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Adenosine stimulation of the proliferation of colorectal carcinoma cell lines
Roles of cell density and adenosine metabolism

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Abstract

Adenosine is a purine nucleoside which is present at micromolar concentrations in the extracellular fluid of solid cancers as a result of tissue hypoxia. Adenosine acts to promote tumor survival by inhibiting the cell-mediated anti-tumor immune response. However, its role in modulating proliferation of the tumor cell population is unclear. Differing results have been obtained using adenosine analogues or by interfering with adenosine metabolism. We examined the effect of adenosine itself on DNA synthesis and cell growth in six different human and mouse colorectal carcinoma cell lines, from different sites and at different stages of differentiation. Adenosine given as a single dose consistently stimulated DNA synthesis and cell proliferation in all cell lines tested, with an EC_{50} of 3.8–30 μM and a maximum stimulation being reached at 10–100 μM. AMP and ATP also stimulated cell proliferation at similar doses. The stimulation by adenosine varied depending upon the culture cell density, with the greatest mitogenic effect at subconfluent densities. Adenosine was metabolized by cellular adenosine deaminase and adenosine kinase. The half-life ($t_{1/2}$) for the decline in adenosine concentration in the medium following a single addition was between 40 min and 3 hr depending on the cell line and culture conditions. The rate of production of endogenous adenosine was low under normoxic culture conditions. Continuous dosing of cultures with adenosine to provide a steady-state concentration showed that proliferation could be stimulated by low micromolar concentrations of adenosine. We conclude that adenosine is stimulatory to the growth of human colorectal carcinoma cells at concentrations present within the tumor extracellular environment.

Keywords: Adenosine; AMP; ATP; Hypoxia; Tumor microenvironment; Colorectal cancer; Cell proliferation

1. Introduction

The tissue environment of solid tumors differs from that of normal tissues because of a compromised vascular supply and the high metabolic rate of the proliferating tumor cell population [1]. As a result, the tumor has local areas of hypoxia [2,3] and increased rates of glucose utilization and lactate release [4–6]. These altered conditions lead to raised levels of the purine nucleoside adenosine, as a consequence of increases in adenosine production and release as a result of cellular ATP depletion or oxygen deprivation [7], due to inhibition of adenosine kinase (AK; EC 2.7.1.20) and activation of the 5′-nucleotidase pathway [8,9]. The enzyme *S*-adenosylhomocysteine hydrolase, which participates in adenosine production, is also upregulated in colorectal cancer [10]. We have previously shown using *in situ* microdialysis of both mouse and human colorectal carcinomas that colorectal tumor tissues have raised extracellular fluid concentrations of adenosine as predicted [11]. The levels of adenosine present in such tumors are sufficient to interfere with the anti-tumor immune response by suppressing T cell activation

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Abbreviations: ADA, adenosine deaminase; AK, adenosine kinase; FCS, fetal calf serum; EC_{50}, single-dose concentration producing half-maximal stimulation of DNA synthesis; NCS, newborn calf serum; $t_{1/2}$, half-life; [^{3}H]TdR, [*methyl*-^{3}H]thymidine.

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[12,13], the interaction of T lymphocytes with tumor cells [14,15], the release of cytolytic effector molecules from killer lymphocytes [16], and target cell lysis [17].

The effect that adenosine in the tumor environment may have on the tumor cells themselves remains unclear. One possibility was raised by observations of a muscle-derived low-molecular weight activity partially due to adenosine, which inhibited the proliferation of tumor cells *in vitro* and *in vivo* [18,19]. The proliferation of normal cells was unaffected or stimulated in the presence of adenosine, leading to the suggestion that adenosine might be a specific inhibitor of the growth of tumor cells. This differential effect appears to be mediated through the A_3 adenosine receptor subtype [20,21]. There are other published reports that adenosine may inhibit the proliferation of virally transformed or neoplastic cells from various sources (e.g. refs. [22–25]) or may trigger apoptosis in cancer cells under certain circumstances (e.g. refs. [26–28]). However, there is also considerable evidence that adenosine may *increase* the proliferation of leukemic [25,29], carcinoma [24,30,31], and astrocytoma [32] cells. Thus, the notion that adenosine might be an inhibitor of tumor cell growth (perhaps offsetting its immunosuppressive actions) remains uncertain.

One reason for these contradictory results may be the different experimental approaches that have been taken. Due to the rapid metabolism of adenosine in many systems, it has often been necessary to use stable synthetic analogues of adenosine in order to see effects on cell growth (e.g. refs. [25,33,34]); or to inhibit adenosine deaminase (ADA; EC 3.5.4.4), the enzyme that metabolizes adenosine to inosine (e.g. refs. [25,28,30,31,34]). However, stable analogues do not have the same relative affinities for different adenosine receptor subtypes as the parent molecule and in most cases are selective for particular receptor subtypes. They may therefore misrepresent the role of adenosine itself, which at appropriate concentrations can concurrently stimulate all adenosine receptor subtypes (A_1, A_{2a}, A_{2b}, and A_3 [35]) which can be co-expressed on the same cancer cell [36]. Similarly, interference with ADA may confound interpretation since ADA itself has signal-modulating properties through binding to ADAcp/DPPIV/CD26 [37], its major receptor at the cell surface, and to A_1 [38] and A_{2B} [39] adenosine receptors.

