Characterization of Adenosine-induced Cytostasis in Melanoma Cells¹

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ABSTRACT

Relative to lymphoid cells and normal fibroblasts, mouse melanoma cells (B16) were moderately sensitive to adenosine, with 80% growth inhibition being observed at 50 μM adenosine instead of at 5 μM as was reported with lymphoid cells or 400 μM as was reported for normal fibroblasts. These differences were not due to adenosine deaminase because lymphoid cells had two to four times more of this activity than did melanoma cells or normal fibroblasts. In melanoma cells, complete adenosine-induced growth inhibition was a gradual process which was observed only after one to two population doublings; after 4 days of treatment, complete recovery was gradual requiring 48 hr. N⁶,O²-Dibutyryladenosine-cyclic-3':5' phosphate and polyadenylic acid were ineffective as growth inhibitors, whereas guanosine exhibited potent growth-inhibiting properties. Homocysteine thiolactone enhanced the cytotoxicity of adenosine but not guanosine; adenosine relieved the cytotoxicity of guanosine. These observations indicated that the two purine nucleosides were exerting their growth-inhibiting effects by different mechanisms. Uridine did not relieve adenosine-induced cytostasis, but at 50 μM adenosine enhanced the incorporation of [³H]uridine into RNA. This suggested that the uridine phosphate pools were depleted at low adenosine concentrations and that exogenous adenosine influences the availability of pyrimidines.

INTRODUCTION

Several laboratories have reported the deleterious effect of exogenous adenosine on mammalian cells in culture. The causative mechanism of adenosine-induced cytostasis and cytolysis has been explained by several mechanisms; these include inhibition of de novo pyrimidine biosynthesis (12, 16), depletion of the 5-phosphoribosyl 1-pyrophosphate pool (25), and disruption of normal pre-rRNA (pre-rRNA) processing and nucleolar function (15). Snyder et al. (25) showed that depletion of the 5-phosphoribosyl 1-pyrophosphate pool did not adequately explain the toxic manifestations of adenosine and adenine. Consequently, with the mouse melanoma as a model, this report investigates inhibition of pyrimidine biosynthesis and disruption of pre-rRNA processing and nucleolar function as mechanisms of adenosine-induced cytostasis.

Inhibition of pyrimidine biosynthesis as a mechanism for adenosine-mediated cytostasis is based on the observation that cells cultured in adenosine had depleted pyrimidine nucleotide pools (12, 16); in these studies, the inhibition of cell proliferation and the eventual cell death were ascribed to pyrimidine starvation. Ishii and Green (16) were the initial advocates of this hypothesis. They observed that cell lethality due to adenosine toxicity could be averted by supplementing the culture medium with exogenous uridine. Although uridine actually stimulated the uptake of adenosine, it inhibited the toxic effects of adenosine as assessed by cell viability and proliferation. Biochemical analysis of de novo pyrimidine biosynthesis with labeled aspartate as a precursor indicated that the pathway which anabolized uridine nucleotides was inhibited at the level of orotate. Further experimentation conducted on lymphoid and fibroblastic cell lines by Green and Chan (12) showed that cells cultured for 3 to 6 hr in adenosine displayed an apparent decrease in the pyrimidine nucleoside diphosphate and triphosphate pools. It was also reported that human and fetal calf serum, components of tissue culture medium, contained adenosine deaminase, an enzyme which converts adenosine to inosine and helps relieve adenosine toxicity (16).

In 1952, Hughes (15) reported that the nucleoli of chick cells became fragmented when adenosine was added to the culture medium. Since that time, other research groups have substantiated his observation (23, 26); these reports constitute the basis of the second mechanism, disruption of pre-rRNA processing, and nucleolar function. Bynum et al. (2, 4) reported experimental results which support this mechanism. Human myeloma cells cultured in 1.9 x 10⁻⁵ M adenosine for 3 and 6 hr had an imbalanced ratio of newly synthesized rRNA. There was no apparent accumulation of precursors, and yet there was a selective decrease in the accumulation of 18S rRNA. The disruption of normal pre-rRNA processing was not relieved by the addition of uridine to the culture medium. Proper pre-rRNA processing is needed to maintain the complement of ribosomes required for anaplerotic protein synthesis and cell proliferation (22, 29). Experiments conducted on HeLa cells showed a 2-fold increase in the labeling of 4S RNA relative to 28S and 18S rRNA (2); however, an alternative interpretation of the data is that 28S and 18S rRNA accumulation was 50% inhibited in the presence of adenosine. Depletion of the pyrimidine nucleotide pool was not observed. Other investigators have also reported the inability of pyrimidine to relieve adenosine-induced cytostasis and cytolysis (3, 20).

