

Inhibition of cancer growth and selective glutathione depletion in Ehrlich tumour cells *in vivo* by extracellular ATP

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We have investigated the effect of extracellular ATP on tumour-cell proliferation and GSH levels in Ehrlich-ascites-tumour-bearing mice. After daily administration of exogenous ATP (1 mmol/kg) during 7 days, we found a 56% inhibition of tumour growth, precisely when controls show the highest rates of cell proliferation and the highest levels of GSH. This effect is accompanied by a decrease in GSH content in the tumour, but not in normal tissues. The decrease in GSH concentration within

the cancer cells is associated with a decrease in γ -glutamylcysteine synthetase activity and in protein synthesis. Growth inhibition is mediated by generation of extracellular adenosine, which subsequently increases intracellular levels of ATP and decreases intracellular levels of UTP in the cancer cells. Our results suggest that inhibition of tumour growth by ATP is due to an adenosine-dependent pyrimidine starvation effect.

INTRODUCTION

GSH, the major non-protein thiol in mammalian cells, is involved in many cellular functions (Meister, 1983; Deneke and Fanburg, 1989). This tripeptide plays a central role in the protection of cells against free radicals, reactive oxygen intermediates and electrophiles, and therefore, in determining the sensitivity of cells to radiation and drug-induced cytotoxicity (Meister, 1983; Arrick and Nathan, 1984; Sies, 1986; Mitchell et al., 1989). GSH depletion can be achieved by using L-buthionine (SR)-sulphoximine (BSO), a non-toxic and selective inhibitor of γ -glutamylcysteine synthetase (Meister, 1983). Recently, we have shown that depletion of tumour GSH by BSO *in vivo* decreases the rate of cellular proliferation and inhibits cancer growth (Terradez et al., 1993). However, a major problem when using BSO, or other GSH-depleting agents (i.e. the $\alpha\beta$ -unsaturated carbonyl compounds diethylmaleate or phorone), is that they decrease GSH levels in tumour and non-tumour cells (Lee et al., 1987; Terradez et al., 1993). Thus, as previously stated (Mitchell et al., 1989), whether GSH-modulating agents will be useful for cancer patients will depend on whether selective depletion of GSH can be achieved in tumour versus normal tissues.

Extracellular ATP has growth-inhibitory properties against different animal and human tumour cells, but not normal cells, in several systems *in vitro* (Rapaport et al., 1983; Chahwala and Cantley, 1984; Weisman et al., 1988). Moreover, intraperitoneal administration of adenine nucleotides into tumour-bearing mice causes inhibition of cancer growth in murine models (Rapaport, 1988). However, it has not been investigated whether this growth inhibition is accompanied by changes in the GSH content of tumour and normal cells. Previously it has been reported that elevation of intracellular GSH is associated with mitogenic stimulation (Shaw and Chou, 1986), that GSH may regulate DNA synthesis (Suthanthiran et al., 1990), that GSH regulates the onset of tumour-cell proliferation by modulating protein kinase C activity and intracellular pH (pH_i) (Terradez et al., 1993), and that GSH content decreases, during tumour growth *in vivo*, when cell proliferation and the rate of protein synthesis in the tumour decrease (Estrela et al., 1992). Therefore, changes in the rate of cancer-cell proliferation must be reflected by changes

in their intracellular GSH levels. In this paper, we have used Ehrlich-ascites-tumour-bearing mice to investigate the effect of exogenous ATP administration on cancer growth and on GSH levels in tumour and normal cells.

MATERIALS AND METHODS

Animals and tumour inoculation

Adult male mice OF1 from IFFA CREDO (Madrid, Spain) were used. The animals, fed *ad libitum* on a stock laboratory diet (Letica, Barcelona, Spain), were kept on a 12 h-light/12 h-dark cycle with the room temperature maintained at 22 °C. All experiments were started between 10:00 and 12:00 h. The Ehrlich ascites tumour was kindly provided by the Department of Pathology (Universidad de Valencia) and inoculated intraperitoneally as previously described (Estrela et al., 1992). All injected animals developed an ascites tumour. The mice died 15.9 ± 0.4 days ($n = 15$) after inoculation of the tumour.

Measurements related to growth of the Ehrlich ascites tumour *in vivo* were carried out as previously described (Terradez et al., 1993).

Isolation and incubation of tumour cells

Cells were collected from tumour-bearing mice, and then isolated, incubated and separated in intracellular and extracellular compartments as previously described (Estrela et al., 1992).

