Retroviral Transduction of Cancer Cell Lines with the Gene Encoding Drosophila melanogaster Multisubstrate Deoxyribonucleoside Kinase*

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Nucleoside kinases from several species are investigated as “suicide genes” for treatment of malignant tumors by combined gene/chemotherapy. We have recently cloned a multisubstrate deoxyribonucleoside kinase of Drosophila melanogaster (Dm-dNK), and we have shown that the enzyme phosphorylates cytotoxic pyrimidine and purine nucleoside analogs. The broad substrate specificity of the enzyme, as well as its very high catalytic rate, makes it a unique member of the human and viral nucleoside kinase enzyme family. In the present study, we evaluated Dm-dNK as a suicide gene by constructing a replication-deficient retroviral vector that expresses the enzyme. The human pancreatic adenocarcinoma cell line Mia PaCa-2 and a thymidine kinase-deficient osteosarcoma cell line were transduced with the recombinant virus. We showed that Dm-dNK can be expressed in human cells, that the enzyme retained its enzymatic activity, and that it is localized in the cell nuclei due to a nuclear localization signal in its C-terminal region. The cells expressing Dm-dNK exhibited increased sensitivity to several cytosotropic nucleoside analogs, such as 1-β-D-arabinofuranosylcytosine, 1-β-D-arabinofuranosylguanine, (E)-5-(2-bromovinyl)-2-deoxyuridine, 2-chloro-2′-deoxyadenosine, and 2′,2′-difluoro deoxycytidine. These findings suggest that Dm-dNK may be used as a suicide gene in combined gene/chemotherapy of cancer.

The transfer of the gene encoding herpes simplex virus type-1 thymidine kinase (HSV-1 TK) into malignant cells and subsequent treatment with ganciclovir is one of the most commonly studied strategies of suicide gene therapy (1–5). The HSV-1 TK gene is introduced into cancer cells using either viral or non-viral vectors. The nucleoside kinase phosphorylates nucleoside analogs such as the guanosine analog ganciclovir to their monophosphate derivatives, and cellular enzymes further phosphorylate the compounds to their triphosphate forms.

Ganciclovir triphosphate interferes with DNA replication (6) and induces cell death, probably by apoptosis (7, 8). In addition to affecting the cells expressing HSV-1 TK, adjacent untransduced cancer cells are killed by the transfer of phosphorylated nucleoside analog between cells via gap junctions (7, 9). This phenomenon, known as the “bystander effect,” results in killing of a larger portion of cells than those transduced with the suicide gene.

One limiting factor for the efficiency of suicide gene therapy is the kinetic properties of the “suicide” nucleoside kinase. Genetically engineered mutants of HSV-1 TK with improved kinetic properties for nucleoside analog phosphorylation show increased efficiencies as suicide genes (10–12). Nucleoside kinases from different members of the herpes virus family, such as herpes simplex virus type 2 and varicella zoster virus, have also been studied for possible use as suicide genes (13–16). The human nucleoside kinases deoxyctydine kinase and deoxyguanosine kinase enhance sensitivity to cytotoxic nucleoside analogs and are also candidate genes for gene therapy (17–19).

Munch-Petersen and co-workers (20) have purified a nucleoside kinase from Drosophila melanogaster that catalyzed the phosphorylation of all the natural pyrimidine and purine deoxyribonucleosides. In addition to its broad substrate specificity, the enzyme also exhibited a high catalytic rate that is 10–100-fold higher than reported for the previously studied nucleoside kinases. The broad substrate specificity of this enzyme, together with its high catalytic rate, makes it a unique member of the human and viral nucleoside kinase enzyme family. We have recently cloned the cDNA of this multisubstrate enzyme, named D. melanogaster deoxyribonucleoside kinase (Dm-dNK), and shown that it also efficiently phosphorylates several anti-viral and anti-cancer nucleoside analogs (21). In the present study, we decided to evaluate the possible use of Dm-dNK as a suicide gene by expressing the enzyme in human cancer cell lines. In summary, we have shown that Dm-dNK can be expressed in human cells with retained enzymatic activity and that it increases the sensitivity of the cells to several cytotoxic nucleoside analogs.

