Adenosine-mediated Killing of Cultured Epithelial Cancer Cells

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ABSTRACT

Because micromolar concentrations of adenosine (Ado) have been documented recently in the interstitial fluid of carcinomas growing in animals, we examined the effects of low concentrations of Ado on the growth of cultured human carcinoma cells. Ado alone had little effect upon cell growth. In the presence of one of a number of Ado deaminase (ADA) inhibitors, Ado led to significant growth inhibition of all cell lines tested. Similar effects were found when ATP, ADP, or AMP was substituted for Ado. Surprisingly, the ADA inhibitor coformycin (CF) had a much greater potentiating effect than did 2'-deoxycoformycin (DCF), although DCF is a more potent ADA inhibitor. The growth inhibition of the Ado/CF combination was not abrogated by pyrimidines or caffeine, a nonspecific Ado receptor blocker. Toxicity was prevented by the addition of the Ado transport inhibitor dipiridamole or the Ado kinase inhibitor 5'-amino 5'-deoxyadenosine. 5'-Adenosylhomocysteine hydrolase is not involved because neither homocysteine thiolactone nor an 5'-adenosylhomocysteine hydrolase inhibitor (adenosine dialdehyde) potentiated toxicity of the Ado/CF combination. Unexpectedly, substitution of 2'-deoxyadenosine (the toxic moiety in congenital ADA deficiency) for Ado, did not lead to equivalent toxicity. The Ado/CF combination inhibited DNA synthesis and brought about morphological changes consistent with apoptosis. Together, these findings indicate that the Ado-mediated killing proceeds via an intracellular route that requires the action of Ado kinase. The enhanced cofactor activity of CF may be attributable to its being a more potent inhibitor of AMP deaminase than is DCF.

INTRODUCTION

Although adenine nucleotides are usually thought of as intracellular compounds, a growing body of literature indicates that they are also found in the extracellular space. Release of ATP (or related compounds) has been demonstrated from ocular ciliary epithelial cells (1), neutrophils (2), erythrocytes (3), nasal epithelial cells (4), endothelial and smooth muscle cells (5), and hepatocytes (6, 7). Extracellular Ado is thought to arise from the sequential dephosphorylation of ATP to ADP and AMP and then to adenosine (or related compounds), up to 50 μM or more, in part because the physiological concentrations of these compounds has been a matter of conjecture. In a recent study (28), microdialysis was used to study the Ado concentrations of interstitial fluid of carcinomas growing in mice. When inhibitors of ADA were used to inhibit Ado breakdown in the microdialysis apparatus, extracellular concentrations of Ado as high as 9 μM were found. Prompted by these observations, we reasoned that low concentrations of Ado might promote tumor cell growth and began experiments to determine whether cultured carcinoma cells would grow more rapidly in its presence. We found instead that Ado, in the presence of ADA inhibitors, inhibited growth of a variety of carcinoma cells. Surprisingly, Ado was substantially more toxic to cells in the presence of CF than DCF, although the latter is a more potent ADA inhibitor. Furthermore, we found that dAdo, which is believed to mediate the lymphoid toxicity characteristic of ADA deficiency, was much less toxic than Ado to the epithelial lines studied.

MATERIALS AND METHODS

Materials. DCF was obtained from SuperGen (San Ramon, CA) and the National Cancer Institute (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment). CF was obtained from Calbiochem (San Diego, CA) as well as the National Cancer Institute. Cell viability testing was performed with a kit (Cell Titer 96 Aqueous One Solution) purchased from Promega Corp. (Madison, WI). Phalloidin-Alexa 488 was obtained from Molecular Probes (Eugene, OR), as was the Prolong reagent to enhance photostability. All other materials were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods. All cell lines were purchased from American Type Culture Collection, except for the OVCAR 3 line, which was provided by Dr. Satya Murthy (Evanston Hospital). The cells were all grown according to American Type Culture Collection specifications. The OVCAR 3 line was grown in DMEM + 10% heat-inactivated fetal bovine serum with penicillin/streptomycin. After passaging, the cells were plated in 96-well plates at a density of 2000–5000 cells/well and were grown overnight. The medium was then removed and replaced with fresh medium containing various concentrations of the compounds to be tested.

Viability Testing. The cells were cultured for 60 h in the presence of drugs, at which time untreated controls were just approaching confluence. All treatments were performed in quadruplicate. Viability was tested using the formazan dye, MTS (29). Reagents were added according to the manufacturer’s specifications, and the plates were incubated at 37°C until the untreated wells exhibited an A570 of −0.7. Wells containing medium alone were used as blanks. Viability was expressed as a percentage of the untreated controls. All experiments were performed at least three times.