Cell culture

Ehrlich ascites-tumour cells were grown in 75 cm² polystyrene flasks (Falcon Labware, U.S.A.) in Dulbecco's modified Eagle's medium (GIBCO Laboratories, U.S.A.), pH 7.4, supplemented with 10% fetal-bovine serum, 10 mM Hepes, 40 mM NaHCO₃, 100 units/ml penicillin and 100 μ g/ml streptomycin. Cell cultures were maintained in a humidified atmosphere of air/CO₂ (19:1) at 37 °C. On day 0 synchronized cells were plated at a density of 2×10^4 cells/cm². The cells were removed from the flasks with 0.05% (w/v) trypsin (Sigma, U.S.A.) in PBS (10 mM sodium phosphate, 4 mM KCl, 137 mM NaCl), pH 7.4, containing 0.3 mM EDTA, and counted in a Neubauer chamber.

To obtain a synchronized population, nocodazole {methyl [5-(2-thienylcarbonyl)-1*H*-benzimidazol-2-yl] carbamate; Sigma} (0.4 µg/ml; from a 4 mg/ml solution in dimethyl sulphoxide, which was stored at -20 °C) was added to the culture medium (Nusse and Egner, 1984) and cells (plated at 5×10^4 cells/cm²) were incubated for 12 h. About 50% of the cells had the highly rounded mitotic morphology after nocodazole arrest, and were detached by rocking and gentle washing of the plates with medium without detachment of the cell monolayer. The detached cells were collected by low-speed centrifugation (4 min, 4 °C) and the cell pellet was washed twice with Dulbecco's modified Eagle's medium supplemented with 10% fetal-bovine serum for further growth (see above).

Drug administration

ATP and adenosine, obtained from Sigma, were in sterile physiological saline solutions (0.9% NaCl) adjusted to pH 7.0. Mice were injected intraperitoneally daily. The volume injected either into controls or into ATP- or adenosine-treated animals was never higher than 0.01 ml/g of mouse.

Preparation of GSH ester

GSH monoisopropyl(glycyl) ester was obtained as previously described (Mårtensson and Meister, 1989).

Sample preparation and metabolite assays

Mice were killed by cervical dislocation. Organs or tissues were processed as previously described (Terradez et al., 1993), and homogenized in 6% HClO₄ containing 1 mM EDTA for GSH determination. GSH was measured as described by Akerboom and Sies (1981).

For measuring ATP levels in whole blood, plasma or erythrocytes, blood (0.5 ml) was collected directly from the heart into 1 ml syringes containing sodium heparin (0.05 ml of a 5% solution in 6.9% NaCl). Plasma was obtained by low-speed centrifugation of whole blood (800 g for 15 min, at 4 °C). Samples (0.1 ml) of plasma were added to 0.9 ml of ice-cold 7% (w/v) trichloroacetic acid. Erythrocytes were obtained by centrifugation of whole blood (1500 g for 5 min at 4 °C), followed by removal of plasma and the buffy coat, and then the pelleted erythrocytes were washed in ice-cold Krebs-Henseleit bicarbonate medium (pH 7.4). After centrifugation, the erythrocytes were resuspended in a volume of Krebs-Henseleit medium to yield the original haematocrit. Samples (0.1 ml) of erythrocyte suspensions or whole blood were added to 0.9 ml of 7% trichloroacetic acid. Extraction of acid-soluble nucleotides and determination of ATP levels by luminometry, using the luciferase luminescent assay (Sigma), were performed as described by Rapaport (1988).

ATP, ADP, UTP and adenosine in tumour cells, grown *in vivo* or *in vitro*, were determined by standard enzymic methods (Bergmeyer, 1974).

Protein content was determined by the method of Lowry et al. (1951).

Assay of γ -glutamylcysteine synthetase and glutathione synthetase activities

Assays in tumour cells were adapted from those previously described by Seelig and Meister (1985) for activities in rat kidney.

γ -Glutamylcysteine synthetase activity was determined, by monitoring the rate of formation of ADP at 37 °C in a reaction

mixture (final volume 1.0 ml) containing 200 mM Tris/HCl buffer (pH 8.2), 100 mM KCl, 5 mM ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L- α -aminobutyrate, 10 mM MgCl₂, 1 mM EDTA, 0.5 mM NADH, 20 µg of pyruvate kinase and 20 µg of lactate dehydrogenase. The A_{340} was monitored. One unit of enzyme catalyses the formation of 1 µmol of product/h at 37 °C.

