cytotoxic effects on the tested lung tumor cell lines (Table 2). In contrast, as seen in the photographs (Fig. 2) ATP

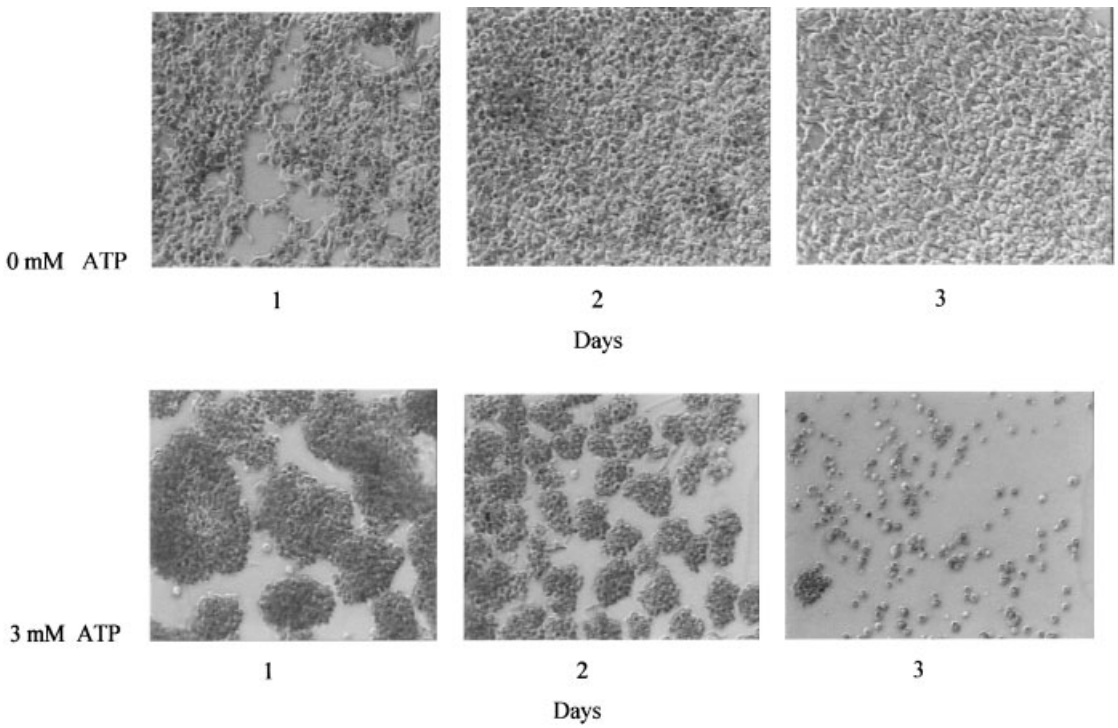

Fig. 2. Growth and morphology changes of small cell lung carcinoma (GLC4) cells at 1, 2, and 3 days after administration of 0 and 3 mM adenosine 5′-triphosphate (ATP).

