Enhancement of Cytarabine Sensitivity in Squamous Cell Carcinoma Cell Line Transfected With Deoxycytidine Kinase

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Background: Cytarabine is the most effective agent known for the treatment of acute myeloid leukemia. Its antitumor effect is expressed by combining with DNA during replication and then destroying the DNA chain. However, cytarabine has only limited activity against most solid tumors, including squamous cell carcinoma of the head and neck. The reason for this is thought to be that in cell lines of solid tumors the expression of cytidine deaminase, an enzyme that degrades cytarabine, is high, whereas the expression of deoxycytidine kinase (dCK), which phosphorylates cytarabine (a prodrug), is weak.

Objective: To determine whether head and neck squamous cell carcinomas can be made more sensitive to the cytotoxic effects of cytarabine by shifting the balance from the degradative to the activation pathway.

Methods: Human SCC-25 squamous carcinoma cells were transfected by either retroviral vector or adenoviral vector containing DCK gene and were identified for dCK expression by Northern blot analysis. In vitro cytotoxic assay after cytarabine exposure was performed using these cells.

Results: Both retroviral and adenoviral vector-mediated transduction of the dCK complementary DNA resulted in marked sensitization of tongue squamous carcinoma cell lines to the cytotoxic effects of cytarabine in vitro.

Conclusion: The dCK-cytarabine system may be a useful approach for gene therapy of squamous cell carcinomas of the head and neck.


Squamous cell carcinomas of the head and neck (HNSCCs) have been increasing as major health concerns around the world. Although considerable advances in surgical techniques, radiation therapies, and chemotherapy have been made during the past several decades, the long-term survival of patients with advanced HNSCC has not been significantly improved. In addition, no effective salvage therapy exists for patients in whom standard treatment fails.

Gene transfer offers the possibility of new approaches to cancer treatment. Head and neck cancers are particularly well suited to gene transduction strategies. Multiple gene transfer strategies that involve the introduction of foreign genes that directly kill tumor cells, that restore a defective tumor-suppressor gene, and induce apoptosis, or that enhance the immune response, are presently under investigation for HNSCC. One gene transduction strategy for the treatment of cancer involves the introduction of a drug sensitivity gene, which encodes an enzyme that can intratumorally activate a prodrug. An example of this strategy is the transfer of the herpes simplex virus thymidine kinase (HSVtk) gene, which encodes the protein that activates ganciclovir, a nucleoside analogue.

Cytarabine is the most effective agent in the treatment of acute myeloid leukemia. It is incorporated into replicating DNA and terminates DNA chain elongation. Cytarabine has limited activity against most solid tumors, including HNSCC. Resistance mechanisms include relatively rapid deamination of cytarabine by cytidine deaminase as compared with phosphorylation to cytidine monophosphate by deoxycytidine kinase (dCK). We hypothesized that HNSCC could be made more sensitive to the cytotoxic effects of cytarabine if the balance is shifted from the degradative to the activation pathway.

The present work demonstrates that the adenovirus-mediated transfer of the...
MATERIALS AND METHODS

TUMOR CELL LINE

Human SCC-25 squamous carcinoma cells (American Type Culture Collection [ATCC], Rockville, Md) were grown as monolayers in Dulbecco modified Eagle minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum; penicillin, 100 U/mL; streptomycin sulfate, 100 µg/mL; 2mM L-glutamine; and hydrocortisone, 0.4 µg/mL. The amphotropic PA317 retrovirus packing cell line (ATCC) was grown in Dulbecco modified Eagle minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum; penicillin, 100 U/mL; streptomycin sulfate, 100 µg/mL; and 2mM L-glutamine.

CONSTRUCTION OF dCK-EXPRESSING VECTORS AND CELL LINES

A 0.8-kilobase (kb) fragment of the human dCK complementary DNA was cloned into the EcoRI site of the pMV7 retroviral vector (pMV7-dCK). The neo gene is transcribed from a thymidine kinase promoter (tk).