Glutathione synthetase activity was determined by measuring the formation of P_i and ADP, in a reaction mixture (final volume 0.2 ml) containing 200 mM Tris/HCl (pH 8.2), 100 mM KCl, 5 mM L- γ -glutamyl-L- α -aminobutyrate, 5 mM ATP, 5 mM glycine, 10 mM MgCl₂ and 1 mM EDTA. The assay mixture was incubated for 20 min at 37 °C. To determine P_i, 0.8 ml of 10% HClO₄ was added, and P_i was measured by fluorimetry as described by Bergmeyer (1974). To determine ADP, we added 0.05 ml of 10% sulphosalicylic acid and 1.8 ml of a solution containing 2 mM phosphoenolpyruvate, 0.5 mM NADH, 10 mM MgCl₂, 100 mM KCl, 0.2 M phosphate buffer (pH 7.0) and 20 µg of pyruvate kinase. The amount of ADP formed is calculated from the change in A_{340} observed after addition of 20 µg of lactate dehydrogenase. One unit of enzyme catalyses the synthesis of 1 µmol of L- γ -glutamyl-L- α -aminobutyrylglycine in 1 h at 37 °C.

Measurement of pH_i

pH_i in tumour cells was determined by using the [¹⁴C]dimethylloxazolidine-2,4-dione (New England Nuclear, U.S.A.) distribution-ratio method (Rottenberg, 1979), as previously described (Terradez et al., 1993).

Measurement of protein synthesis

Fractional rates of protein synthesis were based on the flooding-dose technique of Garlick et al. (1980), and obtained as previously described (Estrela et al., 1992).

Expression of results and statistical significance

The results are expressed as means \pm S.D. for the indicated numbers of different experiments. The statistical significance of differences was assessed by Student's *t* test.

RESULTS

Effect of ATP or adenosine on tumour growth

Rapaport and Fontaine (1989a), using CT26 colon adenocarcinoma, have shown that inhibition of tumour growth *in vivo* requires daily administration of ATP during a period of several days. We studied the dose-response relationship of cancer growth inhibition by ATP in mice bearing an Ehrlich ascites tumour. The growth characteristics for this tumour have been previously reported (Estrela et al., 1992). As shown in Figure 1, we could achieve an approx. 56% inhibition of tumour growth, compared with controls, by daily administration of 1 mmol of ATP/kg for 7 days, starting on day 0 when the tumour is inoculated (see legend to Figure 1). This is important, since, on day 7 after inoculation, the tumour shows the highest rate of cellular proliferation and the highest GSH content (Terradez et al., 1993). We also found that administration of a single dose of ATP (1 mmol/kg) did not result in any significant variation in the rate of tumour cell proliferation (results not shown).

Several studies (e.g. Weisman et al., 1988; Rapaport and Fontaine, 1989b) have suggested that tumour growth inhibition

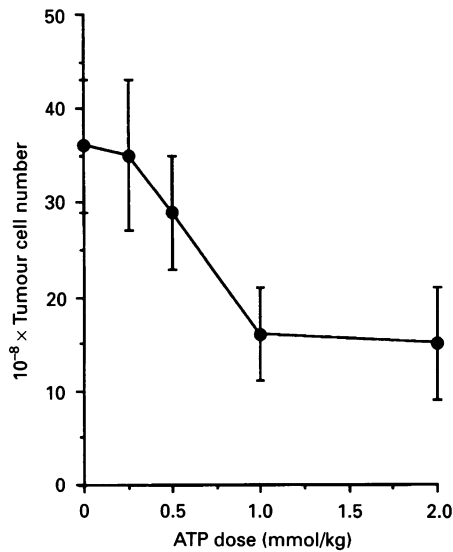


Figure 1 Dose-response relationship of tumour growth inhibition by ATP

An Ehrlich tumour was allowed to grow for 7 days. ATP was administered daily, starting 2 h after inoculation of the tumour. Each value is the mean \pm S.D. for 4 independent experiments: * $P < 0.05$ (when comparing ATP- and saline-treated mice).

by adenine nucleotides may occur via generation of extracellular adenosine. As shown in Table 1, we have compared the effects of ATP and adenosine on tumour growth. ATP-treated mice show a decrease in growth, protein synthesis and pH_i in the tumour (Table 1). However, adenosine-treated mice did not show significant variations in those parameters as compared with controls (Table 1). These results are in agreement with previous studies *in vitro*, where adenosine was shown to be much less effective than ATP, ADP or AMP in affecting tumour growth (Weisman et al., 1988).

Increases in erythrocyte and plasma ATP levels after ATP administration

Single intraperitoneal injections of 1 mmol of ATP/kg resulted

in expansion of erythrocyte ATP pools of tumour-bearing mice (Table 2). This increase in ATP contents was significant 1 h after injection and lasted for 6–9 h. After intraperitoneal injection, ATP (1 mmol/kg) was completely removed from the ascitic fluid in 46–60 min. Adenosine, at concentrations similar to that of ATP, was not effective in expanding erythrocyte ATP pools (results not shown). Plasma ATP levels were also increased in ATP-treated mice (Table 2). These results are in agreement with those found by Rapaport and Fontaine (1989a), who showed that ATP accumulates in red blood cells. In addition, we found that administration of two doses of 1 mmol/kg of ATP per day (one dose each 12 h) did not increase significantly (results not shown) the percentage of cancer growth inhibition obtained (56%; see above) by daily administration of a single dose of 1 mmol of ATP/kg.

