Acta Oncologica Vol. 34, No. 3, pp. 419–422, 1995

EFFECT OF INTRAPERITONEAL ATP ON TUMOR GROWTH AND BONE MARROW RADIATION TOLERANCE

JOHN FROIO, EDWARD H. ABRAHAM, RAJESH SONI, ALAN EPSTEIN and PAUL OKUNIEFF

Transplants of a spontaneous murine fibrosarcoma (FSaII) treated with intraperitoneal ATP were studied in vitro, and in both C3H and *nu/nu* mice. Daily ATP treatment prolonged tumor volume doubling time in vivo and in vitro. Daily ATP treatments at the maximally tolerated dose (2 mmol/kg i.p.) did not significantly affect the pH or the PCr/P_i, or $\beta ATP/P_i$ ratios (measured by MRS). In contrast to the reduced tumor growth rate, there was no change in bone marrow recovery after whole body irradiation. ATP is minimally toxic to animals at active dose levels. It slows tumor growth rate without adversely affecting bone marrow radiation tolerance. ATP might therefore be useful as a biological modifier of chemotherapy or radiation therapy.

Micromolar concentrations of ATP significantly reduce proliferation of both human and murine tumor cell lines in vitro (1–3). Similarly, reductions in growth rate have also been observed in murine tumor models (1). In contrast, adenosine has little or no effect on proliferation while AMP and ADP have variable effects on cellular growth (1, 3). The mechanism of the altered growth rate under either condition is unknown.

The anti-cancer effects of adenine nucleotides measured in vivo may be the result of direct interactions between ATP and tumor cells, or may be secondary to modifications of host physiology. Extracellular adenosine and ATP can affect cardiovascular (4–6), immune (6–9), neurologic (10, 11), and muscular functions (4). Recently, we presented evidence that ATP can slow the growth of a murine mesenchymal tumor, and that the effect was unlikely to be due to physiological alterations (12). In the present report we examined the differential effect of ATP between tumor and bone marrow.

Received 25 September 1994.
Accepted 3 January 1995.
From the Radiation Oncology Branch, National Cancer Institute, Bethesda, MD, USA.
Correspondence to: Paul Okunieff, M.D., Radiation Oncology Branch, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Bldg 10 Rm B3B69, Bethesda, MD 20892, USA.
Paper presented at the Tumour Microenvironment Workshop in Granada, Spain, September 23–25, 1994.

Material and Methods

Sodium adenosine 5'-triphosphate (ATP) was diluted in phosphate buffered saline. Buffered ATP (pH = 7.0 to 7.2) was delivered at doses ranging from 0.5 to 4 mmol/kg i.p./day in volumes of 0.04 ml/g body weight (BW). For in vitro studies, culture solutions consisted of Dulbecco's modified eagles media (DMEM) alone, or DMEM containing ATP.

Experimental animals were 8–12-week-old female and male C3H and *nu/nu* mice. Early generation isotransplants of a spontaneous murine fibrosarcoma (FSaII) were used.

FSaII cells were cultured in 175 mm^3 cell culture flasks (Falcon, Becton Dickinson, Oxnard, CA) until the cells reached confluence. This original solution was diluted so that 5 000 Trypan Blue excluding cells were placed into each well in plastic 24-well trays (Falcon). Cells were cultured in growth media with 0, 50, 100, or 500 μM ATP. Every 24 h, 4 wells from each test group were trypsinized and counted using an electronic cell counter (Electrozone, Particle Data, Inc., Elmhurst, IL). ATP containing medium was refreshed daily.

Tumor volumes and mouse weights were determined at least three times each week. The first dose of ATP was given 5 h after tumor injection. Subsequent daily saline or ATP injections were made based on the most recent mouse weight. Volumes were calculated using an ellipsoid approximation and the three orthogonal diameters measured by caliper [$V = (\pi/6) \times d_1 \times d_2 \times d_3$]. Tumor growth delay (TGD) was defined as the time for test group tumors to

 ISSN 0284-186X

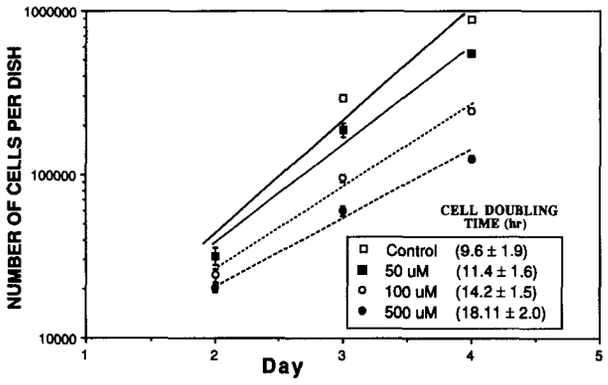

Fig. 1. Cell number per well at various times after inoculation of 5 000 cells per well. Each point represents the mean ± SE of counts from 4 measurements. ATP containing media was refreshed daily. Values differed significantly from control beginning on day 3.

