Retroviral Transfer of Deoxycytidine Kinase into Tumor Cell Lines增强了核苷酸毒性

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ABSTRACT

Deoxycytidine kinase (dCK) phosphorylates a number of nucleoside analogues that are useful in the treatment of various malignancies. Although the level of dCK activity in malignant cells is thought to correlate with chemotherapeutic response, no direct data are available to support this assumption. We have tested this hypothesis by infecting three tumor cell lines, MCF-7, HT-29, and H1437, with the retroviral vector LNPO containing either dCK or LacZ cDNA and measuring the corresponding chemotherapeutic response. No direct data are available to support the hypothesis that dCK activity results in increased sensitivity to drugs that are dependent on deoxynucleosides for DNA synthesis and repair by reutilizing deoxyribonucleotides for DNA synthesis and repair by reutilizing deoxynucleosides. The addition of dCK activity will be a useful adjunct to the treatment of these malignancies.

INTRODUCTION

dCK (EC 2.7.1.74) is a pivotal enzyme involved in the salvage pathway of deoxyribonucleotide biosynthesis. This pathway provides deoxyribonucleotides for DNA synthesis and repair by reutilizing deoxynucleosides taken up via a low-affinity nucleoside carrier (1). Once inside the cell, the deoxynucleotides are phosphorylated to their monophosphate form or exported from the cell. The phosphorylation reaction constitutes the rate-limiting step in this pathway (2) and prevents the efflux of the phosphorylated nucleotides from the cell. In addition to the naturally occurring 2'-deoxyribonucleosides dCyd, dAdo, and dGuo, dCK phosphorylates a number of nucleoside analogue drugs, such as AraC (3), Cda (4), FdAra (5, 6), and FdF (7). FdC has shown considerable activity against solid tumors and leukemic cell lines (7-9), whereas AraC, Cda, and FdAra are all in clinical use for the treatment of leukemias (10-12).

Although several studies (13-14) have suggested that sensitivity to these drugs correlates with the dCK phosphorylating activity of the target cell, none has provided conclusive data that demonstrate a direct dependence of drug sensitivity on dCK activity. Using the cDNA encoding the dCK protein (15), we have asked whether increased expression of dCK in tumor cell lines containing endogenous dCK activity results in increased sensitivity to drugs that are dependent on dCK phosphorylation for their cytotoxic effect.

MATERIALS AND METHODS

Chemicals and Reagents. AraC and adenosine were purchased from Sigma Chemical Co. (St. Louis, MO). Cda was a gift from Ortho Biotech Inc. (Raritan, NJ). FdAra was obtained from Berlex Lab, Inc. (Richmond, CA), and FdF was obtained from Eli Lilly and Co. (Indianapolis, IN). [α-32P]dCTP (3000 Ci/mmol) and [2-3H]adenosine (20 Ci/mmol) were purchased from Amersham Life Science (Arlington Heights, IL). [3H]Cda (20 Ci/mmol) was purchased from Moravak (Brea, CA). Antibody to human dCK was a kind gift of Staffan Eriksson (Swedish University of Agricultural Sciences, Uppsala, Sweden).

Cells and Cell Culture. The colon carcinoma cell line HT-29 and the breast carcinoma cell line MCF-7 were obtained from the University of North Carolina Linberger Comprehensive Cancer Center (Chapel Hill, NC). The small cell lung adenocarcinoma cell line H1437, originating from a lung cancer metastatic to the pleural space, was obtained from Diana Hoganson (University of North Carolina, Chapel Hill, NC). HT-29 cells were maintained in McCoy's 5a medium. All media were supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and antibiotics/antimycotics (Life Technologies, Inc., Grand Island, NY). MCF-7 cells were maintained in Eagle minimum essential medium supplemented with insulin (10 μg/ml; Life Technologies, Inc., Grand Island, NY), non-essential amino acids, and sodium pyruvate. H1437 cells were maintained in RPMI 1640. All cells were kept at 37°C and 5% CO2.