On the other hand, administration of adenosine (1 mmol/kg) did not result in any significant accumulation of ATP in either erythrocytes or the plasma (Table 2; see also Rapaport and Fontaine, 1989a).

GSH contents in different tissues of ATP-treated mice

Recently we have shown that an increase in GSH content within tumour cells is required to increase the rate of cellular proliferation *in vivo* (Terradez et al., 1993). BSO decreases tumour GSH and inhibits cancer growth. However, this inhibitor also decreases GSH in normal tissues at the same time.

We investigated GSH levels in cancer cells and in many different normal tissues of ATP-treated mice bearing the Ehrlich ascites tumour, and found that GSH levels are only decreased significantly in the tumour (Table 3).

Similar control experiments to those described in Table 3 were performed in non-tumour-bearing mice. We investigated GSH levels in all normal tissues displayed in Table 3 in non-treated, saline-treated or ATP (1 mmol/kg)-treated non-tumour-bearing mice, but no significant differences were found when these values were compared with those reported in Table 3 (results not shown).

Effect of ATP or adenosine on GSH synthesis and glutathione efflux in tumour cells

We studied in isolated cancer cells whether the decrease in

Table 1 Effect of ATP and adenosine on growth of Ehrlich ascites tumours

ATP (1 mmol/kg) or adenosine (1 mmol/kg) was administered daily, starting 2 h after inoculation of the tumour. Then the tumour was allowed to grow for 7 days. Abbreviation: FSR, fractional rate of protein synthesis. Results are expressed as means \pm S.D. for 10–12 different animals: *significantly different from control group (mice treated with 0.9% NaCl alone) ($P < 0.05$).

Parameter	Time after inoculation of the tumour (days) ... Administered substance ...	7			
		0	Saline	ATP	Adenosine
Animal wt. (g)		30.5 \pm 0.8	36.8 \pm 0.6	34.5 \pm 0.5*	36.1 \pm 0.7
Carcass wt. (g)			29.8 \pm 1.1	30.3 \pm 0.7	29.5 \pm 0.9
Tumour volume (ml)			6.5 \pm 0.4	4.0 \pm 0.5*	6.3 \pm 0.5
10 ⁻⁶ × tumour cell density (no. of cells/ml)			583 \pm 67	439 \pm 55*	536 \pm 81
FSR in tumour cells (%/day)			65.0 \pm 7.3	47.0 \pm 6.5*	60 \pm 9.5
pH_i			7.36 \pm 0.5	6.95 \pm 0.05*	7.31 \pm 0.06

Table 2 Effect of ATP or adenosine administration on erythrocyte and plasma ATP levels

ATP (1 mmol/kg) or adenosine (1 mmol/kg) was administered on day 6 after inoculation of the tumour. Data are means \pm S.D. for six different animals.

Time after injection (h)	Administered substance ...	ATP (μ M)			
		Erythrocytes		Plasma	
		ATP	Adenosine	ATP	Adenosine
0		765 \pm 152	719 \pm 64	0.96 \pm 0.33	0.88 \pm 0.35
1		1215 \pm 267*	690 \pm 71	3.60 \pm 0.91*	1.01 \pm 0.51
3		1534 \pm 245*	807 \pm 110	4.28 \pm 1.63*	0.90 \pm 0.30
6		1310 \pm 290*	777 \pm 95	3.75 \pm 1.25*	0.91 \pm 0.40
9		957 \pm 130	685 \pm 34	1.09 \pm 0.31	0.86 \pm 0.22
12		742 \pm 161	712 \pm 89	0.84 \pm 0.50	0.85 \pm 0.37
24		751 \pm 163	761 \pm 114	0.91 \pm 0.15	0.93 \pm 0.27

Table 3 Effect of ATP administration on GSH content of several tissues from tumour-bearing mice

The Ehrlich tumour was allowed to grow for 7 days. ATP (1 mmol/kg) was administered daily, starting 2 h after inoculation of the tumour. Data are means \pm S.D. for 10–12 different animals: * $P < 0.05$.

