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Advances in Brief

The Extracellular Fluid of Solid Carcinomas Contains Immunosuppressive Concentrations of Adenosine[1]

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Abstract

The purine nucleoside adenosine (9-β-D-ribofuranosyladenine) inhibits a number of lymphocyte functions *in vitro*, including the ability of activated T lymphocytes and natural killer cells to adhere to and kill tumor targets. Solid tumors, such as adenocarcinomas of the lung and colon, are frequently hypoxic and are, therefore, likely to exhibit increased adenine nucleotide breakdown through the 5′-nucleotidase pathway, yielding adenosine. We examined whether the concentration of adenosine in the extracellular fluid of such tumors is adequate to cause immunosuppression. Murine tumors grown in syngeneic hosts or human tumors grown in immunodeficient *nu/nu* mice were subjected to microdialysis, and adenosine levels in the microdialysate were measured by high-performance liquid chromatography. Treatment of the tumor microdialysates with adenosine deaminase eliminated the adenosine peak. Recovery of adenosine ranged from 15 to 29%, depending on the microdialysis probe, and concentrations of adenosine in tumors ranged from 0.2 to 2.4 μM with a mean of 0.5 μM. In contrast, the adenosine concentration measured s.c. at the same location was 30 ± 5 nM (mean ± SE). Inclusion of the adenosine deaminase inhibitor coformycin (10 μM) and the adenosine kinase inhibitor 5′-iodotubercidin (0.1 μM) in the microdialysis perfusion buffer increased extracellular adenosine concentration in tumors to as high as 13 μM. These data show that extracellular adenosine levels in solid tumors are sufficient to suppress the local antitumor immune response and that interference with pathways of adenosine metabolism causes marked increases in tumor extracellular adenosine concentration.

Introduction

Adenosine is a purine nucleoside that is increasingly being recognized for its ability to modulate inflammatory processes and the immune response. The ability of adenosine to suppress lymphocyte-mediated cytolysis of tumor or leukemic cell targets (1, 2) suggests that it might have the ability to suppress the anticancer immune response. This may be particularly important in hypoxic solid tumors, because hypoxia leads to increased adenosine levels (3). In addition to interfering with cell-mediated lysis of tumor cells, adenosine also inhibits other aspects of the antitumor immune response, including T-killer cell activation (2), adhesion of activated T-killer cells to carcinoma cell targets (4), and granule exocytosis by natural killer cells (5). Its ability to interfere with the initial interaction between T-killer cells and tumor targets is mediated through lymphocyte A_3 adenosine receptors (4). Adenosine also interferes with the host response by suppressing the production (6) and cytolytic action (7) of tumor necrosis factor, again likely acting through A_3 adenosine receptors, this time on macrophages (8).

Our observations of adenosine suppression of T-killer cell function have led us to propose that adenosine may act as a local immunosuppressant within the microenvironment of solid tumors (2, 4). The key question on which such a hypothesis depends is whether the concentrations of adenosine present in the ECF[3] of solid tumors are sufficient to cause immunosuppression. Concentrations of adenosine measured in homogenates of tumor tissue or cultured tumor spheroids[4] may reach 10^{-4} M, but this is likely a gross overestimate of the extracellular level due to the contribution of adenosine from intracellular pools. In this study, we report the results of experiments using microdialysis of tumor tissue *in vivo* to sample the ECF for adenosine measurement. We find that the levels of adenosine in the ECF are sufficient to cause immunosuppression.

Materials and Methods

Materials. Culture media were from ICN-Flow (St. Laurent, Quebec, Canada). Serum and all other culture materials were from Life Technologies, Inc. (Burlington, Ontario, Canada). Female C57 BL/6 and BALB/c *nu/nu* mice, 6–8 weeks of age, were obtained from Charles River Canada (Lasalle, Quebec, Canada). Microdialysis probes were from Chromatography Sciences Company (Montreal, Quebec, Canada). Coformycin was from Calbiochem (La Jolla, CA), and 5′-iodotubercidin was from Research Biochemicals, Inc. (Natick, MA).