To resolve these uncertainties, we have examined the effect of a wide range of concentrations of adenosine itself on six different murine and human colorectal carcinoma cell lines, in the absence of ADA inhibition. In a total of more than 100 separate experiments, we found that adenosine consistently failed to inhibit carcinoma cell growth under a variety of culture conditions. The response to adenosine when observed in our growth assays, whether measured in terms of DNA synthesis or cell number, was exclusively that of a dose-dependent increase in cell growth. We have also directly examined the rate of breakdown of exogenous adenosine and the rate of endogenous production in these cells. When the rate of adenosine breakdown is taken into account, adenosine can be seen to stimulate colorectal carcinoma cell proliferation at concentrations we have shown to exist within colorectal tumors *in situ*.

2. Materials and methods

2.1. Cell culture

Human carcinoma cell lines HT-29, T84, HRT-18, Colo320*HSR*, and Caco-2 were obtained from the American Type Culture Collection. The MCA-38 murine liver-derived colon carcinoma cell line was as used previously in our studies of adenosine [14]. All cell lines were maintained in the absence of antibiotics in Dulbecco's Modification of Eagle's Medium (ICN Biomedicals) containing 2 mM L-glutamine and either 10% (v/v) heat-inactivated NCS (HT-29, T84, HRT-18, and MCA-38 cell lines), 5% NCS (Colo320*HSR*), or 10% FCS (Caco-2). Sera were from Invitrogen Canada. All cell lines were maintained as stock cultures in 80 cm^2 flasks (Nunc, Invitrogen Canada) at 37° in a humidified atmosphere of 90% air/10% CO_2.

2.2. DNA synthesis assay

Cells were seeded in 24-well plates (Nunc), typically at 2×10^4 cells/well in the appropriate culture medium. For studies at different cell densities, cells were seeded at $\sim 6 \times 10^3$ to 100×10^3/well (50–800 per mm^2). Adenosine, AMP or ATP (Sigma), or vehicle control (serum-free medium) were added at the indicated final concentrations, together with [*methyl*-^{3}H]thymidine ([^{3}H]TdR) (final concentrations, 1 μCi/mL, 1 μM; Amersham Biosciences Inc.). Adenosine stock solutions were made fresh for each experiment. The plates were incubated for 36–48 hr to permit the adenosine response and allow incorporation of [^{3}H]TdR into newly synthesized DNA. At the end of this time the plates were placed on ice, washed twice with cold PBS, and treated with 0.5 mL of cold 10% trichloroacetic acid to precipitate DNA. After 60 min at 4° the trichloroacetic acid was removed and the wells rinsed with ethanol. Cellular macromolecules were solubilized in 0.5 mL of 0.1 M NaOH containing 1% SDS and radioactivity was determined using a Beckman LS 5000TA liquid scintillation counter (Beckman Coulter Canada).

2.3. Measurement of cell number

Cells were seeded in 35-mm dishes or 4-well plates (Nunc) and treated with adenosine with medium changes and fresh adenosine added at 2–3 day intervals. Cells were then trypsinized with 0.5 mL of trypsin/EDTA (Canadian Life Technologies), and the diluted cell suspension counted using a Coulter Counter™ ZM30383 (Beckman Coulter).

2.4. Measurements of adenosine breakdown or production

For studies of adenosine breakdown or production, subconfluent cultures of HT-29 cells in 6-well plates were first changed to a serum-free basic medium comprising PBS containing 1.2 mM $CaCl_2$, 0.4 mM $MgSO_4$, and 11.1 mM glucose together with RPMI-1640 vitamins (Canadian Life Technologies). Exogenous adenosine and/or inhibitors of metabolism were then added. Coformycin (Calbiochem) and 5′-iodotubercidin (Sigma-RBI) were dissolved in DMSO and added at final concentrations of 10 and 1 μM, respectively; the final concentration of DMSO in experiments did not exceed 0.1% (v/v). Cultures were incubated at 37° in a CO_2-free atmosphere. Rates of metabolism of exogenous adenosine were measured over 90 min following an addition of 10 μM adenosine. Endogenous adenosine was measured as the accumulation over a 6-hr period. Culture medium (0.5 mL) was collected and the adenosine was derivatized with 0.5% chloroacetylaldehyde for 20 min at 100° to form the fluorescent derivative 1-N^6-ethenoadenosine [40]. Measurement of adenosine was accomplished by HPLC using a Waters 2690 Separations Module and 474 Fluorescence Detector, with a Nucleosil 100 C_{18} column and a mobile phase of 50 mM acetate buffer (pH 4.5), 2.2 mM 1-octanesulfonic acid, and 18% acetonitrile at a flow rate of 2 mL/min. Adenosine was quantitated using excitation and emission wavelengths of 270 and 418 nm, respectively.

3. Results

3.1. The growth response of carcinoma cell lines to adenosine is consistently one of increased DNA synthesis and cell proliferation

For these studies we selected five different human intestinal carcinoma cell lines and one mouse colon carcinoma cell line that (i) derived from colonic primaries and differed in their degree of differentiation: HT-29 (well-differentiated), Caco-2 (moderately well-differentiated), and Colo320*HSR* (poorly differentiated); or (ii) derived from different extracolonic sites: HRT-18 (ileocecal), T84 (lung metastasis), and MCA-38 (mouse liver).

In more than 100 separate experiments observing the responses of these colorectal carcinoma cell lines to adenosine using assays of DNA synthesis or cell number, adenosine consistently produced an increase, rather than a decrease, in tumor cell growth. Representative dose–response curves from assays of DNA synthesis are shown in Fig. 1. The response to adenosine given as a *single dose* at the beginning of the 36–48 hr DNA synthesis assay typically reached a maximum at a concentration of between 10 and 100 μM. Two forms of dose–response curve were observed. Either the rate of DNA synthesis reached a plateau that was maintained up to the highest concentration (300 μM) of adenosine tested, as typically seen for HT-29 and Colo320*HSR* cells (Fig. 1a and d); or, more commonly, there was a biphasic response and the rate of DNA synthesis decreased at higher adenosine concentrations, as seen for T84, HRT-18, Caco-2, and MCA-38 cells (Fig. 1b, c, e, and f). The form of the dose–response curve was generally typical of the particular cell line, although it was not absolutely consistent.