With the adenosine deaminase inhibitor EHNA³, Ullman et al. (28) reported that inhibition of de novo pyrimidine biosynthesis occurred only at extremely low adenosine concentrations and that at higher concentrations some other mechanism was involved. Kredich and Martin (19) used EHNA, adenosine, and HCT to demonstrate that uridine-resistant adenosine toxicity was due to interference with S-adenosylhomocysteine hydrolyase, an enzyme which catalyzes the reversible hydrolysis of S-adenosylhomocysteine to homocysteine and adenosine. By increasing the concentration of the products of the reaction with additional adenosine and HCT, the kinetics was shifted in favor of the reverse reaction, S-adenosylhomocysteine forma-

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³ The abbreviations used are: EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; HCT, homocysteine thiolactone.

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tion; this, in turn, inhibited S-adenosylmethionine-dependent methyltransferase and prevented DNA and RNA methylation.

Most of the previous studies were conducted with transformed lymphoid cells as the experimental model. It has been shown that lymphoid cells possess atypical growth characteristics, particularly as they related to rRNA synthesis and processing (5, 9–11). Consequently, there is a need to explore cell models with applicability to nonlymphoid oncological conditions. An earlier report demonstrated that normal fibroblasts were far less sensitive to adenosine than were transformed lymphocytes and that the insensitivity was not related to proliferative rates or the adenosine deaminase content (1). Consequently, this report will focus on 2 main objectives: (a) to investigate the effect of adenosine on the nonlymphoid cell line, B16 mouse melanoma; and (b) to evaluate pyrimidine nucleolar function via inhibition of RNA methylation as possible mechanisms for adenosine-induced cytostasis.

MATERIALS AND METHODS

Materials. Dulbecco's modified minimum essential medium, fetal calf serum, penicillin-streptomycin, trypsin, and uridine were purchased from Grand Island Biological Company, Grand Island, N. Y.; HCT, N6, O2- dibutyryladenosine-cyclic-3':5' phosphate, inosine, adenosine, polyadenylic acid, and guanosine were purchased from Calbiochem-Behring Corporation, Elkh Grove Village, III.; adenosine deaminase (adenosine amidohydrolase, EC 3.5.4.4) was purchased from Boehringer-Mannheim, New York, N. Y.; and [3H]uridine ([3H]uridine; 21 mCi/mmol) and [14C]adenosine ([8-14C]adenosine; 50 mCi/mmol) were purchased from Schwarz/Mann, Orangeburg, N. Y. Two 100-mg batches of EHNA were generously provided by the National Cancer Institute, Bethesda, Md., and by Dr. Howard J. Schaeffer of Burrough-Wellcome Company, Research Triangle Park, N. C.; 2'-deoxycoformycin was also provided by the National Cancer Institute.

Cell Culture. Mouse melanoma cells (B16) were routinely cultured in complete medium containing Dulbecco's modified minimum essential medium with 15% fetal calf serum, streptomycin (50 µg/ml), and penicillin (50 units/ml), or Eagle's minimum essential medium with 10% fetal calf serum and antibiotics. For subcultivation, confluent cultures in 75-cm² tissue culture flasks were trypsinized for 10 min at 37°C with 9 mℓ of trypsin solution containing 0.05% (500 µg/ml) trypsin and 0.02% (0.54 µM) EDTA (disodium salt) in calcium- and magnesium-free phosphate-buffered saline (0.14 M sodium chloride:0.05 mM dibasic sodium phosphate:1.5 mM mono-basic potassium phosphate:0.1 mM dextrose). One mL of cell suspension was removed for quantitation in a Coulter counter. EHNA at 10 µM was used routinely to inhibit serum adenosine deaminase (24).