Tumor Model. Carcinoma cell lines were cultured in 80-cm^2 flasks in DMEM supplemented with 5% (v/v) newborn calf serum. Cells were detached from confluent monolayer cultures by trypsinization and washed in medium prior to tumor seeding.

Mouse MCA-38 colon carcinoma cells [2–5 × 10^6 cells in 100 μl of RPMI 1640 supplemented with 20 mM HEPES (pH 7.4)] were injected s.c. into the flank of syngeneic (C57BL/6) mice. Human lung (A549) or colon (T-84 and HT-29) carcinoma cells were similarly seeded into the flanks of athymic BALB/c nude (*nu/nu*) mice. C3H/HEJ fibrosarcoma-bearing mice were kindly provided by Dr. Gurmit Singh (Hamilton Regional Cancer Center, Ontario, Canada).

Microdialysis and Adenosine Measurement. Tumor-bearing or control animals for microdialysis were anesthetized with metofane/pentobarbitol and immobilized on a heating pad to maintain body temperature. A small incision was made over the projected implantation site, and the probe (CMA/10 or CMA/20, 0.5-mm diameter; dialysis membrane length, 4 mm) was inserted and held in the appropriate position by a micromanipulator clamp stand. The standard solution for perfusion was PBS (containing 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}) at pH 6.8. This pH was chosen because it more closely represents that which exists within solid tumors (9, 10) than the standard physiological pH of 7.4.

Following implantation, the probes were flushed continuously for at least 30 min at 10 μl/min prior to collecting dialysate for adenosine measurement. This was shown to be sufficient time for a steady state to be attained (Fig. 1). Microdialysis was then carried out at a flow rate of 1–2 μl/min, and samples were taken for adenosine assay. Typically, samples were collected over 10–30-min intervals. Where indicated, coformycin (final concentration, 10 μM) and/or 5′-iodotubercidin (0.1 μM) were included in the microperfusion buffer.

Adenosine was assayed in microdialysates as described (11). Briefly, sam-

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[3] The abbreviations used are: ECF, extracellular fluid; ADA, adenosine deaminase.

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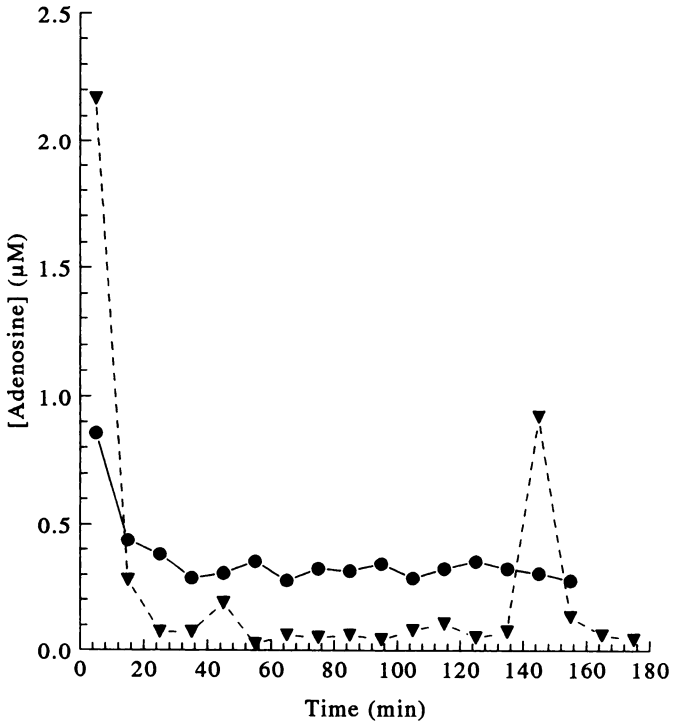

