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Lipid-mediated Protein Delivery of Suicide Nucleoside Kinases[1]

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ABSTRACT

Nucleoside kinases from several species are investigated as suicide genes for treatment of malignant tumors by combined gene/chemotherapy. In the present study, we have investigated a novel strategy where nucleoside kinase proteins are directly delivered to cells without delivery of genetic material. We used a mix of a trifluoroacetylated lipopolyamine and dioleoyl phosphatidylethanolamine (BioPorter) to form protein–lipid complexes containing either recombinant herpes simplex virus type-1 thymidine kinase or *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase. We showed that the nucleoside kinase containing protein–lipid complexes was imported into human osteosarcoma and Chinese hamster ovary cell lines by endocytosis and that the enzymes were delivered to the cytosol and nucleus. The nucleoside kinases imported into the cell lines retained enzymatic activity, and the cells treated with the enzyme–lipid complexes showed increased sensitivity to nucleoside analogues, such as ganciclovir, (E)-5-(2-bromovinyl)-2′-deoxyuridine, and 1-β-D-arabinofuranosylthymine. Our results show that direct delivery of suicide gene proteins to cells may be an alternative approach to conventional suicide gene therapy strategies.

INTRODUCTION

Nucleoside kinases, such as the HSV-1 TK[3] and the multisubstrate deoxyribonucleoside kinase of *Dm*-dNK, are investigated for possible use as suicide genes in gene therapy of cancer (1–6). The suicide gene strategy is based on the introduction of a nucleoside kinase gene into the cancer cells and subsequent systemic treatment with nucleoside analogues activated by the enzyme. The "suicide" nucleoside kinases phosphorylate the nucleoside analogues to their monophosphate derivatives, and cellular enzymes further phosphorylate the compounds to their triphosphate forms. The nucleoside analogue triphosphates interfere with DNA replication and repair, which induces cell death (7, 8). In addition to affecting the cells expressing the nucleoside kinase, adjacent untransduced cancer cells are also killed by the transfer of phosphorylated nucleoside analogue between cells via gap junctions (9, 10). This phenomenon, known as the "bystander effect," results in killing of a larger portion of cells than those transduced with the suicide gene.

The success of suicide gene therapy strategies is dependent on efficient and targeted delivery of the suicide gene to cancer cells. Both viral and nonviral vectors are presently being investigated for gene delivery. Liposome-DNA formulations are among the most commonly used nonviral delivery methods, and several studies show that liposomes may be used for suicide gene delivery (11–13). Recently, Zelphati *et al.* reported the use of liposomes for direct delivery of proteins to cells (14). This method involves the use of a trifluoroacetylated lipopolyamine mixed with dioleoyl phosphatidylethanolamine, known as BioPorter, to form lipid–protein complexes that are imported into cells. Lipid-mediated delivery of proteins to cells represents a novel strategy for protein therapeutics, and we decided to investigate if suicide gene proteins, such as nucleoside kinases, could functionally be delivered to cancer cells using this strategy. In the present study, we report the delivery of recombinant HSV-1 TK and *Dm*-dNK into human osteosarcoma and CHO cell lines. In summary, we showed that the proteins were delivered in a functionally active form and that the sensitivity of the cell lines treated with the enzyme–lipid complexes showed increased sensitivity to cytotoxic nucleoside analogues phosphorylated by the enzymes.

MATERIALS AND METHODS

Expression and Purification of Recombinant HSV-1 TK and Dm-dNK. Dm-dNK cDNA was PCR amplified (5′-AACATATGGCGGAGGC-AGCATCCTGTG and 5′-TTCTCGAGTGGTTATCTGGCGACCCTCTGGC) and cloned into the *Nde*I and *Xho*I sites of the pET-15b plasmid vector (Novagen) to express the protein with an NH_2-terminal poly-histidine tag. The protein was expressed in the HMS *Escherichia coli* strain (Novagen), and the protein was purified using Talon metal affinity resin chromatography (Clontech) as described in the manufacturer's protocol. The HSV-1 TK protein was expressed in fusion with glutathione-*S*-transferase, and the protein was purified by glutathione-agarose affinity chromatography as described (15). The proteins were further purified by gel filtration on a PD-10 column (Amersham Pharmacia Biotech), and the proteins were eluted in 10 mM HEPES (pH 7.0) and 150 mM NaCl. The purity of the protein was verified by SDS-PAGE gel (PhastGel, Amersham Pharmacia Biotech), and the protein concentration was determined with Bradford Protein Assay (Bio-Rad) using BSA as the concentration standard.

Cell Culture. The TK1-deficient human osteosarcoma cell line (gift from Prof. J. Balzarini, Rega Institute, Leuven, Belgium) was cultured in DMEM, and the CHO cell line (American Type Culture Collection) was cultured in McCoy 5A-modified medium. The cell culture medium was supplemented with 10% (volume for volume) FCS (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% CO_2.