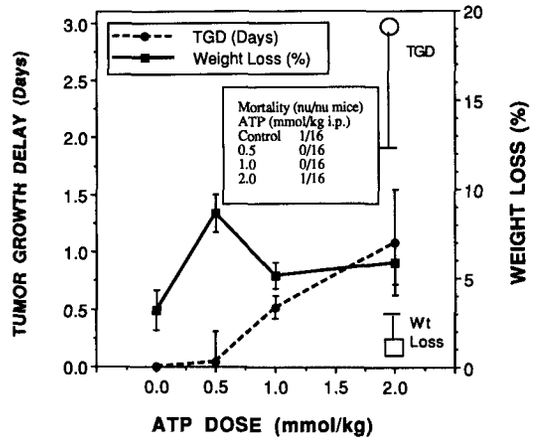

Fig. 2. Tumor growth delay (TGD) were determined as a function of daily i.p. ATP dose. There was a dose dependent increase in TGD with increasing ATP dose in *nu/nu* mice (filled symbols). Weight loss and mortality were also measured in the same groups of animals. Daily ATP doses of 2 mmol/kg or less had no greater weight loss or mortality than daily saline treatments. TGD was measured with ATP 2 mmol/kg/day i.p. in C3H mice (open symbols), and were longer than that observed in *nu/nu* mice. ATP at a daily i.p. dose of 4 mmol/kg (not shown) is toxic. Values are means ± 1 SE.

reach 800 μl volume minus time for control tumors to reach 800 μl volume. ^{31}P NMR were performed using previously described techniques (13). $LD_{50/30}$ experiments were performed on C3H mice. Whole body radiation was delivered 4 h after a first 2 mmol/kg i.p. ATP dose. ATP was then delivered daily. Control groups recieved daily saline injections. Radiation doses ranged from 6.5 to 8.5 Gy. Survival at 30 days was used as the endpoint. Experimental data was fit to a logit regression for calculation of $LD_{50/30}$ values and lethality curves. Student's t-test was used for other statistical comparisons.

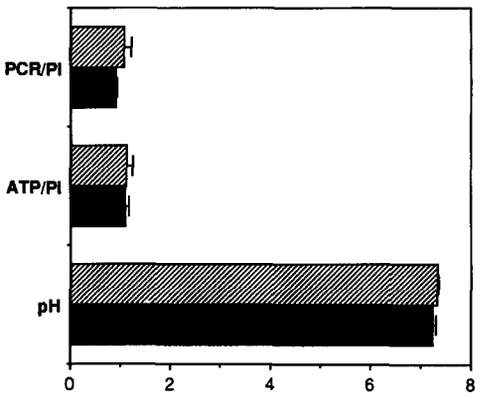

Fig. 3. In vivo ^{31}P NMR of tumors growing in *nu/nu* mice treated with daily ATP (2 mmol/kg i.p.) or saline. There was no reduction of PCr/Pi or βATP/P_i or in tumor pH due to ATP treatment. Values are means ± 1 SE. ▨: APT; ▨: Control.

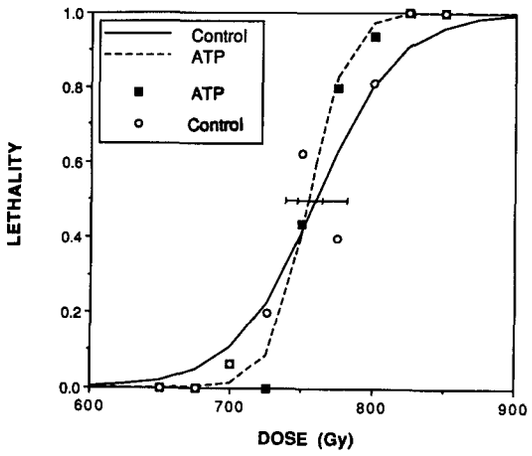

Fig. 4. Calculated curves are the composite of three experiments. The SD of the $LD_{50/30}$ is plotted.

Results

ATP added to the growth media inhibited cellular proliferation; the magnitude of the effect corresponded to the ATP concentration. As shown in Fig. 1, there was a dose-dependent decrease in growth rate when ATP concentrations of 50, 100, and 500 μM were added to the growth media. Cell numbers per tray were significantly reduced by day 3 for all dosages of ATP tested ($p < 0.01$).