Fig. 1. Time course of changes in measured adenosine levels following probe implantation. Adenosine concentrations were measured in the microdialysate from murine tumors over 10-min intervals beginning immediately following insertion and positioning of the probe. The data are from individual animals bearing an MCA-38 tumor (●) or a fibrosarcoma (▼). The increase in apparent adenosine concentration at 145 min for the fibrosarcoma correlates in time with movement of the microdialysis probe.

ples were subjected to $ZnSO_4/Ba(OH)_2$ precipitation and then derivatized with chloroacetaldehyde prior to C_{18} reverse-phase high-performance liquid chromatography and fluorescence detection of the etheno derivative of adenosine. The peak corresponding to adenosine was completely eliminated by pretreatment of the samples with ADA (1 unit for 1 min at room temperature). Chromatography of coformycin and 5′-iodotubercidin confirmed that neither substance caused false-positive results in the adenosine measurement.

Probes were calibrated for recovery of adenosine by dialysis of adenosine standard solutions. Mean recoveries were: CMA/10 probe, 14.7% at 2 μl/min; CMA/20 probe, 16.4% at 2 μl/min and 29.1% at 1 μl/min.

Results

We first set out to determine the appropriate time frame for measurement of adenosine concentrations in tumor ECF following implantation of the microdialysis probe. Fig. 1 shows a typical time course of measured extracellular adenosine for an MCA-38 colon carcinoma and a fibrosarcoma growing as solid tumors in syngeneic mice (C57BL/6 and C3H/HEJ, respectively). Samples were taken with a flow rate of 2 μl/min from the start of dialysis. Measured adenosine concentrations were initially high but fell rapidly to a steady level by about 30 min after initial probe insertion. The initial high level of adenosine is likely due to acute regional release as a consequence of the trauma of inserting the microdialysis probe. The effect of trauma in provoking a transient adenosine release is also evident in the peak at the 145-min time point for the fibrosarcoma (Fig. 1), which correlated with accidental movement of the probe. Again, there was a rapid decline in the adenosine that had been released due to mechanical damage.

In subsequent experiments, probes were perfused for a minimum of 30 min at 10 μl/min before beginning sample collection to exclude the contribution of trauma-evoked adenosine. Fig. 2 shows adenosine measurements from MCA-38 murine tumors and three different human carcinomas grown as xenografts in athymic BALB/c nude (*nu/nu*) mice. The human tumors were A549 (lung), T-84 (colon), and HT-29 (colon). Extracellular adenosine levels measured using a CMA/10 or CMA/20 probe with a 4-mm dialysis membrane length were fairly reproducible for tumors from T-84 and HT-29 human cell lines and less so for A549 tumors. The A549 tumor tissue was less homogeneous than that of the colon carcinomas, consistent with regional variation in the concentration of this metabolic product. The mean adenosine concentrations (± SE) in tumors were: MCA-38, 0.36 ± 0.13 μM; A549, 0.56 ± 0.17 μM; T-84, 0.48 ± 0.02 μM; and HT-29, 0.47 ± 0.04 μM. The mean extracellular adenosine concentration measured using this probe for all human tumors was 0.49 ± 0.05 μM.

Treatment of tumor microdialysates with ADA (1 unit for 1 min at 25°C), which degrades adenosine to inosine, completely eliminated the adenosine (data not shown). Sampling of the s.c. tissue at an equivalent site on the control flank revealed only low levels of adenosine (30 ± 5 nM) by comparison with the tumor tissue.

Comparisons of adenosine concentrations with gross tumor weight for the human carcinoma xenografts (Fig. 3) revealed no significant association between tumor size and adenosine level, either for tumors overall ($r = 0.4$, $P > 0.9$) or for the different tumor types individually.