Formation and Import of Enzyme/BioPorter Complexes. BioPorter lipid reagent containing a trifluoroacetylated lipopolyamine mixed with dioleoyl phosphatidylethanolamine was obtained from Gene Therapy Systems, Inc. (San Diego, CA). Recombinant HSV-1 TK or *Dm*-dNK in 10 mM HEPES (pH 7.0) and 150 mM NaCl was mixed with BioPorter lipids as described by the manufacturer. For the experiments on cells cultured in 96-well plates or chamber slides, 0.25 μg of protein in 10-μl buffer was mixed with 1 μl of dried BioPorter lipids, and the mixture was incubated for 5 min at room temperature. The mixture was diluted in 100 μl of serum-free medium and added to the cells. For the experiments on cells cultured in 24-well plates, 0.5 μg of protein was mixed with 2.5 μl of dried BioPorter lipids, and the mixture was diluted in 250 μl of serum-free medium before being added to the cells. For controls, the amounts stated above of enzyme or BioPorter were added separately to the cells. The cells were washed once with serum-free medium before addition of the protein–lipid complexes, and the medium containing the complexes was replaced with serum-containing medium after 4-h incubation at 37°C.

Imaging of Enzyme Import. Recombinant HSV-1 TK and *Dm*-dNK proteins were fluorescently labeled with Alexa Fluor-488 using the Alexa Fluor-488 Labeling Kit (Molecular Probes). Cells were cultured on Lab-Tech II chamber slides (NUNC) for 24 h. Alexa Fluor-488 labeled enzymes mixed

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[3] The abbreviations used are: HSV-1 TK, herpes simplex virus type-1 thymidine kinase; *Dm*-dNK, *Drosophila melanogaster* deoxyribonucleoside kinase; CHO, Chinese hamster ovary; dThd, thymidine; araT, 1-β-D-arabinofuranosylthymine; BVDU, (E)-5-(2-bromovinyl)-2′-deoxyuridine; GCV, ganciclovir.

with BioPorter reagent was added to the cells as described above. At 4 or 24 h, the cells were washed three times in PBS, and microscopy was performed on the live unfixed cells using a Nikon Eclipse E600 microscope equipped with a SPOT RT digital camera.

Nucleoside Kinase Assays. Cells were cultured in 24-well plates, and the enzyme/BioPorter complexes were prepared as described above. The cells were washed in PBS, and crude cell protein extracts were prepared at 24, 48, 72, and 96 h after the addition of lipid–protein complexes. dThd kinase enzyme assays were performed as described (4), using 3 μM [methyl-^{3}H]-dThd (Moravek Biochem) and 2 μM unlabeled dThd (Sigma) in the enzyme reactions.

Cytotoxicity Assays. The cells were plated at ≈2000 cells/well in 96-well plates. Lipid–protein complexes were added, and the media were replaced with serum-containing media at 4 h. The nucleoside analogues GCV (Sigma), BVDU (gift from Prof. J. Balzarini, Rega Institute, Leuven, Belgium), or araT (Sigma) were added at 24 h, and the medium containing the nucleoside analogues was changed once during the 3-day incubation. Cell survival was assayed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Boehringer Mannheim) after 3 days of drug exposure. Each experiment was performed in triplicate.

The bystander effect was assayed by mixing different ratios of protein-loaded osteosarcoma cells with untreated cells. The cells were detached by incubation with 0.1% EDTA in PBS after 4 h of incubation with the lipid–protein complexes as described above. The lipid—protein-treated cells were mixed with untreated cells and plated at 4000 cells/well in 96-well plates. One-hundred μM GCV were added at 24 h, and cell survival was determined after 3 days as described above.

RESULTS

We decided to investigate if suicide nucleoside kinase proteins, such as HSV-1 TK and *Dm*-dNK, could functionally be delivered to cell lines using liposomes. Recombinant enzymes were fluorescently labeled to visualize the import of the proteins in cultured cells. An osteosarcoma cell line was incubated with the enzyme–lipid complexes for 4 or 24 h (Fig. 1). The cells showed at 4-h incubation predominantly a dotted fluorescent pattern and a weak general fluorescence in both the nucleus and cytosol. This fluorescence pattern suggests endocytosis and endosomal accumulation of the complexes. At 24-h incubation, a relative increase in the general cellular fluorescence in the cytosol and nucleus was observed, but a fluorescent enzyme located in endosomes was also present. Similar results were obtained for CHO cells incubated with the lipid–protein complexes (data not shown). Accordingly, the experiments suggest that enzyme–lipid complexes were internalized by endocytosis and subsequently released into the cytosol. Repeated experiments resulted in 60–80% of the cells exhibiting fluorescence in the cytosol, nucleus, and endosomes. Cells incubated with enzymes without lipids showed no fluorescence, indicating that the lipids were required for cellular import of *Dm*-dNK or HSV-1 TK.