Tissue	Administered substance ...	GSH (μ mol/g)	
		Saline	ATP
Brain		1.5 \pm 0.2	1.3 \pm 0.2
Lung		1.6 \pm 0.4	1.5 \pm 0.2
Heart		0.7 \pm 0.1	0.7 \pm 0.2
Liver		6.0 \pm 1.2	5.1 \pm 0.7
Glandular stomach		5.5 \pm 0.7	5.6 \pm 0.5
Pancreas		0.5 \pm 0.1	0.4 \pm 0.2
Spleen		3.1 \pm 0.3	2.8 \pm 0.4
Kidney		2.3 \pm 0.5	2.4 \pm 0.9
Skeletal muscle		0.3 \pm 0.07	0.3 \pm 0.05
Bone marrow		0.2 \pm 0.05	0.3 \pm 0.1
Testis		3.2 \pm 0.4	3.0 \pm 0.4
Tumour		3.2 \pm 0.5	1.4 \pm 0.3*

tumour GSH in ATP-treated mice could be related to a decrease in the rate of GSH synthesis. As shown in Table 4, in the presence of amino acid precursors (Estrela et al., 1992), neither adenosine nor ATP affects the rate of GSH synthesis by tumour cells. However, those cells isolated from ATP-treated mice show a significantly lower rate of GSH synthesis as compared with that found in controls (cells isolated from saline-treated mice). Moreover, as occurs for the rates of GSH synthesis, rates of glutathione efflux are also lower in tumour cells from ATP-treated mice (Table 4). Therefore, an increase in the rate of glutathione efflux cannot be argued to explain the ATP-induced loss of intracellular GSH.

These results are in agreement with those previously reported showing that tumour cells only have high rates of GSH synthesis and glutathione efflux when they are actively proliferating (Estrela et al., 1992). In fact, as shown in Table 5, γ -glutamylcysteine synthetase activity, the control step in glutathione synthesis (see, e.g., Deneke and Fanburg, 1989), is decreased in two circumstances where tumour cell proliferation is also decreased: (i) in tumour-bearing mice treated with ATP; (ii) in mice bearing the tumour for 14 days, when animals are

Table 4 Effect of ATP or adenosine on GSH synthesis and glutathione efflux in tumour cells

The initial GSH concentration was of 3.3 μ mol/g ($n = 6$). Tumour cells were isolated from mice bearing the tumour for 7 days and incubated as described in the Materials and methods section for 60 min. Rates of GSH synthesis were calculated from total GSH content in incubations at 0, 30 and 60 min. Rates of glutathione efflux were calculated from contents of glutathione in extracellular medium at 0, 30 and 60 min of incubation. AAS (amino acids) = 5 mM glutamine, 2 mM glycine, 1 mM serine and 1 mM *N*-acetylcysteine. In these experiments, only L-amino acids were used. Glucose (5 mM) was present in all incubations. Adenosine or ATP, when present in the incubation medium, was at 1 mM. The significance test refers to the comparison between the saline-treated group and the ATP-treated group (* $P < 0.05$). ATP (1 mmol/kg) was administered daily, starting 2 h after inoculation of the tumour. No significant differences were found when values were compared within their own group (saline- or ATP-treated mice). All values are means \pm S.D. for 6 different observations.

Additions	Treatment of mice ...	Rate (nmol/min per g)			
		GSH synthesis		Glutathione efflux (GSH + 2GSSG)	
		Saline	ATP	Saline	ATP
AAS		37.1 \pm 5.5	19.1 \pm 3.0*	34.6 \pm 4.5	10.5 \pm 1.1*
AAS + adenosine		35.0 \pm 3.9	20.0 \pm 5.2*	35.5 \pm 5.7	9.4 \pm 0.7*
AAS + ATP		35.5 \pm 6.2	16.5 \pm 4.7*	34.9 \pm 5.1	9.1 \pm 1.0*

close to death and the rate of cell proliferation is very low (Terradez et al., 1993).

Intracellular levels of ATP, ADP, UTP and adenosine in the tumour

Studies *in vitro* on transformed mouse fibroblasts (Weisman et al., 1988) show that extracellular adenine nucleotides, by generating adenosine, may elevate intracellular ATP levels and decrease UTP levels. This effect would suppress growth by an adenosine-dependent pyrimidine starvation. Therefore, we tested in tumour-bearing mice whether ATP administration could lead *in vivo* to a similar situation. As shown in Table 6, ATP administration increases intracellular levels of ATP and ADP, whereas it decreases UTP contents. However, adenosine concentration in the tumour cells does not change (Table 6).

Table 5 Effect of ATP administration on γ -glutamylcysteine synthetase and glutathione synthetase activities in tumour cells

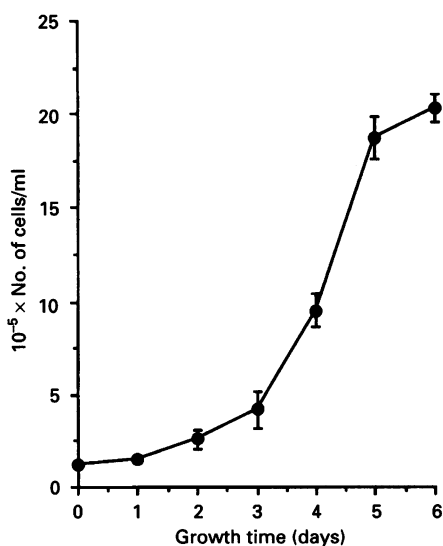
Enzyme activities were measured as described in the Materials and methods section. ATP (1 mmol/kg) was administered daily, starting 2 h after inoculation of the tumour. Data are means \pm S.D. for 5 different animals: *significantly different from control group ('None' treatment and 7 days after inoculation of the tumour; $P < 0.05$).