Supernatant from PA317/pMV7-dCK retroviral producer cells was used to transduce SCC-25 target cells. The cells were incubated for 24 hours and then selected for 2 weeks in the presence of geneticin sulfate 400 µg/mL (Gibco BRL, Gaithersburg, Md). Polyclonal populations of SCC-25/Neo (transduced by pMV7) and SCC-25/dCK (transduced by pMV7-dCK) cells were identified for dCK expression by Northern blot analysis.

NORTHERN BLOT ANALYSIS

Total cellular RNA was isolated as described by others. The RNA (20 µg per lane) was separated in agarose-formaldehyde gels, transferred to nitrocellulose filters, and hybridized to the following phosphorus 32–labeled DNA probes: (1) a 0.8-kb NcoI and BamHI fragment of dCK from the pET3d-dCK plasmid; (2) a 1.3-kb HindIII fragment from the pLaNeo plasmid containing the neomycin 3'-phosphotransferase complementary DNA sequence; (3) a 3.3-kb HindIII/EcoRI lacZ fragment from the pSV-β-galactosidase vector (Promega Corp, Madison, Wis); and (4) a 1.5-kb EcoRI insert of a human β-actin gene purified from the HFBCC49 plasmid (ATCC). Hybridizations were performed as described by others.

RESULTS

EXPRESSION OF dCK IN SCC-25 CELLS

Cells from the SCC-25 squamous cell carcinoma cell line were transduced by the pMV-dCK or control pMV-7 with the use of viral supernatant from the respective PA317 producer cell lines. Polyclonal selection for stable vector integration was performed by adding geneticin sulfate (Gibco BRL) to the culture medium. Northern analysis confirmed that the parental SCC-25 cells (SCC-25/WT) and SCC-25/Neo cells (cells transduced by the control pMV7 vector) did not express dCK mRNA (Figure 1). In contrast, SCC-25 cells transduced by pMV7-dCK (SCC-25/dCK) expressed high levels of the dCK transcript. The neomycin 3'-phosphotransferase gene was expressed in both the SCC-25/Neo and SCC-25/dCK clones (Figure 1). Since transduction of dCK did not affect the cell growth, dCK itself was considered not toxic to the cells.

CYTARABINE SENSITIVITY OF TRANSFECTED CELLS

To determine the sensitivity of SCC-25/WT, SCC-25/Neo, and SCC-25/dCK cells to cytarabine, cytotoxicity curves were generated after 96 hours of drug exposure (Figure 2). After a 96-hour exposure, the concentration of cytarabine that resulted in 50% cytotoxicity (IC50) for the SCC-25/dCK, SCC-25/WT, and SCC-25/Neo cells was 5.0nM, 100nM, and 120nM, respectively (P<.001) (Figure 2). These findings indicate that transduction of dCK increases the sensitivity of cells to cytarabine.

GENE TRANSFER EXPERIMENTS USING AN ADENOVIRAL VECTOR SYSTEM

On the basis of our data demonstrating that DCK gene expression enhanced cytarabine sensitivity to SCC-25 cells in vitro, we hypothesized that DCK might be an effective chemosensitization gene for gene therapy. Accordingly, we constructed a replication-deficient recombinant adenovirus carrying the CMV promoter–CK gene minicassette (Ad.CMV-dCK). To test the activity of this adenovirus, we transfected SCC-25 cells with Ad.CMV-dCK, Ad.CMV-neo, or Ad.CMV-βgal, as previously described. The resulting shuttle plasmid, pCMV-dCK, was cotransfected into 293 cells with the pJM17 plasmid containing the adenoviral type 5 genome as described by others. The calcium phosphate precipitation method was used for DNA transfection. Recombinant adenovirus was isolated from a single plaque, expanded in the 293 cells, and purified by double cesium gradient ultracentrifugation as described by others. The titer of purified adenovirus was determined by means of a spectrophotometer at 260 nm and by plaque assays.
vector, SCC-25/WT cells were transduced with either Ad.CMV-βgal or Ad.CMV-dCK in vitro. Forty-eight hours later, the total RNA was harvested and analyzed for expression of the transgene. The SCC-25 cells transduced with Ad.CMV-dCK expressed high levels of dCK messenger RNA. In contrast, dCK expression was undetectable in Ad.CMV-βgal–infected cells (Figure 3A). The level of dCK expression was dependent on the multiplicity of infection (MOI) (Figure 3B).