**EXPERIMENTAL PROCEDURES**

**Construction of a Retrovirus Vector Expressing Dm-dNK**—We used a retrovirus vector based on the Moloney murine leukemia virus to generate a replication-deficient recombinant retrovirus containing the cDNA of Dm-dNK. Oligonucleotide primers containing engineered EcoRI and XhoI restriction enzyme sites were designed flanking the open reading frame of Dm-dNK cDNA (5′-AAAGATTGGACAGTGGCGGAGGCAGCAGTC-3′ and 5′-TTCTCGAGTGGTTATCTGGCGACCACGGACCT-3′). The primers were used in a polymerase chain reaction, and the DNA fragment was cloned into the EcoRI-XhoI site of the pLXSN plasmid vector (CLONTECH). The plasmid was purified using the NucleoBond plasmid purification kit (CLONTECH). The DNA sequence of the constructed plasmid was verified by DNA sequence determination using an ABI310 automated DNA sequencer (PerkinElmer Life Sciences).
Overexpression of Dm-dNK in Human Cancer Cells

RetroPack PT67 packaging cells (CLONTECH) were cultured at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. The constructed pLXSN plasmid vector was transfected into the packaging cells using LipofectAMINE (Life Technology, Inc.) according to the protocol provided by the supplier. The medium from the transfected cells was collected 48 h after transfection, filtered through a 0.45-μm filter, and diluted 2-fold with fresh medium. The virus-containing medium was subsequently used to transduce the cancer cell lines as described below.

Cell Culture and Retroviral Transduction—TK-deficient osteosarcoma cells was a kind gift from Prof. J. Balzarini, Rega Institute, Leuven, Belgium. MIA PaCa-2 human pancreatic adenocarcinoma cells were obtained from the American Type Culture Collection. All cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were grown at 37 °C in a humidified incubator with a gas phase of 5% CO₂. The cells lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were plated at 2000 cells/well in 96-well plates.

Cell Proliferation Assays—Cell protein extracts were prepared as described (22). The assays were performed in 50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 5 mM ATP, 2 mM dithiothreitol, 15 mM NaF, 100 mM KCl, 0.6 mg/ml bovine serum albumin, and 0.6 μM tritiated DNA nuclear and mitochondrial DNA (23–24). In contrast, the cells expressing Dm-dNK showed a faint dotted autoradiography pattern distributed throughout the cells, indicating phosphorylation of dThd by mitochondrial thymidine kinase 2 and its subsequent incorporation into mitochondrial DNA (23–24). In contrast, the cells expressing Dm-dNK exhibited dark staining of the cell nucleus indicating incorporation of [³H]dThd into nuclear DNA. Approximately 90% of the cells incorporated dThd in nuclear DNA, indicating that the majority of the transduced cancer cells expressed active Dm-dNK.

To quantify the total level of nucleoside kinase activity in the cells, we determined the phosphorylation of dThd, CdA, and araC in cell protein extracts (Fig. 3). Untransfected osteosarcoma cells deficient in cytosolic thymidine kinase 1 activity showed low levels of dThd phosphorylation, probably catalyzed by mitochondrial thymidine kinase 2 (23). The cells transfected with the pLXSN vector alone showed similar levels of dThd phosphorylation as the wild-type cells, whereas the cells transfected with dNK-pLXSN exhibited ~100-fold higher enzymatic activity than the parent cell line. Untransfected MIA PaCa-2 cells exhibited a higher basal level of dThd phosphorylating activity compared with the osteosarcoma cells due to the retained expression of TK1 in the pancreatic cells. However, the Dm-dNK expression increased the dThd kinase activity ~35-fold in the MIA PaCa-2 cells as well. The phosphorylation of CdA and araC in the cell protein extracts were also increased 4–15-fold in the Dm-dNK-transduced osteosarcoma and pancreatic adenocarcinoma cells compared with the untransfected parent cell lines and the cells transfected with pLXSN vector alone (Fig. 3). In summary, these experiments showed that human cancer cells transfected with the dNK-pLXSN retroviral vector expressed enzymatically active Dm-dNK and that the expression resulted in an increase of nucleoside and nucleoside analog phosphorylation.

Expression of Dm-dNK Increased Sensitivity to Nucleoside Analog—We determined the sensitivity of the untransfected cells and the cells transfected with either the retroviral vector alone or the dNK-pLXSN vector for several cytotoxic nucleoside analogs (Table I). The cytotoxicity (IC₅₀) was determined after 4 days of drug exposure as described under “Experimental Procedures.” The difference in sensitivity between the wild-type cell lines and the cells transfected with the pLXSN vector without Dm-dNK was less than 3-fold for all investigated compounds. Both the osteosarcoma cells and the pancreatic cancer

Fig. 1. Retroviral vector (pLXSN) used to insert the Dm-dNK cDNA (dNK-pLXSN). LTR, long terminal repeat; PNeo, SV40 large T-antigen promoter; Neo⁰, neomycin resistance gene.