Adenosine Deaminase Activity. Washed cell pellets were homogenized in 50 mM Tris-Cl (pH 7.4), and the particulate components were sedimented in a Microfuge (Beckman Instruments Inc., Fullerton, Calif.) for 3 min. The supernatant was removed and stored at -20°C until used in the assay. Ten µl of cell homogenate were added to 90 µl of reaction mixture. The final mixture contained 0.25 µCi ([14C]adenosine (47 µCi); 1.2, 2, or 4 mM unlabeled adenosine, 50 mM Tris-Cl (pH 7.4), and 4 to 8 µg of protein. After addition of the homogenate, the mixture was incubated at 37°C for 30 min; the reaction was stopped by placing the tube in a 100°C water bath for 5 min. Twenty µl of the sample were spotted on a polyethyleneimine cellulose thin-layer plate with inosine and adenosine markers, and the nucleosides were resolved by ascending chromatography in n-butyl alcohol:acetone:glacial acetic acid:water (35:10:20:35). The plate was air dried, and the nucleoside spots were visualized with UV light. The spots were scraped from the plate and quantitated in a scintillation counter. Protein was quantitated by the procedure of Lowry et al. (21).

An enzyme unit was defined as µmol inosine produced per min. With this assay system, calf intestinal adenosine deaminase had a specific activity of 2 × 104 ± 0.24 units/mg (S.D.), and calf fetal serum had a specific activity of 42 ± 8 units/mg.

Greater than 90% of the added [14C]adenosine was routinely recovered as [14C]adenosine or [14C]inosine; therefore, the further conversion of inosine to hypoxanthine by nucleoside phosphorylase in the homogenate was not a significant factor under the conditions of the assay.

Analysis of RNA Synthesis. Melanoma cells (2.5 × 10⁵) were grown in 50 mL of complete medium in the appropriate reagents for 4 days. On the fifth day, [3H]uridine was added to 5 µCi/mL; the cells were labeled for 6 hr, harvested, and then frozen.

The cells were homogenized with 10 strokes of a Dounce hand homogenizer in 5 mL of RNA buffer containing 0.01 M sodium acetate (pH 5.2) and polyvinyl sulfate (5 µg/ml) (3, 7). The homogenate was adjusted to 15 mL with RNA buffer, 10% sodium dodecyl sulfate was added to 0.5%, and 15 mL of phenol equilibrated with 0.01 M sodium acetate (pH 5.2) was added. The mixture was shaken for 30 min at 25°C on a Burrell wrist-action shaker. The phases were separated by centrifugation, and the aqueous was removed. The phenolic phase was reextracted with 10 mL of RNA buffer for 15 min. The phases were separated, and the 2 aqueous phases were combined, mixed with 10 mL of phenol, and reextracted for 15 min. The phases were separated, and the RNA precipitated twice in 2 volumes of ethanol and 0.1 volume of 1 M NaCl. The RNA was quantitated by its absorbance at 260 nm, and the radioactivity was quantitated in a scintillation counter.

Scintillation Counting. Each sample was brought to 0.5 mL with water, and then 5 mL of Triton-toluene were added. The scintillation fluid contained 66.7% toluene (v/v), 33.3% Triton X-100 (v/v), 0.01% POPOP (w/v), and 0.55% PPO (w/v).

RESULTS

Effect of EHNA and Cell Number on Adenosine Toxicity. Adenosine deaminase, a constituent of fetal calf serum as well as
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as of cellular proteins, minimizes adenosine-induced growth inhibition by converting toxic adenosine to nontoxic inosine. At low adenosine concentrations, the rapid enzymatic conversion renders the adenosine nontoxic before a growth response can be evoked. Consequently, low-concentration adenosine studies are usually conducted with an adenosine deaminase inhibitor in the culture medium. EHNA and 2'-deoxycoformycin are both effective inhibitors of adenosine deaminase (7, 14); however, while being so, they are also cytotoxic. As measured by growth inhibition, both of the inhibitors exhibited similar levels of cytotoxicity. EHNA was more readily available and therefore was chosen for these studies. To limit the cytotoxic input of EHNA, the inhibitor was used at 10 μM (Chart 1). This concentration usually inhibited cell growth by 15 to 25% with fluctuation of between 10 to 40% being noted with different batches of EHNA and after different storage periods as a solution. However, the studies used in this report were in the 15 to 25% range.

While fetal calf serum contains substantial adenosine deaminase, based on specific activity, the melanoma cells themselves possessed 4 to 5 times more of the enzyme (Table 1). Therefore, the effective adenosine concentration and the duration of cytostasis were clearly functions of the number of cells in culture (Chart 2). To eliminate this variable, the studies were conducted with approximately 5 x 10^4 cells/culture, a value obtained from the nonlinear portion of Chart 2.