Cytotoxic assays were performed to determine whether SCC-25 transduction by Ad.CMV-dCK confers cytarabine sensitivity. Twenty-four hours after transduction, SCC-25 cells were exposed to various concentrations of cytarabine for 96 hours. The SCC-25 cells transduced by Ad.CMV-βgal at different MOIs (MOI = 0, 20) showed no difference in sensitivity to cytarabine (Figure 4). In contrast, SCC-25 cells transduced by Ad.CMV-dCK exhibited a huge increase in sensitivity to cytarabine (MOI = 20). The IC50 values for these cell lines were 100 nM (Ad.CMV-βgal, MOI = 0), 130 nM (Ad.CMV-βgal, MOI = 20), and 0.23 nM (Ad.CMV-dCK, MOI = 20) (Figure 4). We obtained similar results with 2 different tongue squamous cell carcinoma cell lines (SCC-4, KOSC-2 Cl3-43; Human Science Research Resources Bank, Osaka, Japan). The IC50 values for these cell lines were 120 nM (SCC-4: Ad.CMV-βgal, MOI = 0), 100 nM (SCC-4: Ad.CMV-βgal, MOI = 20), and 2 nM (SCC-4: Ad.CMV-dCK, MOI = 20) and 200 nM (KOSC-2 Cl3-43: Ad.CMV-βgal, MOI = 0), 240 nM (KOSC-2 Cl3-43: Ad.CMV-βgal, MOI = 20), and 3 nM (KOSC-2 Cl3-43: Ad.CMV-dCK, MOI = 20).

The standard therapy for HNSCC is a combination of radiotherapy and surgery, while chemotherapy is selected for recurrent or inoperable cases. A representative chemotherapy is a combination of cisplatin and fluorouracil. However, unlike hematologic malignancies, HNSCC is not very sensitive to these chemotherapies, and the development of new therapeutic agents has been hoped for. Accordingly, it would be very advantageous for the treatment of HNSCC if some innovation made it possible to effectively use antineoplastic agents whose safety and antitumor efficacy have already been confirmed in relation to hematologic malignancies.
Transduction of genes that sensitize tumor cells to prodrugs represents a promising strategy for cancer therapy. The following systems are known to increase the chemical sensitivity: HSVtk in combination with ganciclovir as a prodrug, the combination of cytosine deaminase-flucytosine, human platelet-derived endothelial cell growth factor (PD-ECGF)–5-fluorouracil, PD-ECGF–tegafur, and PD-ECGF/fluorouracil. Although there are a few reports about HSVtk-ganciclovir therapy of head and neck cancers, no other systems have been studied. It is known that cytarabine manifests its antitumor effect by combining with DNA during replication and then destroying the DNA chain. However, cytarabine does not have any effect on most solid tumors, although it shows a powerful effect against hematologic malignancies. Cytarabine penetrates cells by a carrier-mediated process using a nucleoside transporter that binds nitrobenzylthioinosine. Cytarabine is converted to its active form via phosphorylation by 3 successive enzymes, consisting of dCK, deoxycytidine kinase, and nucleoside diphosphate kinase. The cytotoxic effects of cytarabine can be abrogated by blocking this incorporation of cytidine triphosphate into DNA. Alternately, cytarabine can be metabolized to inactive intermediates directly through the inactivation of cytidine monophosphate by deoxycytidine deaminase. Thus, cytarabine activation depends on a relative balance between activating and degradative enzymes. The phosphorylation of cytarabine by dCK seems to be a rate-limiting step of the activation of cytarabine. Unlike hematologic malignancies, many solid cancers show little intracellular expression of dCK, and their sensitivity to cytarabine seems to be low for this reason. Moreover, as the reason that cytarabine does not show activity against solid tumors, it has been suggested that these tumors differ from hematologic malignancies in terms of their cell cycle or hemodynamics.