Finally, we studied the influence of blocking elimination pathways on the turnover of adenosine within these solid tumors. To this end, we perfused the microdialysis probes with coformycin (10 μM) to block ADA or 5′-iodotubercidin (0.1 μM), an inhibitor of adenosine kinase. These inhibitors were infused for 60 min prior to adenosine measurements. Neither of these agents interfered with the adenosine assay itself or the recovery of adenosine across the microdialysis membrane (data not shown). Instillation of these inhibitors into the locale of the probe when implanted into HT-29 tumors led to increases in the extracellular adenosine concentration (Fig. 4). The presence of

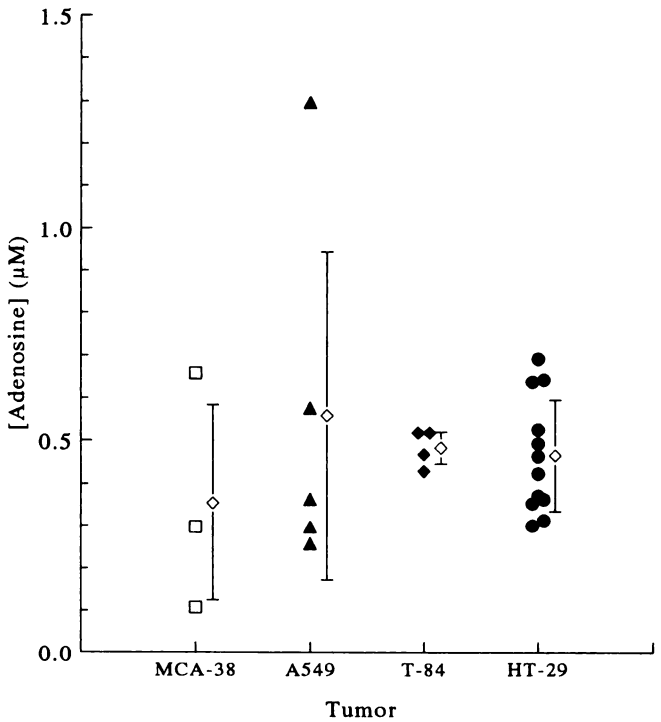

Fig. 2. Extracellular adenosine levels in mouse and human solid adenocarcinomas. Tumors from mouse colon (MCA-38), human colon (T-84 and HT-29), and human lung (A549) carcinomas were implanted with CMA/10 (MCA-38) or CMA/20 (T-84, HT-29, and A549) microdialysis probes, and the steady-state concentration of extracellular adenosine was measured. The points represent measurements from individual tumors. Mean values (◇) and SDs (*bars*) for each group are shown.

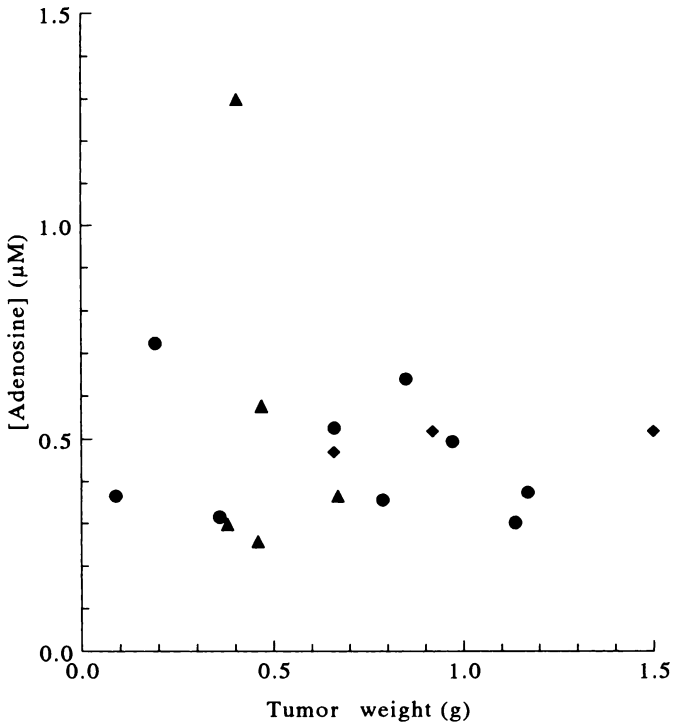

Fig. 3. Relationship between tumor mass and adenosine concentration. Adenosine levels were measured in A549 (▲), HT-29 (●), or T-84 (♦) carcinomas. Following microdialysis, the tumors were carefully excised, dissected free of connective tissue, and weighed.