We determined the sensitivity of the cell lines to the enzyme–lipid complexes to investigate if the complexes alone may induce cytotoxic effects on the cells (Fig. 2). A fixed amount of BioPorter lipids (1 μl) was used to form lipid–protein complexes with different amounts of

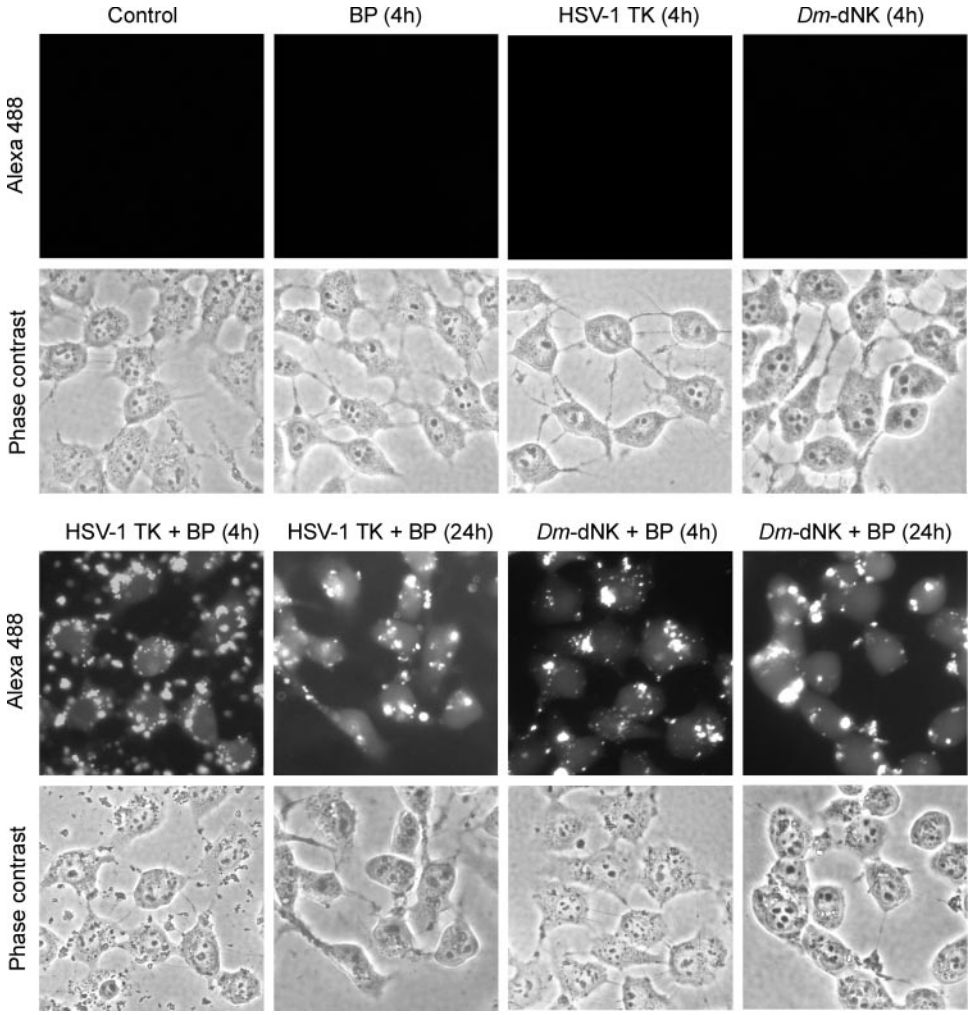

Fig. 1. Liposome-mediated import of nucleoside kinases in a osteosarcoma cell line. The cells were incubated with recombinant fluorescent Alexa Fluor-488-labeled HSV-1 TK or *Dm*-dNK alone or in complex with BioPorter (*BP*) lipids for 4 or 24 h. The cells were thoroughly washed after incubation, and fluorescence microscopy was performed on the live unfixed cells.

