Advances in Brief

The Extracellular Fluid of Solid Carcinomas Contains Immunosuppressive Concentrations of Adenosine

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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 adenosine 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 cytolyis 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 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 A3 adenosine receptors (4). Adenosine also interferes with the host response by suppressing the production (6) and cytolytic activity (7) of tumor necrosis factor, again likely acting through A3 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 ECF1 of solid tumors are sufficient to cause immunosuppression. Concentrations of adenosine measured in homogenates of tumor tissue or cultured tumor spheroids5 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-cm² 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 × 10⁶ cells in 100 μl of RPMI 1640 supplemented with 20% (v/v)] 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/pentobarbital and immobilized on a heating pad to maintain body temperature. A small incision was made over the projected implantation site, and the probe (CMA/20, 0.5-mm diameter; dialysis membrane length, 4 mm) was inserted and held in the appropriate position by a micro manipulator clamp stand. The standard solution for perfusion was PBS (containing 0.9 mM Ca²⁺ and 0.5 mM Mg²⁺) 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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1 The abbreviations used are: ECF, extracellular fluid; ADA, adenosine deaminase.
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3 Received 4/10/97; accepted 5/22/97.
4 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
5 This research was supported by a grant from the Dalhousie Faculty of Medicine Fund for Cancer Research (to J. B. and D. W. H.).
ADENOSINE IN THE EXTRACELLULAR FLUID OF SOLID TUMORS

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.

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.
M@-diethylthioether-N-pyridoximine-5'-phosphate, which has been experiments, to as high as 13 p.M. Similar perfusion of microdialysis coformycin caused a significant (P < 0.05) increase in adenosine, raising the level 12-fold. 5'-Iodotubercidin at 0.1 p.M had little effect by itself but together with coformycin increased the extracellular adenosine concentrations to a mean of 8.9 p.M (Fig. 4), and in other experiments, to as high as 13 p.M. Similar perfusion of microdialysis probes implanted in A549 tumors with coformycin and 5'-iodotuber

**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 p.M) approximates the EC50 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 B6 conjugate adenosine-N4-diethythioether-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 p.M. 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 p.M (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 p.M. The actual concentrations of these inhibitors in the tissue would be significantly less than those delivered to the probe (10 and 0.1 p.M, 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

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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.

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 juxtanuclear 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.

Acknowledgments

We thank Dr. Gurmit Singh (Hamilton Regional Cancer Center) for providing the fibrosarcoma-bearing mice. We are grateful to Dr. Murray Hong for expert help in setting up the technique of microdialysis, and to Willi Talbot for assistance with the tumor model.

References

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