ATP inhibited tumor growth in both C3H and *nu/nu* mice. The TGD were significant for *nu/nu* and C3H mice treated with daily doses of 2 mmol/kg i.p. ($p < 0.05$; Fig. 2). Animal weights and mortality were measured as a means of assessing chronic systemic ATP toxicity. There was no significant weight loss or increase in mortality with ATP doses $\leqslant$2 mmol/kg/day i.p. (Fig. 2). Likewise, daily ATP had no detectable effects on tumor metabolic state as measured by ^{31}P NMR (Fig. 3).

A dose of 2 mmol/kg i.p. ATP had no radiomodifying effect on C3H mice when delivered daily at a dose and

schedule capable of significantly reducing tumor growth rate. Dose response curves for the combined results of 3 experiments are shown in Fig. 4.

Discussion

In the late 1960's it was shown that cAMP and other cyclic nucleotides were inhibitors of tumor cell proliferation both in vivo and in vitro (14–16). Later NAD and other adenine nucleotides were found to be among the most potent purine proliferation inhibitors active at concentrations as low as 50 μM in human tumor cell lines (17). Adenine nucleotides were thought to operate only by cytostasis since, unlike most chemotherapuetic agents, there was no giant cell formation, and ^{3}H-thymidine incorporation and protein synthesis were not impaired. Adenine nucleotides can induce tumor cell apoptosis, alter membrane permeability, and increase intracellular free Ca^{2+}; these effects could result in cell death without the histological changes typical of most therapeutic agents. The antiproliferative effect of adenine nucleotides is seen in vitro (1) and is observed in very low concentrations without being transported intracellularly; hence it has been concluded by several authors that the P2 class of purinoreceptors are responsible for the effect.

Daily ATP injections increase the blood ATP levels chronically by augmenting the total body purine pool. Most intraperitoneally injected ATP is removed during the first pass through the liver. The liver ATP is packaged into the RBC which then produce a chronic increase in blood and presumably tissue ATP levels. Hence, daily ATP therapy acts to chronically increase the level of this nutrient (18).

Interestingly, ATP given at the time of abdominal radiation prevents late radiation damage of the gut (19). In these experiments, blood pressure, body temperature and oxygenation were maintained in a swine model, hence, physiologic alteration (hypoxia) is an unlikely mechanism of the radioprotection. In contrast, our studies featuring radiation tolerance of bone marrow to whole body radiation showed no radioprotection.

In vitro studies of FSaII demonstrated a clear dose-dependent decrease in cell number for exponentially growing murine fibrosarcoma cells even at the lowest ATP concentration (50 μM). There was no apparent increase in floating dead cells for concentrations less than 500 μM. Likewise, histological analysis of tumors in vivo did not show grossly increased regions of necrosis, and metabolic parameters as measured by ^{31}P NMR were not affected. Hence the evidence favors a cytostatic rather than cytotoxic effect in the FSaII tumor model. Of note, the response of FSaII is less dramatic than that of several other in vitro and in vivo tumor models (2, 17, 18).

Tumors of ATP-treated C3H mice demonstrated a 28% increase in volume doubling time over the saline controls ($p < 0.01$), and this corresponded to a modestly longer TGD. For C3H mice the TGD was $\approx$3 days. One can compare this delay to FSaII responses to chemotherapy in the literature. Response to ATP from day of implantation produced a growth delay similar to the response at the maximum tolerated dose of cyclophosphamide (200 to 250 mg/kg; $\approx$3.5 days (20, 21)), and of BCNU (3 to 4 mg/kg; 2 to 4 days (22)), and to 20 Gy radiation in a single fraction (3.6 days (23)). The growth delay was less than the most active agent, cis-DDP (13 mg/kg; $\approx$8.5 days (24)). These comparisons to chronic ATP administration should be interpreted with care, since the published works all featured established tumors.

In conclusion, any systemic toxicity of ATP at doses capable of producing significant growth retardation of FSaII tumors was temporary. Tumor growth rate was reduced in vitro and in vivo. In contrast, proliferative response of bone marrow to radiation did not seem to be affected by ATP. The utility of ATP in an aggressive murine fibrosarcoma was modest compared to reports in other tumor models. Since ATP is minimally toxic to animals at active dose levels it might be useful as a biological modifier of chemotherapy or radiation therapy.