RESULTS

Expression of Dm-dNK in Mammalian Cells—We used a retrovirus vector based on the Moloney murine leukemia virus to create replication-deficient recombinant retroviridae with (dNK-pLXSN) and without (pLXSN) the Dm-dNK cDNA (Fig. 1). A TK-deficient human osteosarcoma cell line and an MIA PaCa-2 human pancreatic adenocarcinoma cell line were transduced with the retrovirus and, polyclonal populations of stably transduced cells were selected.

We used autoradiography to visualize in situ incorporation of [³H]dThd into DNA of the thymidine kinase 1-deficient osteosarcoma cells (Fig. 2). The wild-type untransduced cells and the cells transfected with the pLXSN vector alone showed a faint dotted autoradiography pattern distributed throughout the cells, indicating phosphorylation of dThd by mitochondrial thymidine kinase 2 and its subsequent incorporation into mitochondrial DNA (23–24). In contrast, the cells expressing Dm-dNK exhibited dark staining of the cell nucleus indicating incorporation of [³H]dThd into nuclear DNA. Approximately 90% of the cells incorporated dThd in nuclear DNA, indicating that the majority of the transduced cancer cells expressed active Dm-dNK.

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Cell Proliferation Assays—Nucleoside analogs were added after 24 h, and the medium containing the nucleoside analogs was changed daily. Cell survival was assayed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Roche Molecular Biochemicals) after 4 days of drug exposure. Each experiment was performed in triplicate. The IC₅₀ value of the investigated compounds was calculated as the mean value of these experiments.

Enzyme Assays—Cell protein extracts were prepared as described (22). The assays were performed in 50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 5 mM ATP, 2 mM dithiothreitol, 15 mM NaF, 100 mM KCl, 0.6 mg/ml bovine serum albumin, and 0.6 μM tritiated DNA nuclear and mitochondrial DNA (23–24). In contrast, the cells expressing Dm-dNK showed a faint dotted autoradiography pattern distributed throughout the cells, indicating phosphorylation of dThd by mitochondrial thymidine kinase 2 and its subsequent incorporation into mitochondrial DNA (23–24). In contrast, the cells expressing Dm-dNK exhibited dark staining of the cell nucleus indicating incorporation of [³H]dThd into nuclear DNA. Approximately 90% of the cells incorporated dThd in nuclear DNA, indicating that the majority of the transduced cancer cells expressed active Dm-dNK.

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cells that expressed Dm-dNK showed an increase in sensitivity to several of the nucleoside analogs. The highest increase in sensitivity for the osteosarcoma cell was detected for BVDU, CdA, dFdC, and FdUrd with a 100–400-fold decrease in IC50 compared with the untransduced cells. araC and araT showed a 50-fold reduction in IC50, whereas araA and ganciclovir showed between 3- and 9-fold reduction in IC50 compared with the untransduced cells. araG was not toxic to either cell line at the investigated concentrations. The highest increase in sensitivity for the MIA PaCa-2 cells was observed for BVDU with a 6400-fold increased sensitivity. araT and dFdC increased the sensitivity 30- and 75-fold, respectively. CdA showed a 6-fold increase in sensitivity in the Dm-dNK-expressing cells, whereas the sensitivity of araA, araC, FdUrd, and ganciclovir was not enhanced by Dm-dNK expression in this cell line.

The Dm-dNK Is Localized in the Nucleus of Human Cells—In order to determine the subcellular localization of the Dm-dNK when overexpressed in human cells, we constructed a fusion of Dm-dNK and GFP to visualize the protein in vivo (dNK-GFP) (Fig. 4). The cells transfected with a plasmid encoding the fusion protein exhibited green fluorescence predominantly in the nucleus of the cells. The nuclear localization was verified by contra-staining the cells with DAPI. Based on sequence analysis Munch-Petersen and co-workers (25) suggested two possible nuclear localization signals starting at residue 95 (PT-NKKLK) and 242 (PSKRQRV). We decided to test the role of the putative nuclear localization signal in the C terminus of the protein in an experiment using site-directed mutagenesis. The putative nuclear localization signal was mutated by either