Activity	Time after inoculation of the tumour (days) ... Treatment of mice ...	7			14
		None	Saline	ATP	None
γ -Glutamylcysteine synthetase (units/mg of protein)		3.8 \pm 0.6	3.9 \pm 0.5	1.5 \pm 0.3*	1.7 \pm 0.4*
Glutathione synthetase (unit/mg protein)		0.4 \pm 0.05	0.4 \pm 0.1	0.3 \pm 0.1	0.3 \pm 0.1

Table 6 Effect of ATP administration on intracellular levels (μ mol/g) of ATP, ADP, UTP and adenosine in the tumour cells

The Ehrlich tumour was allowed to grow for 7 days. ATP (1 mmol/kg) was administered daily, starting 2 h after inoculation of the tumour. Data are means \pm S.D. for 7 different animals: * $P < 0.05$.

Administered substance	ATP	ADP	UTP	Adenosine
Saline	3.0 \pm 0.2	0.4 \pm 0.1	1.1 \pm 0.2	0.3 \pm 0.1
ATP	4.8 \pm 0.3*	1.3 \pm 0.1*	0.5 \pm 0.1*	0.3 \pm 0.05

**Figure 2** Proliferation of Ehrlich ascites-tumour cells *in vitro*

Synchronized cancer cells, previously obtained from mice bearing the tumour for 7 days, were cultured as described in the Materials and methods section. All points are means \pm S.D. for 5 independent experiments.

Growth inhibition by extracellular generation of adenosine

We used cultured tumour cells to study whether hydrolysis of extracellular ATP generates adenosine in the medium. Synchron-

ized Ehrlich tumour cells show *in vitro* (Figure 2) a profile of proliferation similar to that found *in vivo* (see Terradez et al., 1993). However, when non-synchronized cells were cultured, no exponential rates of proliferation could be achieved (results not shown). As shown in Table 7, ATP also inhibits growth *in vitro*. This inhibition is accompanied by a decrease in GSH and UTP contents and an increase in ATP contents in the tumour cells. ATP was also a more effective growth inhibitor than other adenine nucleotides, i.e. AMP (Table 7) or ADP (results not shown), probably because hydrolysis of ATP to adenosine is slower than hydrolysis of AMP or ADP (Weisman et al., 1988). Moreover, as shown in Table 7, results obtained in the presence of adenosine were not significantly different from control values.

When dipyrindamole, an inhibitor of the adenosine translocator in mammalian cells (Fox and Kelley, 1978), was added to the medium, extracellular ATP could not exert its growth-inhibitory effect and, moreover, adenosine accumulated in the extracellular space (Table 7). In addition, when uridine was used to prevent the adenosine-dependent pyrimidine starvation, the rate of cellular proliferation obtained was similar to that in controls (Table 7). Furthermore, when GSH ester was used in the presence of ATP, although intracellular levels of GSH could be restored, values of cellular proliferation remained significantly lower than in controls.

For all the experiments displayed in Table 7, previous concentration-dependent experiments for ATP, AMP, adenosine, dipyrindamole, uridine and GSH ester were done to select the concentrations that show maximum effects (results not shown).

We also tested the growth-inhibitory effect elicited by ATP in medium in which phosphohydrolase activity of serum was inactivated by incubating the serum for 1 h with 1 mM dithiothreitol (Weisman et al., 1988). When ATP (100 μ M) is added to the medium on day 3, it is totally catabolized in about 9 h; however, in medium with no phosphohydrolase activity, ATP catabolism required about 12 h to be complete (results not shown). Therefore tumour cells exhibit substantial ecto-ATPase activity and must participate *in vivo* in generating extracellular adenosine.

Finally, we investigated whether purinoceptor activation could be involved in the ATP-induced growth inhibition. For this purpose, we tested the effect of adenosine 5'-[α -methylene]-triphosphate, a non-metabolizable structural analogue of ATP with purinoceptor activity, in cultured tumour cells. The parameters listed in Table 7 were investigated, but no significant differences were found as compared with controls (results not shown). Therefore, our results suggest that hydrolysis of adenine

Table 7 ATP hydrolysis, generation of extracellular adenosine and inhibition of cell growth

Tumour cells were cultured as described in the Materials and methods section (see also Figure 2). After plating the cells, additions were present in the culture medium from day 4. The experiments were finished 24 h after. Substrates listed under 'Additions' were added at the following final concentrations: ATP (100 μM), AMP (100 μM), adenosine (100 μM), dipyrindamole (2 μM), uridine (10 μM), GSH ester (200 μM). Data are means \pm S.D. for 5 independent experiments (n.d., non-detectable): * $P < 0.05$.