Viral Infections. The Moloney murine leukemia/sarcoma-based retroviral vectors LNPO-dCK and LNPO-LacZ containing the Moloney promoter were obtained from John Olsen (University of North Carolina, Chapel Hill, NC; Refs. 16 and 17). LNPO-dCK was constructed by inserting the dCK cDNA (15) into the pLNO vector. MCF-7, HT-29, and H1437 cells were infected with these vectors in the following manner (17). Polyclonal cell populations were established by selecting cells in 1 mg/ml Geneticin (Life Technologies, Inc., Grand Island, NY) for approximately 3 weeks. H1437 cells containing integrated LNPO-dCK and LNPO-LacZ were a gift from Diana Hoganson (University of North Carolina, Chapel Hill, NC). Eight clones were isolated from the HT-29 LNPO-dCK polyclonal cell population by plating cells at low density and isolating individual colonies for expansion.

Enzyme Activity Assays. dCK activity assays were performed as described previously, using Cda as a substrate to ensure specificity (18). Adenosine kinase activity assays (19) were performed with the following modifications: the cell extraction buffer was composed of 50 mM Tris (pH 7.6), 20% glycerol, 0.5% NP-40, 2 mM DTT, 5 mM benzamide, and 0.5 μM phenylmethylsulfonyl fluoride. The assay buffer contained 50 mM Tris (pH 7.6), 2 mM DTT, 5 mM MgCl2, 50 mM KCl, 3 mM GTP, 10 mM NaF, 0.5 μM [3H]adenosine, and 4.5 μM adenosine. Protein concentrations were measured using a standard Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Northern Blots. Total RNA was isolated from each cell line using Trizol reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA (10 μg) from each cell line was electrophoresed on a denaturing formaldehyde/agarose gel and transferred to a nylon membrane (Nytran, Scheich and Schuell, Keene, NH) by standard methods (20). Blots were hybridized overnight at 42°C in High-Intensity Hybridization System with 50% Formamide (Molecular Research Center, Inc., Cincinnati, OH). dCK (15) and human-β-actin (Iso Labs, Inc., Akron, OH) cDNA probes were labeled using the Prime-a-Gene labeling system (Promega, Madison, WI). Blots were washed for 1-2 h at 65°C in 0.1X SSC/0.1% SDS and autoradiographed for 8-24 h at ~70°C. Relative levels of expression were determined by densitometry scanning.

Western Blots. Cell pellets were extracted in buffer with three freeze-thaw cycles and centrifuged at 16,000 × g. Supernatants containing 45 μg protein were electrophoresed for 50 min at 180 V on 12.5% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) by electroblotting as described (21). Blots were incubated at 37°C for 1
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To examine the effects of increased dCK activity on nucleoside analogue drug sensitivity, the tumor cell lines MCF-7, HT-29, and H1437 were infected with the Moloney murine leukemia/sarcoma-based retrovirus vectors LNPO-dCK or LNPO-LacZ. Cells were selected in Geneticin and assayed for dCK activity. As shown in Fig. 1, cells stably transfected with LNPO-LacZ had dCK activity equivalent to that of uninfected cells. In contrast, the mean increase in dCK activity for cells infected with LNPO-dCK was 1.7-fold for the MCF-7 cell line, 2.3-fold for HT-29 cell line, and 16-fold for the H1437 cell line. Adenosine kinase activity, measured as a control for the level of an endogenous nucleoside kinase, was equivalent in all cell lines tested (data not shown).

To determine whether the increase in dCK activity seen in the LNPO-dCK infected cells was due to an increase in both mRNA and protein expression, Northern and Western blots were performed on the three tumor cell lines. As shown in Fig. 2, an endogenous dCK mRNA was detected as a 2.4-kb band in each cell line. A 3.8 kb viral transcript, encoding both the neomycin-resistance and dCK mRNAs, was found in those cell lines infected with LNPO-dCK and was roughly proportional in intensity to the increment in dCK activity. Western blot analysis, shown in Fig. 2B, demonstrates endogenous dCK protein. M, 30,500) in each cell line at low levels. Although no shift was detectable with FTaRA, HT-29 cells demonstrated 5.9-, 7.6-, and 5.9-fold increases in sensitivity to AraC, CdaA, and FTaRA, respectively, whereas H1437 cells demonstrated markedly increased sensitivities of 106-, 21-, and 20-fold for AraC, CdaA, and FTaRA, respectively. Although CdaC was more potent than any of the other compounds for all cell lines, there was a less consistent effect of LNPO-dCK expression on CdaC IC50's.