coformycin caused a significant ($P < 0.05$) increase in adenosine, raising the level 12-fold. 5′-Iodotubercidin at 0.1 μM had little effect by itself but together with coformycin increased the extracellular adenosine concentrations to a mean of 8.9 μM (Fig. 4), and in other experiments, to as high as 13 μM. Similar perfusion of microdialysis probes implanted in A549 tumors with coformycin and 5′-iodotubercidin also raised the measured adenosine levels (mean ± SE, 8.4 ± 2.7 μM).

Discussion

We have shown, using microdialysis, that the extracellular fluid of solid tumors contains significant levels of adenosine. The concentration measured in MCA-38 mouse tumors (0.36 ± 0.13 μM) approximates the EC_{50} of adenosine for inhibition of binding of anti-CD3-activated T killer lymphocytes to MCA-38 monolayers (4), indicating that endogenous adenosine levels in tumors are sufficient to effect immunosuppression. Inhibition of the cell-mediated antitumor immune response by adenosine *in vivo* would serve to remove a restraint on tumor expansion.

Concentrations of adenosine in tumors were 10- to 20-fold higher than those measured s.c. at the same location. Extracellular adenosine concentrations reported for s.c. tissue in humans (12) are similarly low in comparison to the values that we have measured in human tumor xenografts. We do not know whether the increased levels of adenosine in tumors will have any impact on metabolites that are released into the circulation. However, it is noteworthy that: (*a*) the amount of 1-methyladenosine is increased in the serum of mice with solid tumors (13); and (*b*) plasma levels of the vitamin B_6 conjugate adenosine-N^6-diethylthioether-*N*-pyridoximine-5′-phosphate, which has been proposed as a tumor marker, are increased in patients with solid cancers (14).

Adenosine concentrations did not correlate with tumor size, over a range of tumor masses from 0.1 to 1.5 g (Fig. 3). This suggests either that levels of adenosine are not primarily determined by the degree of hypoxia or that the areas of hypoxia in tumors at this stage of growth are determined on a much smaller scale. In fact, the hypoxic regions in such tumors exist around microvessels on the scale of 50–200 micrometers (15), much less than the dimensions of the membrane portion (4-mm length, 0.5-mm diameter) of the dialysis probe used in these studies. The adenosine levels that we have measured in this approach will be mean values for the zone of tumor tissue that surrounds the microdialysis probe. Such mean concentrations will reflect the contributions both from hypoxic areas, within which adenosine levels are likely to be elevated, and from normoxic areas within that volume. Leith *et al.* (16) have estimated the mean hypoxic fraction of HT-29 tumors grown in nude mice, as we have done, to be 16.8%. This would suggest that the concentration of adenosine within hypoxic areas in those tumors might actually be in the region of 2.8 μM. If local concentrations of adenosine are indeed higher in hypoxic areas, then such high local adenosine concentrations should be revealed by improving the resolution of the sampling within the tumor. Using a slightly smaller CMA/11 probe (length, 1 mm; diameter, 240 μm), we have noted that adenosine measurements in tumors are more varied than with the larger probe and yield values (in the absence of metabolic inhibitors) as high as 2.4 μM (data not shown), consistent with our argument.