Additions	$10^{-5} \times$ No. of cells/ml	GSH ($\mu\text{mol/g}$)	Intracellular ATP ($\mu\text{mol/g}$)	Intracellular UTP ($\mu\text{mol/g}$)	Extracellular adenosine (μM)
None	19.2 \pm 1.0	2.7 \pm 0.4	2.4 \pm 0.3	1.0 \pm 0.1	n.d.
ATP	10.6 \pm 0.7*	1.6 \pm 0.3*	3.6 \pm 0.5*	0.3 \pm 0.06*	n.d.
AMP	13.3 \pm 0.9*	2.0 \pm 0.2*	3.1 \pm 0.2*	0.5 \pm 0.2*	n.d.
Adenosine	18.3 \pm 0.9	2.2 \pm 0.4	2.6 \pm 0.3	1.0 \pm 0.2	n.d.
ATP + dipyrindamole	18.5 \pm 1.3	2.5 \pm 0.2	2.3 \pm 0.5	0.9 \pm 0.3	67 \pm 24
ATP + uridine	20.3 \pm 1.8	2.5 \pm 0.4	2.6 \pm 0.4	1.1 \pm 0.2	n.d.
ATP + GSH ester	11.2 \pm 0.5*	2.4 \pm 0.3*	3.5 \pm 0.4*	0.4 \pm 0.05*	n.d.

nucleotides rather than purinoceptor activation is the initial step leading to growth inhibition.

DISCUSSION

Approaches to cancer treatment based on modulation of GSH levels must take into consideration the GSH contents and the rate of GSH synthesis in the tumour (Estrela et al., 1992). Indeed, depletion of cellular GSH by BSO *in vitro* increases the sensitivity to cytotoxic chemotherapy and radiation (Crook et al., 1986; Mitchell et al., 1989). The present results show a methodology *in vivo*, based on administration of exogenous ATP, that can decrease GSH contents in proliferating tumour cells without affecting GSH contents in normal cells (Table 3). Moreover, ATP administration inhibits tumour growth (Figure 1 and Table 1).

Extracellular ATP has multiple roles in cell physiology and pathophysiology (for a review, see Gordon, 1986). ATP administration appears beneficial for the survival of animals suffering endotoxin (Filkins and Buchanan, 1977) and haemorrhagic shock (Chaudry et al., 1974), sepsis (Chaudry et al., 1980), and intestinal (Cikrit et al., 1983), hepatic (Hirasawa et al., 1978) or renal (Sumpio et al., 1987) ischaemia. These beneficial effects have been ascribed to an increase in tissue ATP levels (Chaudry, 1983). On the other hand, studies *in vitro* suggest that various lines of transformed cells, but not of non-transformed cells, grown in culture show passive permeability to normally impermeant compounds, i.e. nucleotides, when inoculated with extracellular ATP (with as little as 150 μM) in an alkaline medium low in bivalent cations (Weisman et al., 1984). Before this event there is a dramatic Na^+ influx and K^+ efflux, and a decrease in the plasma-membrane potential (Weisman et al., 1984). However, these effects could not be reproduced when cells were incubated in growth medium (Weisman et al., 1988). Further, it has been suggested that nuclear ATP pools and ATP/ADP ratios act as S-phase controls, regulating DNA elongation at sites where its synthesis has previously been initiated by cytoplasmic factors (Rapaport et al., 1979).

Weisman et al. (1988) reported experiments *in vitro* showing that growth inhibition of transformed mouse fibroblasts by adenine nucleotides occurs via generation of extracellular adenosine. In agreement with our results (Table 1), Weisman et al. (1988) showed that adenosine had no significant effect on cell growth *in vitro* at concentrations below 1 mM, although ATP, ADP or AMP effects appeared to be mediated by serum- and cell-associated hydrolysis of the nucleotides to adenosine (see

also Table 7). As shown in Table 2, administration of ATP, but not of adenosine, expands erythrocyte and plasma ATP pools during several hours. Then, as suggested by Rapaport and Fontaine (1989a), red blood cells may release ATP slowly into the plasma, and growth inhibition of tumour cells could be explained by the slow generation of adenosine derived from nucleotide catabolism on cell surfaces and in serum. However, adenosine was undetectable in plasma, either in controls or in ATP-treated mice. The expansion of erythrocyte ATP pools after ATP administration *in vivo* is presumably the result of its dephosphorylation, followed by the erythrocyte uptake of the adenosine that is generated *in situ* (Rapaport and Fontaine, 1989a). Interestingly, as compared with murine models, human blood and tissues have less soluble and ecto-enzymic activities capable of degrading ATP (Trams et al., 1980). Thus, perhaps in humans it will be possible to achieve effective ATP levels in erythrocytes or the plasma by administering less than 1 mmol of ATP/kg.