To determine whether there was a direct relationship between the amount of dCK expressed and the sensitivity of that cell to the nucleoside analogue drugs, a number of clones from the polyclonal population of HT-29-dCK cells were isolated and expanded and the dCK activities were determined. Eight clones were found to have a range of dCK activities from 19.6 to 47.3 nmol/mg/h and were tested for sensitivity to each drug. As shown in Fig. 4, a strong correlation exists between dCK expression on dCIC50's.

DISCUSSION

A strong relationship between dCK activity and nucleoside toxicity has been postulated since the initial observations that the naturally occurring nucleosides 2'-deoxyadenosine and 2'-deoxyguanosine were far more toxic to human thymocytes and cultured lymphoblast
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Fig. 3. Representative dose-response curves for the H1437 cell line. Cells were treated with either AraC (A), CdA (B), FAraA (C), or dFdC (D) for 4 days and assayed for cell survival using the MTT assay. Data points, means of six determinations; bars, SE. Values are plotted relative to control values in the absence of drug. ○, H1437; ■, H1437-LacZ; □, H1437-dCK.

Table 1 Decrease in IC50s for nucleoside analogues in tumor cell lines infected with LNPO-dCK vector as compared to LNPO-LacZ-infected controls

<table>
<thead>
<tr>
<th>Cell line</th>
<th>AraC</th>
<th>CdA</th>
<th>FAraA</th>
<th>dFdC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>2.2 ± 0.1^cd</td>
<td>2.5 ± 0.2^cd</td>
<td>0.9 ± 0.3^b</td>
<td>7.6 ± 6.6</td>
</tr>
<tr>
<td>HT-29</td>
<td>5.9 ± 2.1^b</td>
<td>7.6 ± 2.9^cd</td>
<td>5.9 ± 0.9^d</td>
<td>1.7 ± 0.7^c</td>
</tr>
<tr>
<td>H1437</td>
<td>105.7 ± 19.5^cd</td>
<td>21.0 ± 4.6^b</td>
<td>20.0 ± 5.1^d</td>
<td>20.3 ± 15.1^b</td>
</tr>
</tbody>
</table>

a Sensitivity assays were done as outlined in "Materials and Methods." IC50s were determined from replicate values in each experiment (n = 6) and the fold decrease in IC50 determined as IC50 LacZ/IC50 dCK.

b Mean ± SE (n = 3-5 experiments).

c Values were determined using the parental cell line as the control.

d Experimental values in each LNPO-dCK group were significantly different from control values at the P < 0.05 level.

Although several studies have addressed the relationship between dCK activity and drug sensitivity in lymphoid cells, they have required an indirect correlative approach. Kawasaki et al. (13) examined the activities of dCK and 5'-NT in cells from patients with hairy-cell leukemia and chronic lymphocytic leukemia and correlated the dCK: 5'-NT ratio with response to chemotherapy with CdA. Those patients cell lines than they were to other cell types (24). Human thymus and lymphoblasts have been demonstrated to contain high levels of dCK (25-27), as well as low levels of cytosolic nucleotidase (5'-NT) responsible for the phosphorolysis of nucleoside monophosphates. The combination of these two characteristics has been thought to be of at least ancillary importance in the susceptibility of T lymphoblasts to nucleoside analogue toxicity and in the T-cell depletion related to adenosine deaminase and purine nucleoside phosphorylase deficiency states (4, 13, 28–30). Conversely, there are definitive data from many reports that cells lacking dCK activity are resistant to a variety of drugs, including AraC, CdA, FAraA, and dFdC (4, 5, 31, 32) and that drug sensitivity to AraC can be restored by expressing functional dCK protein (33).

Fig. 4. Correlation of dCK activity with mean IC50 for HT-29 cell lines exposed to nucleoside analogues. Included are WT, LacZ, and dCK polyclonal and eight monoclonal cell lines. Each IC50 represents the mean of 2–10 determinations, and dCK activity levels represent the mean of 3–10 determinations. Correlation coefficients are as follows: □, FAraA (r = 0.727); ●, CdA (r = 0.822); ○, AraC (r = 0.865); and ■, dFdC (r = 0.636).
who responded tended to have higher dCK and lower 5'-NT activities, but other variables, such as heterogeneity in other nucleoside- or nucleotide-metabolizing enzymes and in the components of the apoptotic pathway, could not be taken into account in such a study. Spasokoukotskaja et al. (14) examined the relationship between dCK and CdA phosphorylation in normal lymphoid cells and a variety of tumors by analyzing dCK protein by Western blot analysis and demonstrating a good correlation with CdA phosphorylating activity. These studies laid the foundation for a direct determination of the role of dCK activity per se in nucleoside-analogue-induced cytotoxicity to tumor cells.