We therefore hypothesized that cytarabine metabolism might be shifted from intracellular deamination toward phosphorylation and activation after overexpression of dCK. That is, we surmised that the sensitivity to cytarabine might be improved to the level of that shown by hematologic malignancies by introducing the DCK gene.

Figure 3. Northern blot analysis of deoxycytidine kinase (dCK) expression in SCC-25 cells transduced with Ad.CMV-dCK. A, Forty-eight hours after transduction (multiplicity of infection [MOI]=20), cells were harvested, and the total RNA (20 µg) was analyzed for the expression of transgenes. B, SCC-25 cells were transduced at the indicated MOIs to assess the effect of the viral titer on the level of gene expression. Hybridization to the β-actin probe demonstrated equal loading of the lanes.
into the HNSCC cells. Our previous study had shown that transduction of brain tumor (glioma) cells by retroviral and adenoviral vectors expressing the DCK gene increased the sensitivity of these cells to the cytotoxic effects of cytarabine in vitro and in vivo.17

Cancer cells of the central nervous system show low activity of cytidine deaminase, an enzyme that degrades cytarabine, resulting in a longer half-life of cytarabine in those cells. Moreover, cytarabine can be administered into the medullary cavity. For these and other reasons, cytarabine has proved to be comparatively easy to use in the treatment of brain tumors. However, cell lines of other types of solid tumors show strong expression of cytidine deaminase, reported to be 100 to 1000 times greater than that in the cells of hematologic malignancies.33 The results of the present study clearly demonstrated that it is possible to intensify the antitumor effect of cytarabine even in relation to HNSCC by transducing the DCK gene into HNSCC cells.

The dCK-cytarabine system has potential advantages for cancer gene therapy for 4 reasons. First, cytarabine has been used for many years, as a result of which its pharmacokinetics have been extensively studied and a high-dose regimen has been established. Moreover, the adverse reactions caused by cytarabine, of which bone marrow suppression is representative, have been thoroughly investigated, and thus the level of safety of this drug is also very high.

Second, an immune response is induced when foreign genes are introduced in vivo by means of an adenovirus, and it is said that the targets of cytotoxic T lymphocytes are the proteins that are produced by the transduced foreign genes.34 However, unlike HSVtk and other currently used chemosensitization genes such as cytosine deaminase, DCK is a human gene and thus limits the likelihood of a significant anti-dCK immunologic response in humans.

Third, in HNSCC the cause of death is almost always local recurrence, while cases said to be due to distant metastasis are rare. Accordingly, for unresectable cases and cases with local recurrence, administration of an adenoviral vector directly into the tumor can be expected to result in efficacy of the transduced genes. In addition, such direct administration of an adenoviral vector into a tumor is easy to accomplish, and the level of expression of the adenovirus in the tumor is thought to be high. It is also thought that an immune response against the adenovirus is less likely to occur in intratumoral administration of the adenoviral vector than in intravenous administration.

Fourth, the usual clinical dosage of cytarabine is approximately 20 mg/kg per day, which results in a plasma drug concentration in the range of 10^4 nM to 10^5 nM. In the present study, the cytarabine ΙC50 for the SCC25 cells transfected with the DCK gene by means of adenoviral infection was 2.3 × 10^4 nM, which is much lower than the stated plasma drug concentration range achieved in actual clinical therapy.

For these reasons, although further studies including in vivo experiments will be required, we believe that the dCK-cytarabine system may be a useful approach for gene therapy of HNSCC, as well as possibly other solid tumors.

Accepted for publication October 26, 2001.

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