BrdUrd Incorporation. A colorimetric ELISA kit was used (Roche Molecular Biochemicals, Indianapolis, IN) to determine BrdUrd incorporation into cells. Cell lines were plated out in 96-well plates as described above and allowed to adhere and grow overnight. Ado, CF, and/or DCF were added for 6 and 12 h at a final concentration of 10 μM each, in quadruplicate. BrdUrd label was added for the last 2 h of each treatment time, and the manufacturer’s instructions were followed for performing the immunoassay and measurement steps. The experiment was performed three times on each cell type.

Morphological Evaluation. Cells were grown on chamber slides overnight. Drugs were added in fresh media. At the times indicated, the medium was removed, and the cells were rinsed with PBS and fixed in 3.7% paraformaldehyde in PBS for 10 min. After rinsing three times with PBS, the fluorescent dye Hoechst 33258 (0.5 μg/ml in 10 mM phosphate, 0.15 M NaCl, pH 7.4) was added to the cells for 2 min. The cells were rinsed, dried thoroughly, and
then coverslipped with an aqueous mounting medium. Cells were then evaluated by fluorescent microscopy. Alternatively, cells grown on coverslips were fixed, permeabilized, and stained with phalloidin-Alexa 488 as per the manufacturer’s protocol and evaluated by fluorescent microscopy.

RESULTS

Various (1–500 μM) concentrations of Ado or its possible precursors, adenine, AMP, ADP, or ATP (30), were added to the culture medium used to grow lines derived from human epithelial cancers of breast (MCF-7 and MDA MB-231), colonic (HT-29), and ovarian (OVCAR-3 and OVCAR-5) origin. The cells’ growth was assessed after 60 h of incubation with the added compounds (Fig. 1). Instead of observing growth stimulation from the addition of these compounds, we noticed a trend toward growth inhibition at higher concentrations.

To determine the effects of Ado in the absence of the protective effects of ADA, we grew the cells in the presence of various ADA inhibitors.

One to 10 μM concentrations of Ado were added to the cells in the presence of low (3–20 μM) concentrations of the ADA inhibitors EHNA or CF. As shown in Table 1, the addition of EHNA to Ado resulted in only modest degrees of growth inhibition. The addition of low (5–20 μM) concentrations of CF, however, resulted in a much greater growth inhibition, especially of the two ovarian cancer cell lines. Neither CF nor Ado alone inhibited the proliferation of any of the cells tested (Table 1). Prostate cancer lines (PC3, LNCaP, TSU-Pr1, and DU145) were also sensitive to the Ado/CF combination.

<table>
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<tr>
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<th>MCF-7</th>
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<tr>
<td>3 μM EHNA</td>
<td>101 ± 11</td>
<td>ND</td>
<td>97 ± 11</td>
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<tr>
<td>10 μM EHNA</td>
<td>111 ± 3</td>
<td>96 ± 8</td>
<td>99 ± 7</td>
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<td>76 ± 7</td>
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<td>104 ± 10</td>
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</tr>
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<td>10 μM Ado + 3 μM EHNA</td>
<td>107 ± 7</td>
<td>79 ± 2</td>
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<tr>
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<td>96 ± 13</td>
<td>76 ± 4</td>
<td>84 ± 8</td>
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Table 1 Comparison of the ADA inhibitors EHNA and CF on the viability of human epithelial cancer cell lines alone and in combination with Ado

Cells were grown in the presence of drugs for 60 h, and viability was assessed with the MTS assay. Values shown are percentages of viable cells compared with untreated controls.

Bar, SD.
Substituting ATP, ADP, or AMP for Ado led to the same degree of growth inhibition (Fig. 2). Substitution of 10 μM adenine for Ado did not lead to growth inhibition (data not shown).

Surprisingly, much less growth inhibition was seen when the more potent ADA inhibitor DCF was substituted for CF (Fig. 3). The same results were obtained when either CF or DCF obtained from a second source was used (data not shown).

A dose response relationship was seen when either the Ado (Fig. 4A) or CF (Fig. 4B) concentrations were varied and the CF or Ado concentrations, respectively, held constant (10 μM). Although a 24-h exposure to the drugs was necessary to see the maximal drug effect (data not shown), the effects on cell growth became more evident with increasing observation times (Fig. 4C).

To determine whether the site of action of the Ado/CF was extra- or intracellular, cells were grown in Ado/CF-containing medium in the presence of the nonspecific Ado receptor antagonist, caffeine. Caffeine (100 μM) had no effect upon the Ado/CF-mediated toxicity (data not shown). Toxicity was largely abrogated, however, by the nucleoside transport inhibitor, dipyridamole (Fig. 5A), suggesting that the Ado/CF combination is acting intracellularly and not via cell surface receptors. This conclusion was supported by the finding that the Ado kinase inhibitors 5′-amino-5′-deoxyadenosine (Fig. 5B) or iodothyricidin (data not shown) protected cells from the Ado/CF combination.

