Retroviral Transfer of Deoxycytidine Kinase into Tumor Cell Lines Enhances Nucleoside Toxicity

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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 sensitivity to nucleoside analogues. DCK activity was increased by 1.7-, 2.3-, and 16-fold in MCF-7, HT-29, and H1437 cells, respectively. Northern and Western blots demonstrated a similar increase in mRNA and protein levels. As a result of dCK expression, MCF-7 cells demonstrated a 2.5-fold increase in drug sensitivity to 1-ß-D-arabinofuranosylcytosine (AraC) and 2-chloro-2'-deoxyadenosine (Cda). HT-29 cells had a 7-fold increase in sensitivity to AraC, Cda, and 2-fluoro-9-ß-D-arabinofuranosyladenine, whereas H1437 cells demonstrated a 20- to 106-fold increase. For all three drugs, there was a linear relationship between dCK activity and drug sensitivity in cell lines. Because many tumors have relatively low levels of dCK, it is possible that dCK gene transfer 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 deoxyribonucleosides for DNA synthesis and repair by reutilizing deoxyribonucleosides taken up via a low-affinity nucleoside carrier (1). Once inside the cell, the deoxyribonucleosides are either 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 nucleosides from the cell. In addition to the naturally occurring 2'-deoxyribonucleosides dCd, dAdo, and dGuo, dCK phosphorylates a number of nucleoside analogue drugs, such as AraC (3), Cda (4), AraA (5, 6), and dFdC (7). dFdC has shown considerable activity against solid tumors and leukemic cell lines (7-9), whereas H1437 cells demonstrated a 20- to 106-fold increase. For all three drugs, there was a linear relationship between dCK activity and drug sensitivity in cell lines. Because many tumors have relatively low levels of dCK, it is possible that dCK gene transfer will be a useful adjunct to the treatment of these malignancies.

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). FARA was obtained from Berlex Lab, Inc. (Richmond, CA), and dFdC 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/antimyotics (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.

Virai Infections. The Moloney murine leukemia/sarcoma-based retroviral vectors LNPO-dCK and LNPO-LacZ containing the MMLV 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 BanHI site of the pLNPO vector. MCF-7, HT-29, and H1437 cells were infected with these vectors as described previously (17). Polyclonal cell populations were established by selecting cells in 1 µg/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 mM glycerol, 0.5% NP-40, 200 µM DTT, 5 mM benzamide, and 0.5 µM phenylmethylsulfonyl fluoride. The assay buffer contained 50 mM Tris (pH 7.6), 200 µM DTT, 5 mM MgCl2, 50 mM KCl, 3 mM GTP, 10 mM NaF, 0.5 µM [3H]adenosine, and 45 µM adenosine. Protein concentrations were measured using a standard Bradford assay (BioRad Laboratories, Hercules, CA).

Northern Blots. Total RNA was isolated from each line using the TRIzol/agent (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 (Promega, Madison, WI). Blots were washed for 1-2 h at 65°C in 0.1 X 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 x g supernatants containing 45 µg protein were electrophoresed for 50 min at 180 V on 12.5% SDS-polyacylamide 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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2 To whom requests for reprints should be addressed, at CB #7365, 905 FLOB, Department of Pharmacology, University of North Carolina-Chapel Hill, Chapel Hill, NC 27599.
3 The abbreviations used are: dCK, deoxycytidine kinase; AraC, 1-ß-D-arabinofuranosylcytosine; Cda, 2-chloro-2'-deoxyadenosine; FARA, 2-fluoro-9-ß-D-arabinofuranosyladenine; dFdC, 2'-difluorodeoxycytidine; TBS, Tris-buffered saline; RT, room temperature; MTT, 3-(4,5-dimethylthiazol-2-yI)-2,5-diphenyltetrazolium bromide.

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h in blocking buffer (TBS with 0.2% Tween 20, 0.5% gelatin, and 5% dry milk), followed by overnight incubation at 4°C. Incubation with rabbit anti-human dCK antibody (22) at a dilution of 1:250 was performed for 1 h. Blots were then washed 5 times for 10 min at RT in TBS with 0.2% Tween 20 and developed using the ECL Western blotting detection kit (Amersham Life Science, Arlington Heights, IL). Goat antirabbit antibody (conjugated with horseradish peroxidase) was added at a dilution of 1:1000 in blocking buffer and incubating at RT for 1 h. Blots were washed 3 times in TBS with 0.2% Tween 20 and twice in TBS for 10 min and autoradiographed. Relative levels of expression were determined by densitometry.