Table 1
Potency of adenosine in stimulating DNA synthesis in human colorectal carcinoma cells

Cell line	EC_{50} (μM) (mean ± SEM)
HT-29	5.7 ± 0.6 (N = 6)
T84	3.8 ± 1.0 (N = 4)
Colo320*HSR*	6.8 ± 0.4 (N = 4)
Caco-2	30.0 ± 7.7 (N = 4)
HRT-18	17.5 ± 1.3 (N = 4)

Cultures of carcinoma cells were treated with single doses of adenosine over the concentration range 1–300 μM and the effect on DNA synthesis determined. Individual dose–response curves were determined using four to eight replicate cultures at each adenosine concentration. The EC_{50} within each experiment was defined as the concentration of adenosine that produced a half-maximal stimulation of DNA synthesis. The table shows mean ± SEM of values for each cell line derived from four to six completely separate experiments.

The EC_{50} values (single-dose concentration producing half-maximal stimulation of DNA synthesis) for the different human colorectal cancer cell lines varied from 3.8 to 30 μM (Table 1). Significant variability in the measured EC_{50} was only observed for Caco-2 cells, which showed marked progressive changes in cell phenotype as the cells differentiated in culture. No obvious differences were seen in the EC_{50} values for any cell line over a range of passage numbers (e.g. for HT-29 cells, passages 8–25 from initial receipt).

The dose–response relationship for adenosine stimulation of DNA synthesis did not change if the addition of [^{3}H]TdR was delayed to 24 hr after adenosine dosing (data not shown), at which time the added adenosine would have been completely metabolized (see below). This excludes potential artifacts of adenosine in the thymidine incorporation assay. We also measured the effect of adenosine on growth in longer term culture by measuring cell number directly. Figure 2 shows the result for HT-29 cells. Adenosine again stimulated proliferation. The maximum effect was reached at a similar adenosine dose (30 μM) as for DNA synthesis, and with a similar EC_{50} value (10.8 ± 1.8 μM, three separate experiments).

Inhibition of ADA with coformycin (1–10 μM) did not block the adenosine mitogenic response in colon carcinoma cell cultures (data not shown). This is consistent with the view that the effects seen are due to adenosine itself rather than its breakdown product inosine.