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**TABLE I**

Sensitivity (IC50) of osteosarcoma and MIA PaCa-2 pancreatic adenocarcinoma cell lines to nucleoside analogs

| Osteosarcoma (µM) | MIA-PaCa (µM) |
|-------------------|---------------|
|                    | wt pLXSN Dm-dNK | wt pLXSN Dm-dNK |
| AraA              | 1.4 26 4.5      | 6.4 4.2 1.3      |
| araC              | 0.28 0.31 0.0051 | 0.09 0.09 0.075  |
| araG              | >50 >50 >50      | >50 >50 >50      |
| araT              | 17 11 0.32      | 16 27 0.0053    |
| BVDU              | 2.0 1.9 0.022   | >50 >50 >50      |
| CdA               | 5.0 9.0 0.22    | 4.9 4.9 0.82     |
| dFdC              | 0.067 0.077 0.00036 | 0.071 0.065 0.00094 |
| FdUrd             | 5.8 3.5 0.054   | >50 >50 >50      |
| GCV               | 9.0 15 3.2      | >50 >50 >50      |

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FIG. 2. [3H]dTdr autoradiography of TK-deficient osteosarcoma cells transduced with pLXSN (A) or dNK-pLXSN (B).

FIG. 3. [3H]dTdr, [3H]CdA, and [3H]araC phosphorylating activity in cell protein extracts. A, osteosarcoma cells; B, MIA PaCa-2 pancreatic adenocarcinoma cells. Wild-type cells (open bars), pLXSN-transduced cells (gray bars), and dNK-pLXSN-transduced cells (black bars).

~50-fold reduction in IC50, whereas araA and ganciclovir showed between 3- and 9-fold reduction in IC50 compared with the untransduced cells. araG was not toxic to either cell line at the investigated concentrations. The highest increase in sensitivity for the MIA PaCa-2 cells was observed for BVDU with a >6400-fold increased sensitivity. araT and dFdC increased the sensitivity 30- and 75-fold, respectively. CdA showed a 6-fold increase in sensitivity in the Dm-dNK-expressing cells, whereas the sensitivity of araA, araC, FdUrd, and ganciclovir was not enhanced by Dm-dNK expression in this cell line.

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replacing Arg-247 with a serine residue (Δ247/dNK-GFP) or by replacing Lys-244 and Arg-245 with glutamic acid and serine amino acid residues, respectively (Δ244–245/dNK-GFP). When expressed in the osteosarcoma cell line, Δ247/dNK-GFP and Δ244–245/dNK-GFP were both retained in the cytosol and did not translocate to the cell nucleus (Fig. 4). In summary, these experiments showed that the nuclear localization of Dm-dNK in human cells is mediated by the C-terminal signal sequence.

**DISCUSSION**

Here we have shown that the multisubstrate nucleoside kinase of D. melanogaster retains its enzymatic activity when expressed in human cells and that the activity results in an enhanced sensitivity to several cytotoxic pyrimidine and purine nucleoside analogs. These results suggest that Dm-dNK may be used as a suicide gene in combined gene/chemotherapy of cancer. Two of the most promising candidate nucleoside analogs to use in conjunction with Dm-dNK gene transfer are the pyrimidine analogs dFdC and BVDU. dFdC is currently used in the treatment of several types of solid tumors such as pancreatic adenocarcinoma and non-small cell lung cancer (26). It may be possible to increase the efficiency of this therapy by transduction of tumor cells in vivo with the Dm-dNK gene. BVDU, the second candidate nucleoside analog, was initially developed as an anti-herpetic agent, and the compound has been used to treat patients with varicella zoster infections (27). BVDU shows low toxicity to untransduced cells, but the cells expressing Dm-dNK are highly sensitive to the compound. The concurrent use of both a nucleoside analog that is restricted to Dm-dNK for activation, such as BVDU, and a nucleoside analog that already has shown clinical efficacy in treatment of malignant tumors, such as dFdC, is one possible approach to develop new treatment modalities for tumors with combined gene and chemotherapy. Combining the therapy with other anti-metabolites to achieve synergistic effects may be another approach to enhance the efficiency of nucleoside kinase suicide gene therapy. Recent studies on HSV-1 TK-transduced cancer cells suggest that synergistic cytotoxicity and enhanced bystander effects may be achieved when combining ganciclovir with the ribonucleotide reductase inhibitor hydroxyurea (28).

Inhibition of ribonucleotide reductase results in decreased de novo dNTP synthesis, which is favorable for the incorporation of nucleoside analogs into DNA. Furthermore, a decrease of the dNTP pool may also increase the phosphorylation of nucleoside analogs because several nucleoside kinases are feedback-inhibited by dNTPs. The nucleoside analogs dFdC and CdA are both potent inhibitors of ribonucleotide reductase once phosphorylated to their diphosphate forms. Because Dm-dNK phosphorylates both of these compounds, it is possible that they will inhibit ribonucleotide reductase and thereby enhance the phosphorylation of BVDU or other nucleoside analogs that are activated by Dm-dNK. We are presently initiating studies on tumor models to investigate further the possible use of Dm-dNK as a suicide gene in vivo to evaluate the possible benefits of combining multiple nucleoside analogs to improve this therapeutic regime.