Ado toxicity in fibroblasts or lymphoblasts can be abrogated by supplementing the growth media with pyrimidines (30, 31).
addition of up to 100 μM thymidine, uridine, cytidine, or deoxycytidine (data not shown), or a combination of 100 μM thymidine, cytidine, and uridine (Fig. 5C) did not prevent toxicity of the Ado/CF combination to these carcinoma lines.

Elevation of dAdo concentrations, an irreversible inhibitor of the enzyme SAHH (32), is believed to play an important role in mediating lymphoid toxicity in patients with congenital ADA deficiency (33). To determine whether the effects of Ado and CF on carcinoma cells might be attributable to the intracellular conversion of Ado to dAdo, we treated cells with equimolar concentrations of dAdo and CF. As shown in Fig. 6, the combination of dAdo and CF caused much less toxicity than Ado/CF. This result implies that the toxicity of the...
Ado/CF treatment is not attributable simply to the generation of dAdo or its nucleotides.

In some circumstances, Ado toxicity is thought to occur because homocysteine and Ado can be enzymatically converted to S-adenosylhomocysteine by SAHH, thereby interfering with a number of cellular methylation reactions (34). The importance of this reaction has been inferred from experiments in which the toxicity of low concentrations of Ado is potentiated by the addition of homocysteine (or its thiolactone; Refs. 35–38). We therefore treated cells with 1 mM Ado, 10 μM CF, and 100 μM homocysteine thiolactone. As shown in Fig. 7A, homocysteine thiolactone did not potentiate the toxicity induced by this low concentration of Ado, suggesting that the observed toxicity was not attributable to the formation of SAH. To further explore the possible involvement of the SAH pathway in the toxicity we found, we added an SAHH inhibitor, Ado dialdehyde. As shown in Fig. 7B, 100 μM Ado dialdehyde did not inhibit the toxicity of the Ado/CF combination, further arguing that the toxicity we observed was not mediated, directly or indirectly, by SAHH.

Although the number of cells in Ado/CF-treated cultures does not begin to differ from untreated cultures until after 24 h of drug exposure, we observed morphological changes in the cells at earlier time points. We therefore used an immunoassay of BrdUrd uptake to determine the effects of the Ado/CF combination upon DNA synthesis. After 6 h of drug exposure, DNA synthesis was inhibited by 60% and after 12 h was inhibited by >90%, as compared with untreated cultures. In contrast, treatment of cells with DCF and Ado resulted in only a slight effect upon DNA synthesis after 12 h (Fig. 8). Thus, inhibition of DNA synthesis after 6 h exposure predicted the growth inhibition seen after 60 h of treatment.

Microscopic examination of cells treated with Ado/CF showed a notable change in cell morphology within the first 6 h of addition. Treated cells pulled away from neighboring cells and became more cuboidal in shape. After 8 h of drug treatment, the Ado/CF-treated cells showed a loss of cytoskeletal organization, as evidenced by a loss of actin stress fiber staining with phalloidin. By 24 h, phase contrast microscopy revealed the cells to be smaller and more refractile, and membrane blebs were seen (data not shown). Staining of the cells with the fluorescent dye Hoechst 33258 (Fig. 9) revealed increased nuclear uptake and fragmentation, as compared with control cells, changes consistent with the induction of apoptosis (39).

DISCUSSION

The present study provides the most extensive analysis of the effects of Ado upon human epithelial cancer lines performed to date. Initiated by the observation that Ado could be detected in the interstitial fluid surrounding a carcinoma (28), the experiments described here demonstrate the toxic effects of Ado and the ADA inhibitor CF upon multiple representatives of this important group of cancers. Although many others have found Ado to be toxic to a variety of cell types (12, 40–46), this report documents toxicity at low micromolar Ado concentrations, without apparent involvement of the usual mechanisms cited to explain such toxicity: Ado receptor stimulation, depletion of intracellular pyrimidine stores, alteration of cellular methylation because of involvement of SAHH, and/or elevation of increased concentrations of dAdo.

Because inhibition by the Ado/CF combination was blocked by the nucleoside-transport inhibitor dipyridamole (47) but not the nonspecific Ado receptor blocker caffeine (48), an intracellular rather than extracellular site of action was established, in contrast to recent reports of growth inhibition or killing via stimulation of extracellular Ado receptors (44–46).

Although under some conditions Ado toxicity appears to be attributable to a depletion of intracellular pyrimidine stores, which can be ameliorated by the addition of one or more members of this family (30, 49, 50), the addition of a 10-fold molar excess of thymidine, cytidine, and/or uridine did not protect the cells.