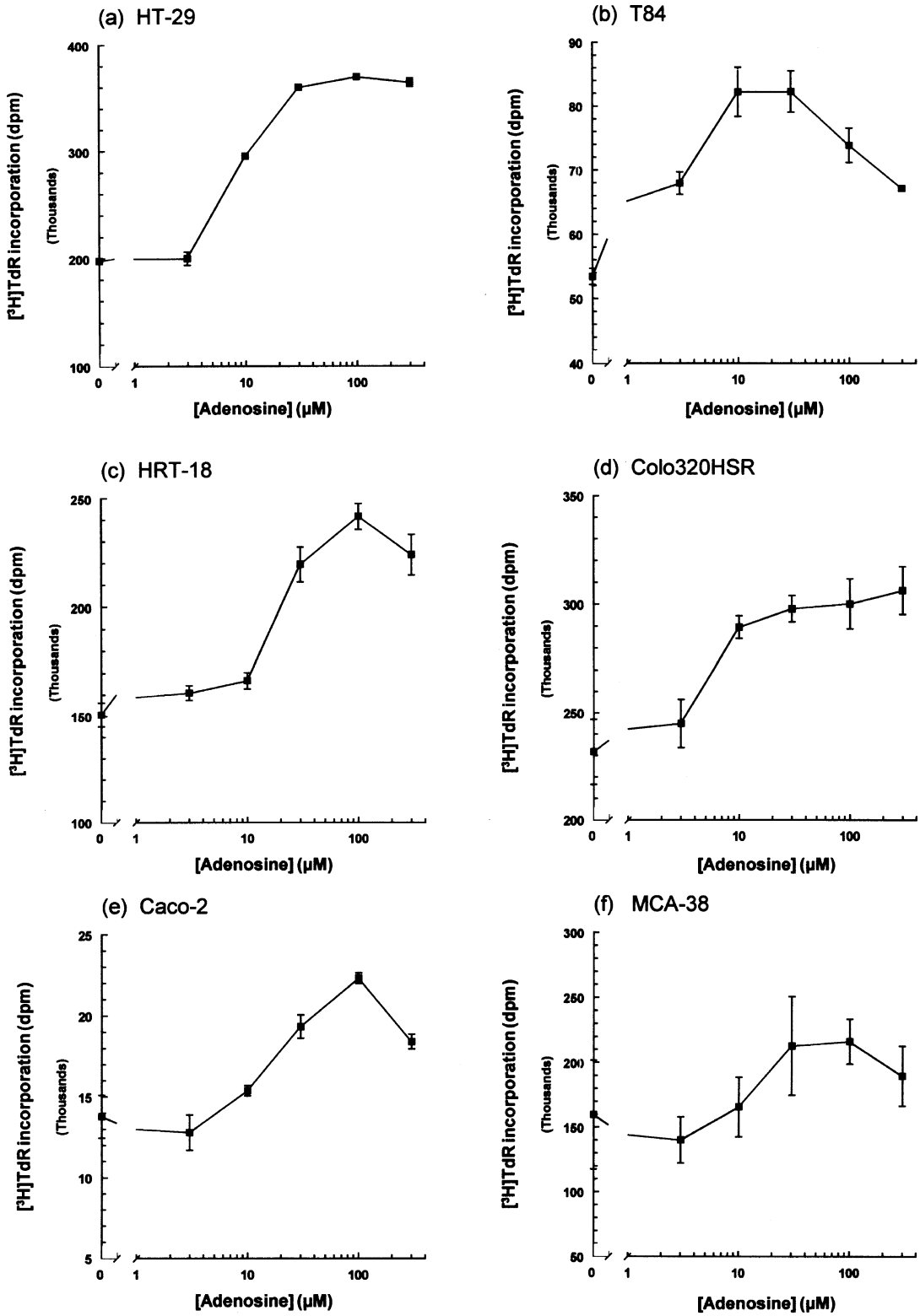

Fig. 1. Effect of adenosine on DNA synthesis in human and mouse colorectal carcinoma cell lines. Data points and error bars represent mean ± SEM for quadruplicate determinations. Each panel is representative of four to six independent experiments.

3.2. Density dependence of the adenosine response

Figure 3a shows the dose dependence of the adenosine response for HT-29 cells at a range of cell densities. The magnitude (but not the concentration dependence) of the adenosine response varied up to 4-fold as the cell number increased between 5 and 100% of confluence, even though the cells were in excess fresh culture medium. The lack of a

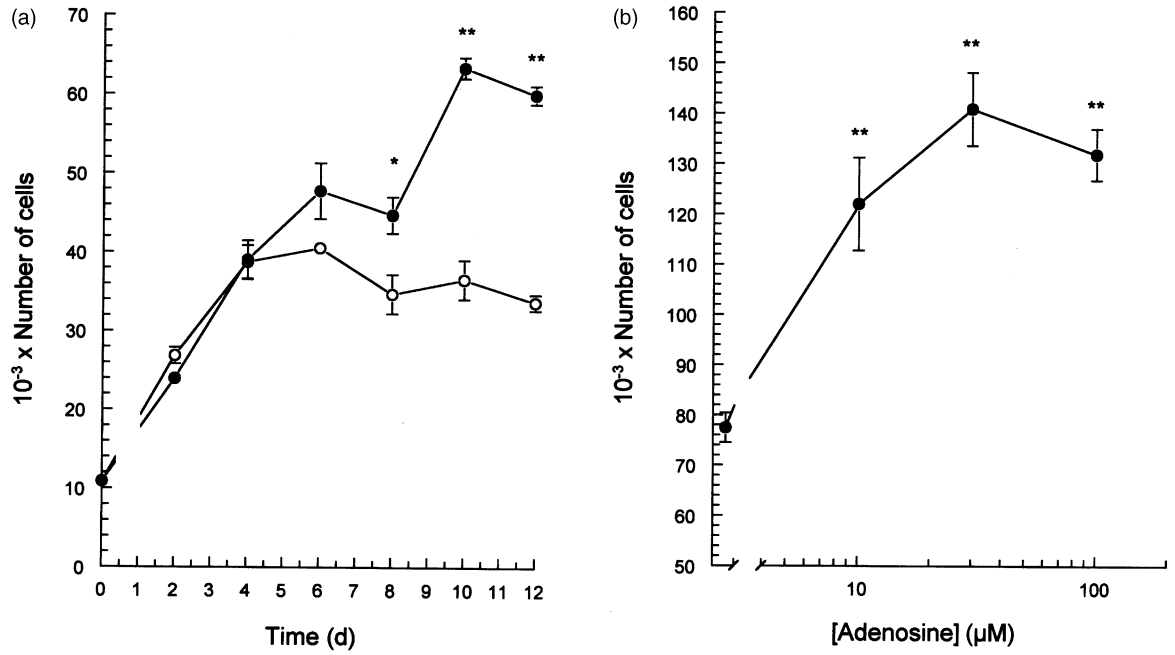

Fig. 2. Effect of adenosine on the proliferation of HT-29 cells. (a) Time course. Cells were cultured without (○) or with (●) 30 μM adenosine. (b) Concentration dependence. Cells were counted after 10 days of growth in the presence of adenosine at the concentrations indicated. The panels show mean ± SEM of quadruplicate cultures. Significant increase over control: $^{*}P < 0.05$, $^{**}P < 0.01$.

lateral shift in the overall dose–response relationships implies that differences in adenosine supply were not a contributing factor. The maximum adenosine response declined steadily as cell number increased (Fig. 3b). A consistent observation in this and other cell lines was that at the lowest cell density examined, the shape of the adenosine dose–response relationship was different at lower adenosine concentrations (Fig. 3a), and single addition of low doses (1–10 μM) produced an additional enhancement of DNA synthesis that was not observed at higher cell densities.