Cessation of melanoma growth in adenosine was not an immediate phenomenon; even the cells in 200 μM adenosine experienced 1 to 2 doublings before complete inhibition was observed (Chart 3); even then, after 6 days, the cells would again start to proliferate. Consequently, even in the presence of EHNA:adenosine-containing medium, adenosine was being depleted. This was confirmed by the addition of fresh adenosine-containing medium which resulted in further growth inhibition. Because of these constraints, growth studies were conducted within a 4-day period (88 to 96 hr). While cells in 200 μM adenosine were doubling 1 to 2 times during this period, control cells were experiencing 4 to 5 doubling. Therefore, under these conditions, up to 90% growth inhibition could be observed (Chart 4). Increasing the adenosine concentration to 800 μM did not increase growth inhibition significantly; however, it did extend the duration of cytostasis. Similarly, replacing the medium on the fifth day with fresh adenosine-containing medium prolonged the inhibition, and no new lag phase was observed; when replenished, cells in high concentrations of adenosine (100 to 200 μM) displayed a decrease in cell number (cytolysis), indicating that adenosine exposure was actually

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**Table 1**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Adenosine deaminase activity (units/mg)</th>
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</thead>
<tbody>
<tr>
<td>Mouse melanoma (B16)</td>
<td>228 ± 55*</td>
</tr>
<tr>
<td>Fetal lung fibroblast (IMR-91)</td>
<td>171 ± 40</td>
</tr>
<tr>
<td>Burkitt's lymphoma</td>
<td>597 ± 63</td>
</tr>
<tr>
<td>Human myeloma (RPMI-8226)</td>
<td>1320 ± 170</td>
</tr>
</tbody>
</table>

*Mean ± S.D. of 3 determinations at different substrate concentrations.

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**Charts**

Chart 1. Cytotoxicity of the adenosine deaminase inhibitor, EHNA. EHNA in different concentrations was incubated with 5 x 10^6 mouse melanoma cells. The cells were harvested after a 4-day period as stated in "Materials and Methods." Bars, S.D. of 3 determinations.

Chart 2. Effect of cell number on adenosine-induced growth inhibition. Melanoma cells (5 x 10^6 to 5 x 10^7) were cultured in 200 μM adenosine and 10 μM EHNA for 4 days, and then the cells were quantitated. Control cultures lacking adenosine or EHNA but containing the same number of cells were grown for the same periods. Different numbers of cells grown in 50 μM adenosine and 10 μM EHNA gave a similar profile. Maximum inhibition occurred at adenosine levels greater than 2 μmol/10^5 cells.

Chart 3. Inhibition of melanoma proliferation by adenosine. Cells (5 x 10^6) were grown in 25 to 200 μM adenosine and 10 μM EHNA. The cells were quantitated, as stated in "Materials and Methods."
lethal. If, however, after 4 days of incubation, the cultures in adenosine concentrations of 50 μM and greater were replenished with medium lacking adenosine, 48 hr were required before the cell once again achieved log-phase growth. Consequently, in high concentrations of adenosine there was a 4- to 5-day lag period before complete cytostasis was observed, and after this exposure, a 2-day lag period was required before complete recovery was apparent.

These experiments characterized the conditions for analyzing adenosine-induced growth inhibition in melanoma cells and demonstrated that adenosine not only caused cytostasis but, under prolonged periods of growth inhibition, also caused cytorecrosis.

Effects of Adenosine-containing Compounds and HCT. Lymphoid cells have been reported to be extremely sensitive to adenosine with 80% growth inhibition being evident at 5 μM concentration after 3 days (28); in contrast, normal human fibroblasts were reported to be relatively insensitive with 400 μM adenosine being required to invoke a significant inhibitory response in 6 days (1). Melanoma cells were 80% inhibited in 50 μM adenosine after 4 days of incubation (Chart 4). Quantitation of the adenosine deaminase activity in these 3 tissue types showed that the reported adenosine sensitivities were not directly related to the cellular content of this enzyme (Table 1). The most sensitive cells, the lymphoid cells, possessed the highest adenosine deaminase activity, while the activity in the least sensitive fibroblasts and moderately sensitive melanoma cells was 2 to 6 times less.