**Drug Sensitivity Assays.** Cytotoxicity assays were performed using the MTT assay (23). Cells were plated in 96-well plates at a density of 1 × 10^4 cells/well and exposed to a range of nucleoside analogue drug concentrations for 4 days. Cells were then assayed for survival by adding MTT (Sigma, St. Louis, MO) for 2.5 h, and absorbance was measured using an automated microplate reader at a wavelength of 540 nm. Statistical analysis was performed using the Student’s paired t test.

**RESULTS**

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 HI437 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. 2A, 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 an increase in dCK protein was not apparent in extracts from MCF-7 LNPO-dCK infected cells, a 3- and 42-fold increase was present in HT-29 and H1437 LNPO-dCK infected cells, respectively.

To determine how increased dCK activity affects nucleoside analogue drug sensitivity, each cell line was exposed to four nucleoside analogues that are substrates for the enzyme. Fig. 3 demonstrates a representative experiment. Exposure of H1437 cells to increasing concentrations of each drug for 96 h resulted in similar sensitivities of wild-type and LacZ cells to AraC (Fig. 3A), Cda (Fig. 3B), FaraA (Fig. 3C), and dFdC (Fig. 3D). However, the IC50 for H1437-LNPO-dCK cells decreased on the order of 10-15-fold for each drug. Table 1 shows the mean alteration in IC50 due to infection with LNPO-dCK for each drug in the three tumor cell lines. MCF-7 cells had a 2.2- and 2.5-fold increase in sensitivity to AraC and Cda, respectively, although no shift was detectable with FaraA. HT-29 cells demonstrated 5.9-, 7.6-, and 5.9-fold increases in sensitivity to AraC, Cda, and FaraA, respectively, whereas H1437 cells demonstrated markedly increased sensitivities of 106-, 21-, and 20-fold for AraC, Cda, and FaraA, respectively. Although dFdC was more potent than any of the other compounds for all cell lines, there was a less consistent effect of dCK expression on dFdC IC50s.

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 between dCK activity and IC50 was seen for AraC (r = 0.863), Cda (r = 0.822), and FaraA (r = 0.727). In contrast, a lesser level of correlation exists between dCK activity and IC50 for dFdC (r = 0.636).

**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 lymphoblasts

![Fig. 1. dCK activity in MCF-7, HT-29, and H1437 tumor cell lines. Mean dCK activity levels were determined using Cda as the substrate, as described in "Materials and Methods." Columns, mean; bars, SE; n = 4–7 determinations in duplicate.](image-url)
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. O, H1437; , H1437-LacZ; □, H1437-dCK.

Table I 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>
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<tbody>
<tr>
<td>MCF-7</td>
<td>2.2 ± 0.1&lt;sup&gt;bc&lt;/sup&gt;d</td>
<td>2.5 ± 0.2&lt;sup&gt;bc&lt;/sup&gt;d</td>
<td>0.0 ± 0.8&lt;sup&gt;c&lt;/sup&gt;c</td>
<td>7.6 ± 6.6&lt;sup&gt;c&lt;/sup&gt;c</td>
</tr>
<tr>
<td>HT-29</td>
<td>5.9 ± 2.1&lt;sup&gt;bc&lt;/sup&gt;d</td>
<td>7.6 ± 2.9&lt;sup&gt;c&lt;/sup&gt;c</td>
<td>5.9 ± 0.6&lt;sup&gt;c&lt;/sup&gt;c</td>
<td>1.7 ± 0.7&lt;sup&gt;c&lt;/sup&gt;c</td>
</tr>
<tr>
<td>H1437</td>
<td>105.7 ± 19.5&lt;sup&gt;bc&lt;/sup&gt;d</td>
<td>21.0 ± 4.6&lt;sup&gt;c&lt;/sup&gt;c</td>
<td>20.0 ± 5.1&lt;sup&gt;c&lt;/sup&gt;c</td>
<td>203.0 ± 15.1&lt;sup&gt;c&lt;/sup&gt;c</td>
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* 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.

<sup>a</sup> Mean ± SE (n = 3-5 experiments).

<sup>b</sup> Values were determined using the parental cell line as the control.

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

<sup>d</sup> n = 2 experiments.

Although several studies have addressed the relationship between dCK activity and drug sensitivity in lymphoid cells, they have required an indirect correlational 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...
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 FAraA 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 FAraA by 6–7-fold, whereas the 16-fold increase in dCK activity in H1437 cells increased sensitivity to CdA and FAraA 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 FAraA, 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 FAraA 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 could conceive of manipulation of endogenous dCK gene expression in leukemic cells 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 IC50d 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

OVEREXPRESSION OF dCK IN TUMOR CELLS


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