3.3. Persistence of adenosine within the culture medium and the contribution of cell-derived adenosine

We examined the fate of the single dose of adenosine in the culture medium. Adenosine concentrations in the medium of subconfluent (60–70%) HT-29 cultures declined

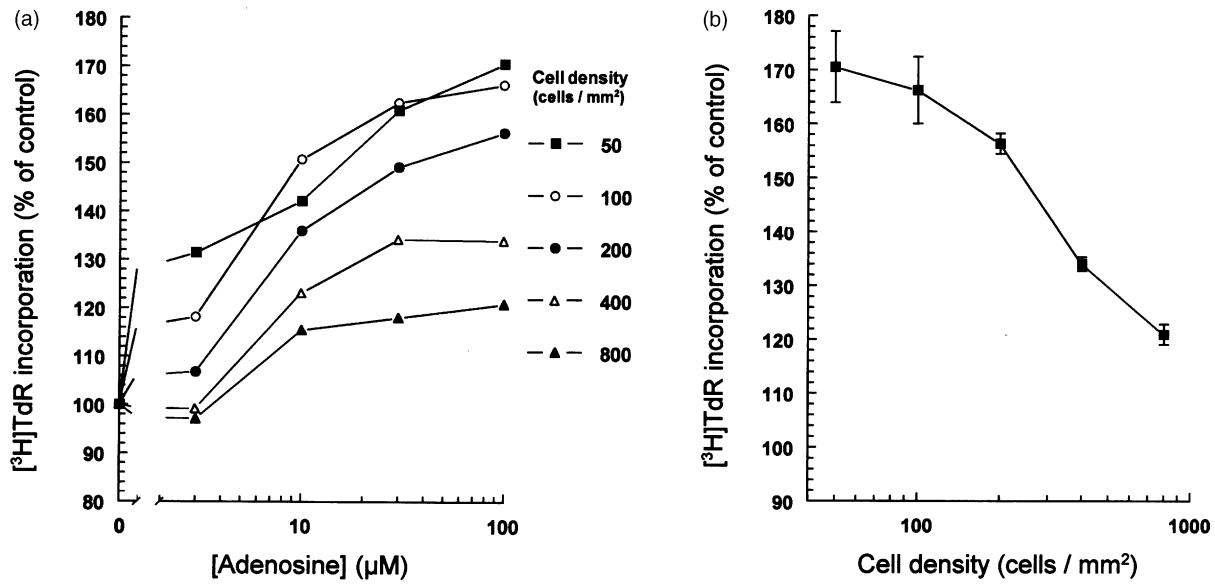

Fig. 3. Effect of cell density on the magnitude of the adenosine growth response of HT-29 cells. (a) Concentration dependence. (b) Density dependence of the maximal adenosine growth response. Data are mean ± SEM of quadruplicate cultures.

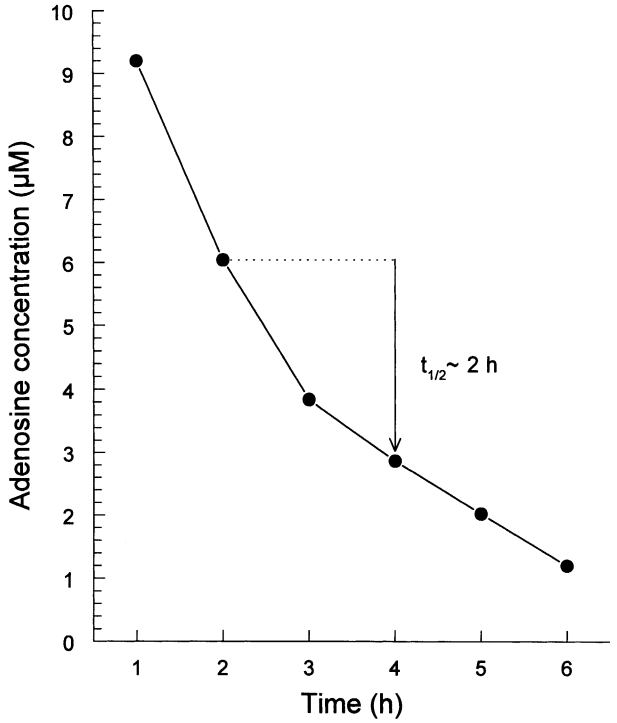

Fig. 4. Rate of metabolism of adenosine by HT-29 cells in culture. HT-29 cells were given a single initial adenosine dose of 10 μM. Data are mean determinations from duplicate cultures.

from the initial concentration of 10 μM with a $t_{1/2}$ of approximately 2 hr (Fig. 4). Similar studies with other culture conditions and cell lines showed a range in $t_{1/2}$ of between 40 min and 3 hr depending on the cell line and culture conditions.

The degradation of adenosine was enzyme-mediated rather than through spontaneous chemical breakdown under the conditions of incubation. The rate of metabolism was decreased in the presence of coformycin (10 μM) to eliminate breakdown *via* ADA, or 5′-iodotubercidin (1 μM), to prevent phosphorylation through AK (Fig. 5a) and completely suppressed in the presence of both of these agents together, showing that both deaminase and kinase activities play a part in its removal by the cells at this adenosine concentration.

We also examined the endogenous production of adenosine by the cells themselves. The initial production of adenosine was relatively low in control cultures, about 60 pmol/hr for subconfluent HT-29 cells. The rate of adenosine production was significantly enhanced (more than 10-fold) when AK was blocked with 5′-iodotubercidin (Fig. 5b), showing that much of the adenosine produced under basal conditions is routed through to nucleotide pools. Inhibition of ADA had no effect on the endogenous production of adenosine (Fig. 5b).

3.4. Repeated dosing with adenosine leads to proliferative responses at low micromolar concentrations

Given the rapid breakdown of adenosine in culture, we examined the effect of adding adenosine in smaller, repeated doses instead of a bolus dose. Adding adenosine over 2-hr intervals (a dose separation equivalent to one half-life) during a 12-hr period yielded a dose–response curve that was shifted to the left compared with that resulting from single dosing with adenosine (Fig. 6). The response was essentially unaltered for the same overall dose of adenosine given as a bolus or in divided doses over the 12-hr interval. The single-dosing approach produced the same result whether additions were made 48 hr after seeding according to our usual approach, or at the time of cell plating, an approach favored by others [41] (Fig. 6).