REFERENCES

1. Rapaport E. Mechanisms of anticancer activities of adenine nucleotides in tumor-bearing hosts. Ann NY Acad Sci 1990; 603: 142–50.
2. Rapaport E, Fishman RF, Gercel C. Growth inhibition of human tumor cells in soft-agar cultures by treatment with low levels of adenosine 5′-triphosphate. Cancer Res 1983; 43: 4402–6.
3. Weisman GA, Lustig KD, Lane E, Huang N, Belzer I, Friedberg I. Growth inhibition of transformed mouse fibroblasts by adenine nucleotides occurs via generation of extracellular adenosine. J Biol Chem 1988; 263: 12367–72.
4. Gaba SJM, Prefaut C. Comparison of pulmonary and systemic effects of adenosine triphosphate in chronic obstructive pulmonary disease—ATP: A pulmonary controlled vasoregulator? Eur Respir J 1986; 233: 309–19.
5. Burnstock G, Kennedy C. A dual function for adenosine 5′-triphosphate in the regulation of vascular tone. Circ Res 1986; 58: 319–30.
6. Gordon JL. Extracellular ATP: Effects, sources and fate. Biochem J 1986; 233: 309–19.
7. Sung SJ, Young JD, Origlio AM, Heiple JM, Kaback HR, Silverstein SC. Extracellular ATP perturbs transmembrane ion fluxes, elevates cytosolic [Ca^{2+}], and inhibits phagocytosis in mouse macrophages. J Biol Chem 1985; 260: 13442–9.
8. Cowen DS, Lazarus HM, Shurin SB, Stoll SE, Dubyak GR. Extracellular adenosine triphosphate activates calcium mobilization in human phagocytic leukocytes and neutrophil/monocyte progenitor cells. J Clin Invest 1988; 83: 1651–60.
9. Redegeld F, Filippini A, Sitkovsky M. Comparative studies of the cytotoxic T lymphocyte-mediated cytotoxicity and of extracellular ATP-induced cell lysis. J Immunol 1991; 147: 3638–45.
10. Gomaa AA. Characteristics of analgesia induced by adenosine triphosphate. Pharmacol Toxicol 1987; 61: 199–202.

11. Inoue K, Nakazawa K, Fujimori K, Takanaka A. Extracellular adenosine 5'-triphosphate-evoked norepinephrine secretion not relating to voltage-gated Ca channels in pheochromocytoma PC12 cells. Neuroscience Letters 1989; 106: 294–9.
12. Okunieff P, Froio J, Soni R, et al. Tumor growth retardation and pathophysiological effects of adenosine 5'-triphosphate therapy measured by thallium washout, ^{31}P NMR, laser Doppler flowmetry, and intradermal angiogenesis. Radiat Oncol Invest 1994; 1: 346–60.
13. Okunieff P, Kallinowski F, Vaupel P, Neuringer LJ. Effects of hydralazine-induced vasodilation on the energy metabolism of murine tumors studied by in vivo ^{31}P-nuclear magnetic resonance spectroscopy. JNCI 1988; 80: 745–50.
14. Nolde S, Hilz H. The cytostatic action of extracellular NAD in tumor-bearing mice. Br J Cancer 1972; 26: 299–303.
15. Gericke D, Chandra P. Inhibition of tumor growth by nucleotide cyclic 3',5'-monophosphates. Hoppe-Seyler's Z Physiol Chem 1969; 350: 1469–71.
16. Ryan WL, Heidrick ML. Inhibition of cell growth in vitro by adenosine 3',5'-monophosphate. Science 1968; 162: 1484.
17. Fang W, Pirnia F, Bang Y, Myers CE, Trepel JB. P_2-Purinergic receptor agonists inhibit the growth of androgen-independent prostate carcinoma cells. J Clin Invest 1992; 89: 191–6.
18. Rapaport E, Fontaine J. Anticancer activities of adenine nucleotides in mice are mediated through expansion of erythrocyte ATP pools. Proc Natl Acad Sci USA 1989; 86: 1662–6.
19. Senagore AJ, Milsom JW, Walshaw RK, Mostoskey U, Dunstan R, Chaudry IH. Adenosine triphosphate-magnesium chloride in radiation injury. Surgery 1992; 112: 933–9.
20. Urano M, Kim MS, Kahn J, Kenton LA, Li ML. Effect of thermochemotherapy (Combined cyclophosphamide and hyperthermia) given at various temperatures with or without glucose administration on a murine fibrosarcoma. Cancer Res 1985; 45: 4162–6.
21. Urano M, Kim MS. Effect of hyperglycemia on thermochemotherapy of a spontaneous murine fibrosarcoma. Cancer Res 1983; 43: 3041–4.
22. Urano M, Majima H, Miller R, Kahn J. Cytotoxic effects of 1,3 bis (2-chloroethyl)-N-nitrosourea at elevated temperatures: Arrhenius plot analysis of tumour response. Int J Hyperthermia 1991; 7: 499–510.
23. Okunieff PG, Suit HD. Toxicity, radiation sensitivity modification, and combined drug effects of ascorbic acid with misonidazole in vivo on FSaII murine fibrosarcomas. J Natl Cancer Inst 1987; 79: 377–81.
24. Urano M, Kahn J, Kenton LA. The effect of cis-diamminedichloroplatinum(II) treatment at elevated temperatures on murine fibrosarcoma, FSa-II. Int J Hyperthermia 1990; 6: 536–70.