The unique features of Dm-dNK as a suicide gene compared with HSV-1 TK and other nucleoside kinases are its broad substrate specificity and high catalytic rate. Pyrimidine deoxyribonucleosides have in vitro higher affinity to the enzyme compared with purine deoxyribonucleosides (20, 21, 25). The difference in affinity between purine and pyrimidine nucleosides is also observed for some, but not all, nucleoside analogs. Enzymatic assays performed with recombinant Dm-dNK show that pyrimidine nucleoside analogs such as BVDU, 5-FC, araC, araT, araA, araG, and araT compete more efficiently with the natural pyrimidine substrates dThd and dCyd compared with most of the investigated purine nucleoside analogs such as araG and araA (21). However, one of the exceptions is the clinically important purine nucleoside analog CdA, which efficiently competes with both dThd and dCyd for phosphorylation by Dm-dNK. This compound did also show a marked increase in cytotoxicity in the Dm-dNK-transfected cells. Studies on HSV-1 TK-transfected cells suggest that the purine phosphorylating activity in vivo may be increased by mutations that decreases the enzymes affinity to pyrimidine nucleoside analogs (29). We are currently initiating studies to investigate if a similar approach can be used to enhance purine nucleoside analog phosphorylation catalyzed by Dm-dNK.

Dm-dNK is sequence related to the human nucleoside kinases deoxyuridine kinase, deoxyguanosine kinase, and thymidine kinase 2 (21). The human enzymes differ in their subcellular location by being located in either the nucleus, cytosol, or mitochondria (23, 30–32). In the present study we have shown that Dm-dNK is targeted to the cell nucleus when expressed in human cell lines. Two putative nuclear localization signal sequences have been identified in the Dm-dNK sequence (25). We showed that two independent mutants of the putative nuclear localization signal located in the C terminus of the protein completely abolished its nuclear translocation. These experiments showed that this signal is the physiologically important signal mediating the nuclear localization. We do not yet know whether the nuclear location of the enzyme is restricted to the mammalian cells used in this study or whether the enzyme is also located in the nucleus of D. melanogaster cells as well. Interestingly, HSV-1 TK is also located in the cell nucleus when expressed in human cells (33). The physiological importance of the nuclear location of nucleoside kinases is, however, yet unclear, since studies suggest that phosphorylated nucleoside analogs may freely traverse the nuclear envelope, and no differences in nucleoside analogs sensitivity have been detected when nucleoside analogs are phosphorylated in either of the two subcellular compartments (30).

The success of suicide gene therapy is dependent on efficient gene delivery systems and the selective expression of the suicide gene in tumor cells. Most of the in vivo studies on nucleoside kinase suicide gene therapy have been performed using first generation retro- or adenovirus vector systems (2–5). These systems show insufficient vector distribution and low transduction efficiencies. Recently, significant achievements in vector developments have been made for both viral and non-viral systems (34). Progress has also been made in achieving selective and targeted expression of the suicide gene in tumor cells. Several tissue-specific promoters have been shown to direct transgene expression to a certain tissue or cell type. These promoters include the tyrosinase promoter for transgene expression in melanocytes that may be used for targeting melanoma cells (35), the prostate-specific antigen promoter for selective gene expression in prostate and prostate cancer tissue (36), and the glial fibrillary acidic protein promoter for gene expression in brain tumors of glial origin (37). Several promoters of tumor marker proteins, such as the carcinoembryonic antigen (38) and the α-fetoprotein (39), can also be used to control selective transgene expression in tumor cells. Novel vector systems also utilizes receptors specific for tumor cells or other phenotypic alterations of the cancer cells to restrict vector transduction or replication to tumor cells (34). The recently developed oncolytic adenovirus ONYX-015 utilizes the loss of p53 function in many types of human tumors for selectivity. The ONYX-015 adenovirus cannot replicate in cells with intact p53 (40), and the virus thus only kills cells deficient in p53 expression, whereas normal cells are not killed by the virus. Initial clinical studies using the ONYX-015 virus for local treat-
ment of tumors show promising results (41), and it has been suggested that the efficacy may be further enhanced by the concomitant use of a suicide gene expressed by the virus (41–42). In the future, it will be important to evaluate these novel vectors and expression system using the optimal prodrug/suicide gene combination to achieve the most efficient killing of transduced tumor cells as well as killing of untransduced tumor via bystander effects.

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