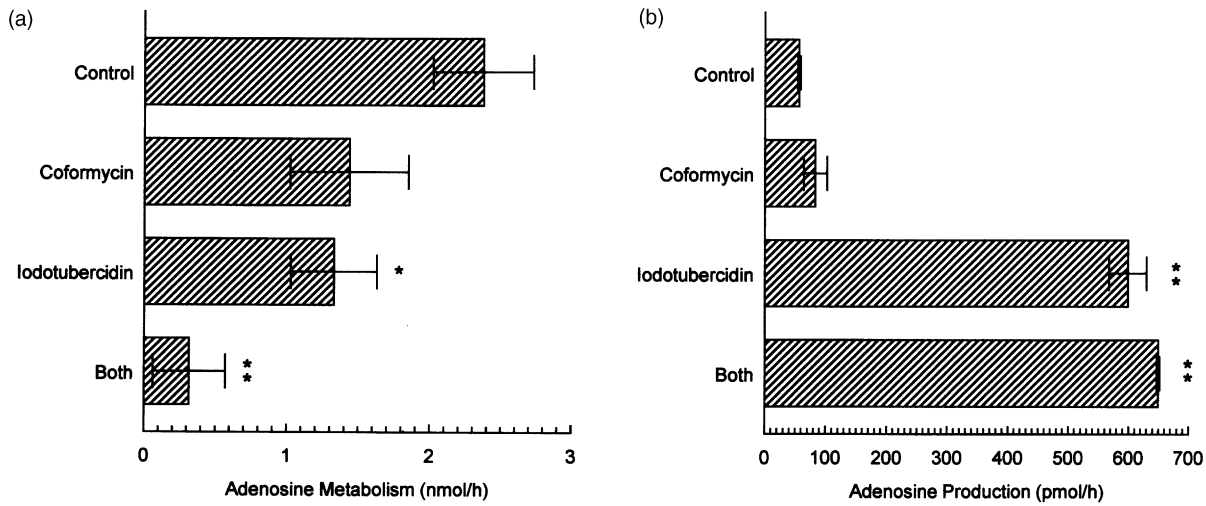

Fig. 5. Involvement of adenosine deaminase and adenosine kinase in modulation of adenosine levels by HT-29 cells. (a) Metabolism of exogenous adenosine. (b) Endogenous adenosine production. Points represent mean ± SEM of determinations from triplicate cultures. Significantly different from control: $^{*}P < 0.05$, $^{**}P < 0.01$.

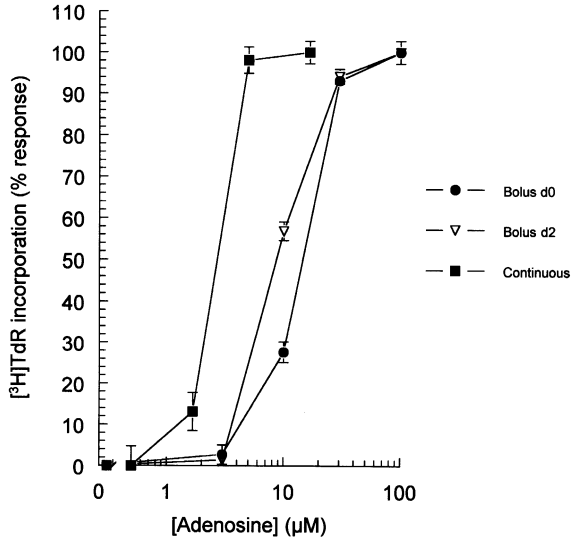

Fig. 6. Effect of timing and schedule of *in vitro* dosing on the adenosine growth response of HT-29 cells. Adenosine was given as a single dose at the same time as plating of the cells (“Bolus d0”), 2 days after plating (“Bolus d2”), or divided between six fractional doses given at 2-hr intervals (“Continuous”). Points represent mean ± SEM values for quadruplicate cultures.

3.5. Adenine nucleotides also stimulate colorectal carcinoma cell growth

Given that adenosine may be derived extracellularly from adenine nucleotides within the tumor environment, we compared the effects of AMP and ATP with adenosine in all six colorectal carcinoma cell lines. We found that AMP and ATP consistently stimulated DNA synthesis at similar doses to adenosine. Figure 7 shows a typical result for HT-29 cells.

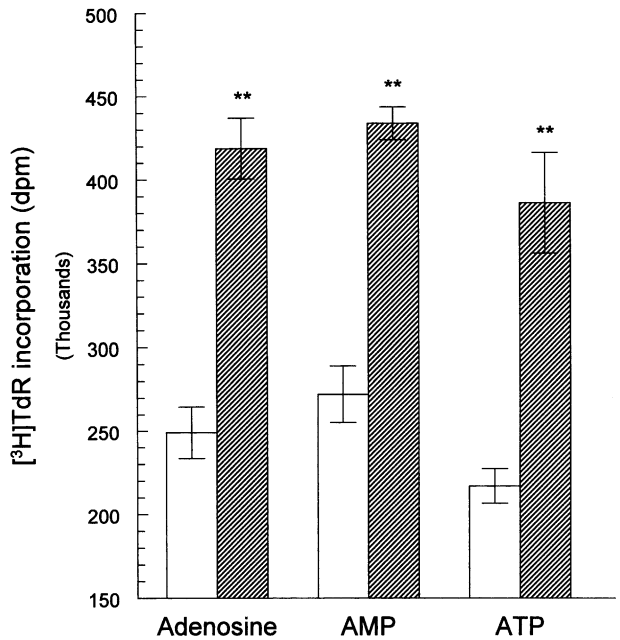

Fig. 7. Effect of adenosine and adenine nucleotides on DNA synthesis in HT-29 cells. Cells were cultured without (open bar) or with (hatched bar) 30 μM adenosine, AMP, or ATP, as indicated. Data are mean ± SEM for quadruplicate determinations. Significant increase over control: $^{**}P < 0.01$.