Inclusion of the ADA inhibitor coformycin and the adenosine kinase inhibitor 5′-iodotubercidin in the microdialysis probe perfusion buffer increased the measured adenosine levels to greater than 8 μM. The actual concentrations of these inhibitors in the tissue would be significantly less than those delivered to the probe (10 and 0.1 μM, respectively) because of: (*a*) the fractional transfer across the dialysis membrane; and (*b*) diffusion outward from the probe. Nevertheless, these data do confirm the presence of enzyme activities within tumors for both the deamination and phosphorylation of adenosine and suggest that the regulation of such activities at a cellular level has the

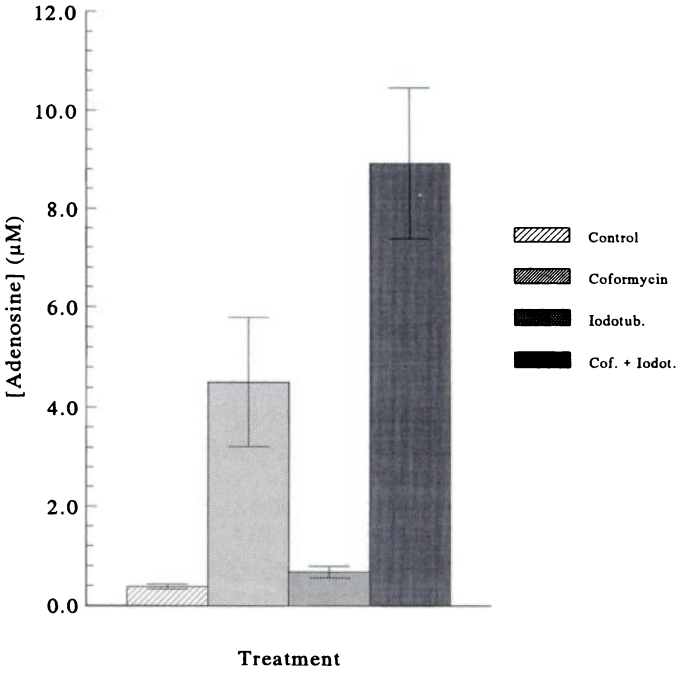

Fig. 4. Effects of coformycin and 5′-iodotubercidin on tumor extracellular adenosine levels. HT-29 tumors were implanted with CMA/20 probes as before. Following 30 min of perfusion/washing at 10 μl/min, the probes were further perfused for 60 min with buffer alone (*Control*) or buffer containing coformycin (final concentration, 10 μM) and/or 5′-iodotubercidin (0.1 μM) as indicated. At the end of this time, samples were collected for adenosine measurement. The data show means for $n = 3$; *bars*, SE.

potential to modulate concentrations of adenosine in the pericellular space.

Adenosine may play a key role in regulating the activity of cytotoxic T cells against tumor targets. Although T cells in general have a high level of ADA, cytotoxic T cells have relatively little ADA activity (17), potentially making them more sensitive to the inhibitory effects of the nucleoside. Adenosine and ADA likely are important in determining the ability of T cells to become activated. ADA is found on the surface of T cells and colocalizes with CD26, a T-cell activation antigen (18). CD26 is an ectoenzyme with dipeptidyl peptidase IV activity, which plays an important part in its costimulatory role (19). ADA released into the extracellular milieu binds to cell surface CD26 and confers resistance to the inhibitory effects of adenosine (18), presumably by reducing the local juxtamembrane concentration that is available to act on adenosine receptors. T cells also display on their surface a series of coordinately regulated ectoenzyme activities that may serve to recycle nucleotides with the concomitant production of adenosine (20, 21). Thus, the potential for marked adenosine production exists both generally within the tumor and specifically at the T-cell surface. Interestingly, antibodies against CD73, which has 5′-nucleotidase activity, have been shown to trigger T-cell proliferation (22), providing further evidence that enzymes in the adenosine pathway are directly or indirectly coupled to costimulation events and adding weight to the suggestion that adenosine is an important player in the autoregulatory control of T-cell activity.

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