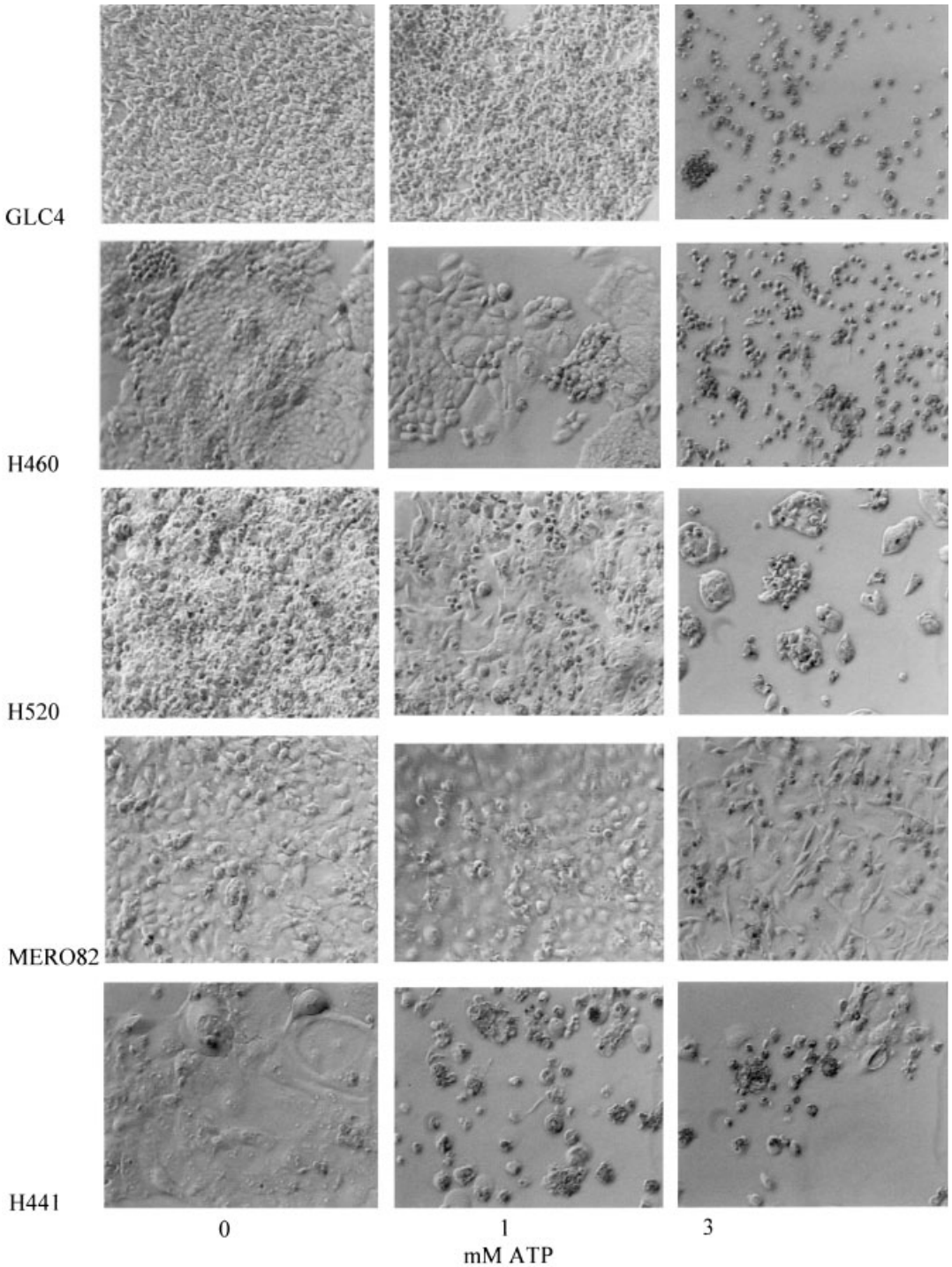

Fig. 3. Effects of administered adenosine 5′-triphosphate (ATP) on the growth and the morphology of small cell carcinoma (GLC4), large cell carcinoma (H460), squamous cell carcinoma (H520), mesothelioma (MERO82), and papillary adenocarcinoma (H441) cell lines at concentrations of 0, 1, and 3 mM at 3 days incubation period.

appears not only to exert cytostatic effects on lung tumor cell lines but also cytotoxic effects. Three 3 days after 3 mM ATP administration, the number of the GLC4 cells decreased compared to days 1 and 2. This finding was also observed in H460 cells (data not shown).

TABLE 2. Percentage Dead or Damaged Cells on the Total Cell Count of the Different Lung Cancer Cell Lines 3 Days After ATP Administration*

	mM ATP				
	0	0.5	1	2	3
GLC4	11±2	16±2	13±1	11±2	9±1
H460	16±1	13±1	15±1	11±1	14±1
H520	18±1	13±1	15±2	18±1	18±3
MERO82	11±1	9±1	9±1	13±1	25±3
H441	20±3	9±2	13±2	12±2	18±3

*Values are mean of 27 measurements. Results are expressed as mean±SEM.

DISCUSSION

In the present study, we investigated the effects of extracellular ATP on the growth and morphology of five human lung cancer cell lines, i.e., a small cell lung carcinoma (GLC4), a large cell lung carcinoma (H460), a squamous cell lung carcinoma (H520), a mesothelioma (MERO82), and a papillary lung adenocarcinoma (H441).

Incubation with 0.5 to 3 mM ATP resulted in a dose-dependent growth inhibition of GLC4, H460, H520, and MERO82 cell lines. In general, the most pronounced growth inhibitory effects (38–65% inhibition depending on cell type) were demonstrated at 3 mM ATP after an incubation time of 3 days. At this concentration, GLC4 cells were most sensitive, whereas MERO82 cells were least sensitive. It may be noted that small cell lung carcinomas (GLC4) also have a high sensitivity to chemotherapy [Carney, 1995], whereas mesotheliomas (MERO82) are almost completely resistant to this type of treatment [Bowman et al., 1991]. In H441 cells, ATP induced significant growth inhibition at both high (3 mM) and low (0.5 mM) concentrations.