N°O°-Dibutyryladenosine-cyclic-3':5' phosphate was reported to inhibit the proliferation of normal and transformed lymphocytes (14, 20). As an adenosine-containing analog, it was evaluated for its ability to mimic adenosine-induced growth inhibition in melanoma cells; it had no growth inhibiting effects on these cells. When complexed with polyuridylic acid, polyadenyllic acid was reported to stimulate lymphocyte proliferation and enhance the immune response (8, 17, 27). The adenosine containing homopolymer had essentially no effect on melanoma proliferation. These observations suggest that melanoma cells are not as sensitive to adenosine-containing compounds as lymphocytes are and thus may be less complex in their metabolic response to adenosine.

Kredich and Martin (19) reported that HCT, a stable analog of homocysteine, enhanced the cytotoxicity of adenosine when added to cultures of transformed lymphocytes. They hypothesized that adenosine in conjunction with cellular homocysteine reverses the activity of S-adenosylhomocysteine hydrolase and causes S-adenosylhomocysteine formation; this in turn inhibits S-adenosylmethionine-dependent methyltransferase and nucleic acid methylation. In agreement with their hypothesis, HCT greatly enhanced the cytotoxicity of adenosine in melanoma cells (Table 2), while EHNA and HCT in combination had only the expected effect of EHNA alone.

Effects of Guanosine and Uridine. In consecutive studies, guanosine, the companion purine nucleoside of adenosine, was only slightly effective in relieving adenosine-induced growth inhibition (Chart 5). However, guanosine alone was very effective as a growth inhibitor (Chart 5). The inability of HCT to enhance guanosine toxicity suggested that the 2 purine nucleosides were exerting their growth-inhibiting effects by different mechanisms. A surprising response was observed when the 2 were used in combination; instead of being additive, adenosine relieved the growth-inhibiting effect of guanosine (Chart 5). This observation has not been reported for lymphoid cells.

Ishii and Green (16) postulated that adenosine-induced cytostasis and cytolysis were the result of pyrimidine starvation, and accordingly, growth inhibition could be relieved by adding uridine to the culture medium. In melanoma cultures, uridine did not diminish the extent of adenosine-induced growth inhibition. However, when the cells were grown for 4 days in a different adenosine concentration and the RNA was labeled with [3H]uridine and extracted, the specific activity of the RNA from cells maintained in 50 to 100 μM adenosine was greater than the cells in the control culture or 25 μM adenosine (Chart 6). Such an observation could be the result of the UTP pool being depleted before the labeled uridine was added, accelerated RNA synthesis, or merely the stimulation of uridine uptake by certain adenosine concentrations. Accelerated RNA synthesis is not likely to occur in growth-inhibited cells. At 200 μM, the specific activity was less than that of the control and clearly demonstrated a toxic state or cellular degeneration. These results are comparable to those reported by Ullman et al. (28) who stated that at low adenosine toxicity and at higher concentrations uridines were ineffective as a rescuing agent. The results also indicate that adenosine influences the availability of pyrimidine nucleotides.

DISCUSSION

Complete adenosine-induced growth inhibition in melanoma cells occurred after 1 to 2 doublings and implied a gradual shutdown of metabolic activity more so than an abrupt shutoff of a major biochemical event such as protein or RNA synthesis. One can speculate that either inhibition of de novo pyrimidine biosynthesis or inhibition of nucleic acid methylation with grad-
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Chart 5. Cytotoxicity of guanosine and of combined guanosine and adenosine. Melanoma cells (5 x 10^6) were grown in 25 to 200 μM guanosine and 10 μM EHNA; 25 to 200 μM guanosine, 10 μM EHNA, and 50 μM adenosine; and 25 to 200 μM adenosine, 10 μM EHNA, and 50 μM guanosine. The control cultures lacked adenosine, guanosine, and EHNA. The cells were quantitated after 4 days incubation.

Chart 6. Effect of different concentrations of adenosine on [3H]uridine labeling of melanoma RNA. The cells were treated, and the RNA was extracted as stated in "Materials and Methods."

In general, the melanoma cells are quite responsive to adenosine cytotoxicity. Although they are not as sensitive as lymphoid cells, they lack many of the metabolic complications and regulatory constraints that the immune system imposes on lymphocytes. In addition, they retain the ability to produce melanin under proper stimulation, and because of this they would be an appropriate model for investigating the effect of adenosine on cytodifferentiation.

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