We have infected three tumor cell lines with endogenous dCK activities ranging from approximately 2 (H1437) to 10 (MCF-7, HT-29) nmol/mg/h, with a retroviral vector containing the dCK cDNA and increased mean dCK activity in each of three polyclonal tumor cell lines. This approach has allowed us to control for other variables in drug metabolism and apoptotic response within each cell line. The results indicate that a less than 2-fold increase in dCK activity in the MCF-7 cell line resulted in a small but significant increase in sensitivity to AraC and CdA, although not to FARA or dFdC. In contrast, a slightly more than 2-fold increase in dCK activity in the HT-29 cell line increased sensitivity to AraC, CdA, and FARA by 6–7-fold, whereas the 16-fold increase in dCK activity in H1437 cells increased sensitivity to CdA and FARA by 20-fold and to AraC by over 100-fold. These results confirmed in a general way that increases in dCK activity can increase drug sensitivity for a given cell line. What remained unclear was whether a threshold effect exists in which a specific level of dCK is required for substrate activation or there is a linear relationship between activity and drug sensitivity at all levels of dCK activity. It should be noted in this regard that dCK activity in thymus tissue is on the order of 58 nmol/mg/h, whereas that in normal lymphoid cells and B cell CLL falls within the 13–30 nmol/mg/h range (14, 18). The data on the clonal cell lines expressing a range of dCK activities (Fig. 4) clearly demonstrate that, for HT-29 cells, there is a linear relationship between this activity and IC50 for the drugs tested. Thus, for AraC, CdA, and FARA, a 5-fold increase in dCK activity lowers the IC50 for each drug by approximately 1–1.5 logs, with the highest level of dCK activity achieving approximating the activity found in human thymus.

In summary, we have demonstrated that the level of dCK activity, independent of other variables, is an important parameter in predicting nucleoside toxicity for malignant cells. Additional factors that are important in mediating the sensitivity of malignant cells to nucleoside analogues include: nucleoside transport (34), the intracellular half-life of the corresponding triphosphate (34, 35), and downstream effector mechanisms leading to apoptosis. From a therapeutic standpoint, the more practical question of whether dCK gene transfer could play a role in enhancing chemotherapeutic response remains open to question. The drugs AraC, CdA, and FARA are primarily useful in the treatment of a variety of leukemias and lymphomas. Current limitations in gene transfer technology and the requirement for cellular proliferation for effective retroviral-mediated enhancement of gene expression make leukemias, in particular, an unlikely target in the near future for gene therapy approaches. Although one might conceive of manipulation of endogenous dCK gene expression in leukemias as an alternative approach, recent studies have failed to elucidate tissue-specific mechanisms of dCK transcription that could be exploited (36). In contrast, dFdC has shown significant promise in the treatment of refractory solid tumors of the lung, colon, and ovaries (7, 9), where gene therapy might be more applicable. It should be noted, however, that dFdC cytotoxicity for each tumor cell line used here showed less of a dependency of IC50 on dCK activity than each of the other drugs showed. This observation, in conjunction with the significantly greater potency of dFdC for all cell lines, may be explained by the multiple mechanisms of action of this drug. Thus, the ability of dFdC to incorporate into RNA (37), inhibit ribonucleotide reductase (38), and inhibit DNA synthesis via competitive inhibition of DNA polymerase and chain termination (39) could lessen the dependency on initial phosphorylation for its cytotoxicity. On the other hand, a number of tumors have been demonstrated to have very low levels of dCK activity, including brain and colon (18). It remains possible that dCK gene transfer could be used in conjunction with dFdC in selected solid tumors, where dCK represents a major limitation to the activation of this drug. In addition, dFdC has been demonstrated to have a radiosensitizing effect (40). Whether enhanced phosphorylation by dCK would lead to local enhancement of this effect remains open to evaluation.

REFERENCES
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