Our results and those of Weisman et al. (1988) are also in agreement on the adenosine-dependent pyrimidine starvation effect. As shown in Table 6, ATP administration decreases UTP contents and increases adenine nucleotide contents in tumour cells. This effect may be reversed, in the presence of ATP, by uridine (Table 7). Elevation of intracellular ATP and ADP levels may inhibit the orotate phosphoribosyltransferase (Snyder and Seegmiller, 1976), decreasing UTP and DNA synthesis (Fox and Kelley, 1978). Uridine would prevent the growth-inhibitory effect of ATP by avoiding the inhibited step in pyrimidine biosynthesis (Fox and Kelley, 1978; Table 7). Finally, the fact that the adenosine content in the tumour cells does not change (Table 6) can be explained either by the rapid utilization of adenosine for the synthesis of adenine nucleotides or by its rapid catabolism to inosine and hypoxanthine (Weisman et al., 1988; Asensi et al., 1991).

As shown in Table 7, when added to the culture medium, adenosine appears unable to affect cell growth. This can be explained by the following: (i) adenosine is transported into cells via an adenosine translocator and metabolized by different enzymes, including adenosine kinase and adenosine deaminase (Lum et al., 1979); (ii) adenosine is more rapidly metabolized than ATP (Weisman et al., 1988); (iii) adenosine kinase is substrate-inhibited at concentrations exceeding several micromolar, allowing most adenosine to be metabolized via adenosine deaminase (Archer et al., 1985). Indeed, the K_m for adenosine kinase is in the low-micromolar range (< 10 μM) (Mills et al., 1976; Perrett and Dean, 1977). In the case of added ATP (Table

7), which requires a long period (about 9 h) to be catabolized, a constant flux of adenosine in the very-low-micromolar range must be essentially metabolized through adenosine kinase (Rapaport and Fontaine, 1989a). These facts would explain the inability of adenosine, when administered, to inhibit growth *in vivo*.

Further, our experimental set-up suggests that ATP concentrations of several millimolar will be reached upon intraperitoneal injection in the ascites fluid, and such concentrations have been reported to disrupt membrane integrity and destroy cells (see, e.g., Gordon, 1986). However, high concentrations of extracellular ATP are not immediately cytotoxic and need to be present for at least 60–120 min to produce irreversible cell damage (Nagelkerke et al., 1989). In fact, as mentioned in the Results section, ATP is removed from the peritoneum after 40–60 min of the intraperitoneal administration. Part must be degraded within the peritoneal cavity, but part enters the blood-stream, expanding erythrocyte and plasma pools (Table 2). Thus, possible ATP-mediated cytotoxic effects on tumour cells do not seem responsible for growth inhibition.

Finally, we propose the following mechanism to explain the ATP-induced GSH depletion in cancer cells. (i) Extracellular ATP is catabolized, adenosine is taken up into cells, and then tumour growth is inhibited by an adenosine-dependent pyrimidine starvation effect, leading to a decrease in the intracellular levels of UTP. (ii) Growth inhibition is associated with lower rates of protein synthesis and, consequently, lower γ -glutamylcysteine synthetase activity. (iii) This leads to lower rates of GSH synthesis, promoting a decrease in the intracellular contents of GSH in cancer cells. Therefore, by inhibiting adenosine uptake, we block the first step and GSH remains as in controls (see Table 7). Thus, in this case, our results suggest that GSH depletion is a consequence of growth inhibition, and not the primary cause.

In conclusion, the relevant facts reported in this paper are: (1) exogenous administration of ATP inhibits cancer growth and decreases GSH contents in the tumour, but not in normal cells; (2) growth inhibition appears to be mediated by hydrolysis of the nucleotide to adenosine, which subsequently will increase intracellular ATP and will decrease UTP levels in the cancer cells; (3) the decrease in GSH levels accompanying inhibition of cell proliferation is a consequence of the decrease in γ -glutamylcysteine synthetase activity; (4) extracellular ATP leads to the expansion of erythrocyte ATP pools, which may ensure a maintained adenosine flux to the plasma during several hours; (5) exogenous administration of adenosine is much less effective than ATP in inhibiting tumour growth, presumably because it is very rapidly metabolized; (6) this is the first time that selective GSH depletion in proliferating tumour cells has been achieved under conditions *in vivo*.

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