4. Discussion

We have carefully explored the adenosine growth response in six different colorectal carcinoma cell lines, from two species (human and mouse) that encompass different primary and metastatic sites as well as a range of degrees of cellular differentiation. In more than 100 experiments measuring cell proliferation using cell counts, measurements of DNA synthesis and MTT assay (data not shown), we have failed to find evidence for either an overall inhibitory or cytotoxic effect of pathologically relevant ($\sim 10^{-4}$) levels of adenosine itself on cancer cell growth, in contrast to other published reports [19,20,22–25,28]. One explanation for the disparities between the effects of adenosine on tumor cells might be the tissue origin of the neoplastic cell. Most reports of an inhibitory effect of adenosine on cancer cell growth or survival have evaluated the response of cells of leukemic or lymphoma origin [19,20,26,27,42] whereas our focus has been on cells of solid carcinomas. However, there are also several descriptions of growth inhibition in epithelial cancer cells [22,24,25] and, conversely, adenosine stimulation of growth in MOLT 4 and HL-60 leukemia cells [25,29]. The disparity between responses cannot therefore be due to the different cell types studied.

The proposed beneficial effect of adenosine on the growth of “normal” (nontumorigenic) cells is also ambiguous. Adenosine has certainly been shown to stimulate DNA synthesis or cell growth in cell lines and normal cell populations as diverse as LLC-MK_2 monkey kidney endothelial [43], COS-7 monkey kidney fibroblast [43], mouse mammary epithelial [44], human umbilical vein endothelial [45,46], and chick astrocytic [32] cells. However, adenosine conversely *inhibits* the growth of human and rat aortic smooth muscle cells [47,48], rat cardiac fibroblasts [49], mouse T cells [13], mouse 3T3 fibroblasts [22], and rat RIE-1 intestinal epithelial cells.[1] The role of adenosine in regulating cell growth may not be simple and may require that we first understand how the mitogenic stimulus intersects with other signals due to adenosine, which is capable of affecting cellular interactions with the substratum [50] and regulating interactions with other cells in the environment [14,15].

We have been careful to evaluate the effect of adenosine itself in the absence of inhibitors of its metabolism, which can greatly alter the fate of exogenous adenosine (Fig. 5a) and its endogenous production (Fig. 5b). This circumvents problems that may be encountered using stable analogues

[1] Blay, unpublished data.

and manipulations of ADA. A stable analogue, such as NECA, may produce very different effects than adenosine on the same cell type, even under the same assay conditions [22,25,33]. Similarly, certain older agonists may not allow the tonic cytoprotective effect of adenosine believed to be exerted through low level activation of A_3 adenosine receptors [42,51]. Similarly, the effects of adding ADA or inhibiting its activity may not be consistent with the observed response to adenosine in the same system (e.g. refs. [25,34]). It is therefore important to study the authentic effect(s) of adenosine.

Our findings show that the inherent response of colorectal carcinoma cells to adenosine is one of *increased* proliferation. With one exception, the EC_{50} value for adenosine stimulation (single dose) was narrowly defined in each cell line, consistent with an effect of adenosine on adenosine receptors with a characteristic binding affinity and not through nonspecific effects of adenosine on purine metabolism. Interestingly, the one cell line that exhibited substantial variability in EC_{50} values (Caco-2; range, 15–55 μM) is a cell line that shows significant heterogeneity in phenotype due to its capacity to differentiate spontaneously in culture. This may suggest that adenosine receptor expression and/or coupling to signal transduction pathways alters as cells differentiate.

Our investigations of the effects of adenosine on cell growth, as with essentially all similar studies, involve single dosing of adenosine at the start of the experiment, as in the DNA synthesis assays described above, or doses spaced over 2–3 days intervals, as for the growth curves. This differs somewhat from the situation that would be more usual *in vivo*, in which adenosine levels may be raised to a higher equilibrium concentration over a period of at least hours (e.g. acute injury), days (e.g. sites of inflammation), or months (e.g. hypoxic solid tumors). Single dosing may have a relatively close counterpart *in vivo* in the response to simple mechanical trauma, which we have shown produces a spike in tissue extracellular fluid concentrations of adenosine which is of less than 20-min duration [11]. However, for the most part we would expect the adenosine signal to be much more persistent *in vivo* than represented by a single dose *in vitro*. The adenosine-mediated stimulation of growth with a single dose was typically seen at adenosine concentrations above 3 μM, with an EC_{50} below 20 μM for most cell lines. This *in vitro* approach significantly overestimates the concentration of adenosine necessary to produce a growth stimulation. When we provided the adenosine as separate additions over a 12-hr period, a leftward-shifted dose–response relationship was evident relative to single dosing (Fig. 6). The repetitive dosing approach takes into account the steady degradation of adenosine in culture, which we estimated to occur with a $t_{1/2}$ of between 40 min and 3 hr depending on the cell line and culture conditions. The steady-state adenosine concentration that is required to stimulate growth is likely at least one order of magnitude less than the concentration indicated by the single addition approach, and would suggest that at steady state the EC_{50} would typically be less than 3 μM and a maximum response would be elicited in the 10 μM range. These levels correspond very closely to the range of adenosine concentrations that are likely to be present within the extracellular fluid of solid carcinomas *in vivo* [11].