The lack of involvement of SAHH is inferred from the results of
several experiments: (a) in lymphoblasts, Ado toxicity may proceed in the absence of Ado kinase activity (51) because of inhibition of cellular methylation reactions, acting through SAHH (35, 52). Because the toxicity of Ado/CF seen here was blocked by the Ado kinase inhibitors 5'-amino-5'-deoxyadenosine and iodotubericidin, a phosphorylation event is apparently involved in Ado/CF toxicity; (b) the addition of homocysteine thiolactone, which can increase SAH levels when coupled to Ado by SAHH, thereby potentiating Ado toxicity (35, 37, 38, 52), did not alter the toxicity of 1 μM Ado and CF; (c) inhibition of SAHH with Ado dialdehyde had no protective effect.

Fig. 9. Uptake and distribution of the fluorescent dye Hoechst 33258 in human carcinoma cell lines cultured in the absence (A, C, and E) or presence (B, D, and F) of 10 μM Ado and 10 μM CF for 18 h at 37°C. A and B, OVCAR-3; C and D, MCF-7; E and F, HT-29.
upon the toxicity of the Ado/CF combination, arguing against a build-up of SAH levels because of increased Ado concentrations; and (d) substitution of Ado by dAdo, a potent SAHH inhibitor (32), did not lead to comparable toxicity.

This last finding was particularly surprising because much of the pathophysiology of congenital ADA deficiency, which has often been modeled in vitro by adding ADA inhibitors such as CF, has been thought to be attributable to elevated levels of dAdo (33, 53–56). Furthermore, in vitro studies have shown dAdo to be toxic to a variety of cell types (13), including lymphoid cells (57, 58), fibroblasts (59), adrenal chromaffin cells (60), sympathetic neurons (61), and mono-cytioid leukemia cells (62). Taken together, these experiments argue against the involvement of either SAHH or dAdo in low-dose Ado-mediated toxicity to carcinoma cell lines.

Because the Ado kinase inhibitor 5′-amino-5′-deoxyadenosine protected the cells, it seems likely that phosphorylation of Ado with a resultant elevation in AMP, ADP, and/or ATP is required for growth inhibition. It is possible that both Ado and CF must be phosphorylated intracellularly for toxicity to occur. Although DCF can be phosphorylated by enzymes other than Ado kinase and incorporated into cellular DNA (63), it is not known whether CF is similarly phosphorylated and incorporated into DNA or RNA.

EHNA, a synthetic ADA inhibitor (64), was significantly less toxic to the human epithelial cancer cell lines tested than CF. This was not surprising because EHNA is a less effective inhibitor of ADA ($K_i = 6.5$ nm; Ref. 65) than is CF ($K_i = 0.01$ nm; Ref. 66). Of greater interest, however, was that CF potentiated the toxic effects of Ado to a greater degree than DCF, a yet more potent ADA inhibitor ($K_i = 0.0025$ nm; Ref. 65). Intracellular concentrations of the two drugs were likely comparable because others have shown that CF and DCF are transported across cell membranes equivalently (67).

The difference in their cofactor activity in these experiments might be attributable to the fact that neither compound is totally specific for ADA, and each may have inhibitory effects upon other enzymes. In fact, all of the ADA inhibitors used in this study also inhibit AMPDA. Although DCF is a more potent ADA inhibitor than EHNA or CF, its AMPDA inhibitory activity is greater than that of EHNA (68) but 50-fold less potent than that of CF (69). Because deamination, rather than dephosphorylation, is the primary route of AMP catabolism (33), the enhanced toxicity of the CF-containing combination may be attributable to a greater combined blockade of ADA and AMPDA than is seen with DCF.

The growth inhibition seen when AMP, ADP, or ATP was substituted for Ado is likely attributable to extracellular conversion of these nucleotides to Ado by ecto-ATP diphosphohydrolase and 5′-nucleotidase. These results corroborate the work of others who have found that adenine nucleotides kill various types of cancer cells (reviewed in Ref. 70).

At this time, we cannot provide an explanation for the inhibition of DNA synthesis and induction of apoptosis seen after the cells were incubated with Ado/CF. Studies are in progress to confirm our suspicions that high intracellular concentrations of ATP arise as a result of treatment with this combination and to explore potential mechanisms whereby such elevations might trigger cell death.

To date, Ado analogues have not been useful in treating epithelial malignancies. We note that unlike either Ado or CF, the three Ado analogues in clinical use (fludarabine, DCF, and 2-chloro-2′-deoxyadenosine) all lack hydroxyl groups at the 2′ position of the ribose ring and are only active against lymphoid malignancies. Further study is required to determine whether Ado analogues with an intact ribose ring might be more useful against epithelial malignancies than are their 2′-deoxy counterparts.
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