It is intriguing that ATP also influenced the morphology of the lung cancer cells in a dose-dependent manner. Changes in morphology were related to the level of growth inhibition. Incubation with ATP induced changes in cell shape, membrane movement, cell agglutination, and attachment of cells to the substratum. It has been reported that extracellular ATP influences the cytoskeleton [Kitagawa and Akamatsu, 1983; Zheng et al., 1991]. Possibly, ATP induces changes in cytoskeletal structures which may contribute to increased permeability of transformed cells [Zheng et al., 1991]. In this connection, it could be worthwhile to investigate effects of ATP on cell adhesion molecules including E-cadherin, integrins, and vimentins, which may explain directly the spheroid nature of the cultured cells. It should be noted that loss of cell attachment by incubating the cells in 3 mM ATP during 3 days resulted not only in inhibition of growth, but also in cell death and cell loss as shown in Figure 2. The reason for not detecting ATP-induced cell death using the Trypan Blue test may be removal of dead cells that were detached from the substratum together with the supernatant. Possibly, as the photographs seem to suggest, there may have been more dead cells than were actually counted.

Several mechanisms have been proposed to explain the ATP-induced growth inhibition and death of tumor cells. Firstly, exposure of human adenocarcinoma cells to extracellular ATP has been reported to cause intracellular accumulation of ATP and arrest of tumor cells in the S-phase of cell replication, followed by cell death [Rapaport, 1983]. A similar ATP-induced growth inhibition, due to prolonging of the S-phase, was found in human breast cancer cells [Spungin and Friedberg, 1993]. Secondly, ATP-induced tumor growth inhibition is associated with a decrease in glutathione (GSH) content of the tumor, but not of normal tissues [Estrela et al., 1995; Lasso de la Vega et al., 1994]. Thirdly, in various transformed cells ATP administration contributed to increased membrane permeability [Chahwala and Cantley, 1984; Di Virgilio et al., 1990; Filippini et al., 1990; Kitagawa and Akamatsu, 1986; Kitagawa et al., 1988; Mure et al., 1992; Wiley and Dubyak, 1989; Zheng et al., 1991]. Increased cell permeability after exposure to extracellular ATP may be due to activation of $P2X_7$ receptors [Franseschi et al., 1996; Surprenant et al., 1996] that have been found on the cell surface of several tumor cells [Bretschneider et al., 1995; Kaiho et al., 1998]. Activation of the $P2X_7$ receptors causes opening of intrinsic ion channels, which leads to massive efflux of K^+, and influx of Ca^{2+} and Na^+, resulting in a decrease of the plasma membrane potential [Heppel et al., 1985; Pizzo et al., 1991]. Activation of $P2X_7$ receptors further results in formation of non-selective pores, which induces an increase in nonselective membrane permeability for aqueous solutes that ordinarily do not cross the cell membrane [Chiozzi et al., 1996; Dubyak and el-Moatassim, 1993; Macino et al., 1996]. These effects have been seen in many transformed cells [Belzer and Friedberg, 1989; Chahwala and Cantley, 1984; Fang et al., 1992; Rapaport, 1983; Rapaport et al., 1983; Spungin and Friedberg, 1993; Vandewalle et al., 1994] but not in untransformed cells [Di Virgilio et al., 1989; Hatta et al., 1994; Kitagawa et al., 1988; Rozengurt et al., 1977; Weisman et al., 1984].

Preclinical studies have shown that ATP administration not only induces growth inhibition of tumor

cells, but also potentiates the cytotoxic effects of several chemotherapeutic agents [Kitagawa and Akamatsu, 1983; Maymon et al., 1994] and radiotherapy [Estrela et al., 1995]. It would be interesting to explore effects of combinations of chemotherapeutic agents and ATP on lung carcinoma cell lines.

In summary, our results show that ATP induces a dose-dependent growth inhibition in four out of five lung cancer cell lines: GLC4, H460, H520 cells, and MERO82 cells. Lung adenocarcinoma cells (H441) show already maximal (55%) inhibition at the lowest ATP dose tested. There is a relationship between growth inhibition and morphology. Although not demonstrated by the Trypan Blue exclusion test, the photographs suggest that it seems that ATP may induce cell death at higher concentrations. Experiments to explore underlying processes contributing to ATP-induced cytostatic and cytotoxic effects, and morphology changes are warranted. Based on the marked growth inhibition of human lung adenocarcinoma cells by ATP at low dosage, further study with this tumor cell type would appear especially relevant.

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