We compared the effects of AMP and also ATP with adenosine, in all six colorectal carcinoma cell lines. We found that AMP and ATP also stimulated DNA synthesis. The effects of both AMP and ATP were only partially inhibited by AMP-CP, an inhibitor of 5′-nucleotidase (data not shown). This implies that such adenine nucleotides act to some extent through being converted to adenosine, but that they may also stimulate growth without being dephosphorylated—in the case of ATP perhaps through action on P_2 purinergic receptors as noted elsewhere [51].

The precise pathway through which adenosine itself (as opposed to synthetic analogues) mediates its effects on carcinoma cell proliferation is not clear. RT–PCR analysis of mRNA expression for each of the adenosine receptor subtypes (A_1, A_{2a}, A_{2b}, A_3) shows that all four receptor subtypes may be expressed in different cultures of the five human colorectal carcinoma cell lines (data not shown). Expression of the receptor subtypes and coupling to the growth response seems to vary depending on culture conditions and growth state. A_{2b} and A_3 receptors are most consistently expressed on these cell lines, and antagonists to these particular receptor subtypes (alloxazine and MRS1191 or MRS1523, respectively) block the proliferative response to adenosine in about 20–25% of the experiments. However, the block is not consistent between different cell populations. We believe that the precise adenosine signaling pathway involved may be determined by the cellular context, including cell cycle phase, cell population density, and extracellular matrix. Since A_{2b} and A_3 receptors are oppositely coupled to adenylate cyclase, it is likely that the proliferative response is mediated through pathways other than those dependent on adenylyl cyclase and cAMP.

In most of the cell lines studied, we observed a decline in the adenosine stimulation from its maximum at adenosine concentrations that exceeded 100 μM (Fig. 1). High concentrations of adenosine in this range [22,27,44], or lower adenosine concentrations in the presence of reduced ADA activity [22,27,28], have been observed to trigger cellular apoptosis. This raises the possibility that the decline in adenosine stimulation of cell growth at higher concentrations might be due to cytotoxicity. Indeed, it was possible that the overall effect on cell number (Fig. 2) might be the net effect of both a stimulation of cell proliferation and induction of cell death through apoptosis. However, using the JAM assay of DNA fragmentation [52], we have found no evidence for induction of apoptosis in these intestinal carcinoma cell lines by adenosine at concentrations below 1 mM (data not shown). Furthermore, using

the more sensitive MTT assay for cellular metabolic activity and viability [53], we have found no detrimental effect on mitochondrial succinate dehydrogenase activity until adenosine concentrations reach 600 μM (HT-29, Colo320*HSR*), 2 mM (T84), or 5 mM (Caco-2, HRT-18, MCA-38), concentrations that are well above the maximum concentration that we studied in this work (300 μM). The adenosine response of colorectal carcinoma cells does not include cell death at these lower concentrations. Moreover, the more moderate levels of adenosine that seem to be present within the growing tumor are very unlikely to stimulate apoptosis, and adenosine is more likely to facilitate growth of the carcinoma cell population. Furthermore, it is interesting that two of the cell lines which we studied (HT-29, Colo320*HSR*) did not show a decrease in the growth response at high adenosine concentrations (Fig. 1a and d), raising the possibility that cancer cells may develop mechanisms to be resistant to the decline in effects of adenosine at high doses.

We do not yet know what is the explanation for the difference in adenosine growth stimulation depending on cell density. It is not likely that the reduced response is due to increased adenosine metabolism at higher cell densities. Enhanced adenosine degradation would produce a rightward shift in the adenosine dose–response curve, with the same maximum being reached as higher levels of exogenous adenosine overwhelm the capacity for degradation. However, the curves shown in Fig. 3a do not show a trend to a rightward shift in the adenosine dose–response curve at higher cell densities, but rather a decline in the maximum adenosine response itself.

Overall, our data point to a relatively small endogenous production of adenosine under standard cell culture conditions in the absence of inhibitors of adenosine metabolism (about 60 pmol/hr in these cultures; Fig. 5b). Although such cultures are ''normoxic'', unstirred monolayer cell cultures may show a reduction in the ambient oxygen tension depending on the cell density [54]. The finding (Fig. 5b) that in monolayer culture it is AK rather than ADA that is limiting for the endogenous production of adenosine contrasts with our earlier finding in solid tumors *in vivo*, for which adenosine breakdown is effected principally by ADA and much less by AK [11]. A similar prominent role for AK is however seen in other cell cultures [49,55]. This is consistent with the relative kinetic properties of the two enzymes [56]. Under the normoxic conditions in monolayer culture phosphorylation of intracellular adenosine through AK, which has a lower K_m (40 nM), would take preference. Under hypoxic conditions, as in tumors *in vivo*, the generation rate of adenosine through AMP nucleotidase would be much higher, shifting the major role in intracellular breakdown over to ADA, which has a much higher K_m (70 μM) but far greater capacity for adenosine metabolism than AK [56].

We have previously shown that adenosine can dramatically suppress the anti-tumor immune response [12–17]. Adenosine may also act to promote tumor cell migration [57] and stimulate angiogenesis [58,59], as well as increase intratumoral blood flow [60], and confer protection against radiotherapy [61]. Our current data argue that adenosine confers a further survival advantage on the tumor by stimulating the growth of the tumor cells themselves. The concept that adenosine might be a suppressor of tumor cell proliferation and therefore an anti-tumor agent is not supported by our studies. Adenosine seems rather to be a multifactorial promoter of tumor growth.

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