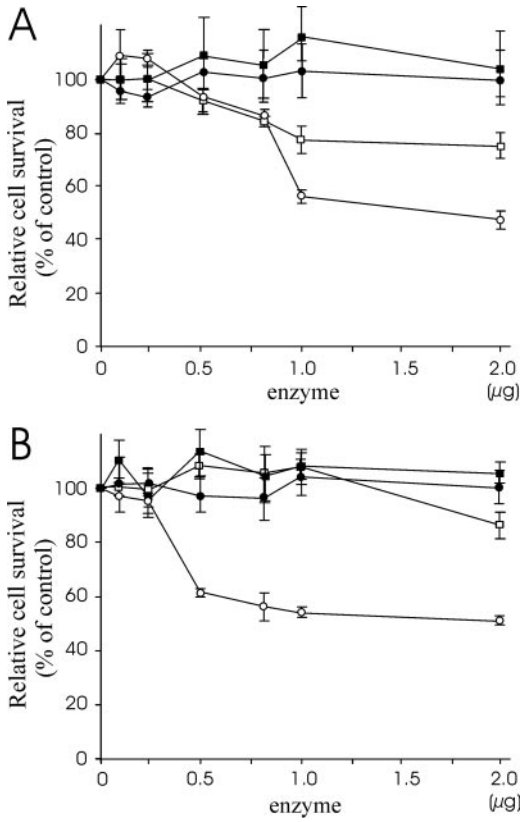

Fig. 2. Sensitivity of osteosarcoma (*A*) or CHO (*B*) cells to HSV-1 TK or *Dm*-dNK lipid-enzyme complexes. The cells were incubated with HSV-TK alone (●), HSV-1 TK in complex with BioPorter lipids (○), *Dm*-dNK alone (■), or *Dm*-dNK in complex with BioPorter lipids (□). Data points indicate mean ± SD.

recombinant nucleoside kinases (0.1–2 μg), and these complexes were administered to cultured osteosarcoma cells and CHO cells. Cells incubated with the enzymes alone, without lipids, showed no decrease in viability compared with controls. Incubation with BioPorter lipids alone neither affected cell viability (data not shown). In contrast, cells incubated with lipid–protein complexes with a high protein/lipid ratio showed a decrease in viability (Fig. 2). These experiments suggest that the lipid–protein complexes may induce cytotoxic effects. We decided to use a combination of lipids and protein that by itself did not affect cell survival in the subsequent experiments to study the cells' sensitivity to nucleoside analogues phosphorylated by the enzymes (0.25 μg of recombinant enzyme and 1 μl of BioPorter lipids).

We determined the dThd kinase activity in crude cell extracts of cells incubated with the lipid–protein complexes to verify that the enzymes retained enzymatic activity when imported into the cells (Fig. 3). The cell lines were incubated with the enzyme–lipid complexes for 24, 48, 72, or 96 h. At 24-h incubation, dThd kinase activity in the osteosarcoma cells was increased ≈20–30-fold compared with control cells. At 96 h, the dThd kinase activity in the osteosarcoma cells had decreased and was ≈8-fold and ≈3-fold increased compared with control cells for the cells treated with HSV-1 TK or *Dm*-dNK lipid–protein complexes, respectively. The dThd phosphorylation activity for both HSV-1 TK and *Dm*-dNK was 2–5-fold lower in the CHO cells compared with the osteosarcoma cells treated with the lipid–protein complexes. At 96 h after addition of lipid–protein complexes, no increase in dThd kinase activity compared with control cells was detected in the CHO cells. The general lower enzyme activity in the CHO cells suggested that the nucleoside kinases were less effectively delivered to the CHO cells compared with the osteosarcoma cells under the investigated conditions. The specific dThd kinase activity of the purified recombinant *Dm*-dNK was ≈64 nmol/μg/min. Assuming that the specific activity of the recombinant enzyme was unaffected by the formation of lipid–protein complexes and the internalization into endosomes, ≈1–3% of the enzyme activity added to the cells was retained after 24 h, and <0.2% of the enzyme activity added was retained after 96 h. In summary, the dThd kinase assays showed that the nucleoside kinases imported into cells in lipid–protein complexes retained enzyme activity, although the retained activity is only a small fraction of the total activity of the recombinant enzyme added to the cells.

We determined the nucleoside analogue sensitivity of the cell lines to investigate if the nucleoside kinases imported into the cells could phosphorylate nucleoside analogues within the cells and induce cytotoxic effects. Unphosphorylated nucleoside analogues are imported into cells by nucleoside transporter proteins in the cell membrane, but phosphorylated nucleoside analogues cannot cross the cell membrane, and nucleoside analogues are accordingly only cytotoxic if phosphorylated by an enzyme located in the cells. The purine nucleoside analogue GCV and the pyrimidine nucleoside analogues BVDU and araT were used in the experiments. BVDU and araT are substrates of both HSV-1 TK and *Dm*-dNK, whereas GCV is only phosphorylated by HSV-1 TK. The osteosarcoma cells incubated with the HSV-1 TK and *Dm*-dNK lipid–protein complexes showed increased sensitivity to BVDU and araT compared with cells incubated with lipids or proteins separately (Fig. 4). The cells showed also increased sensitivity to

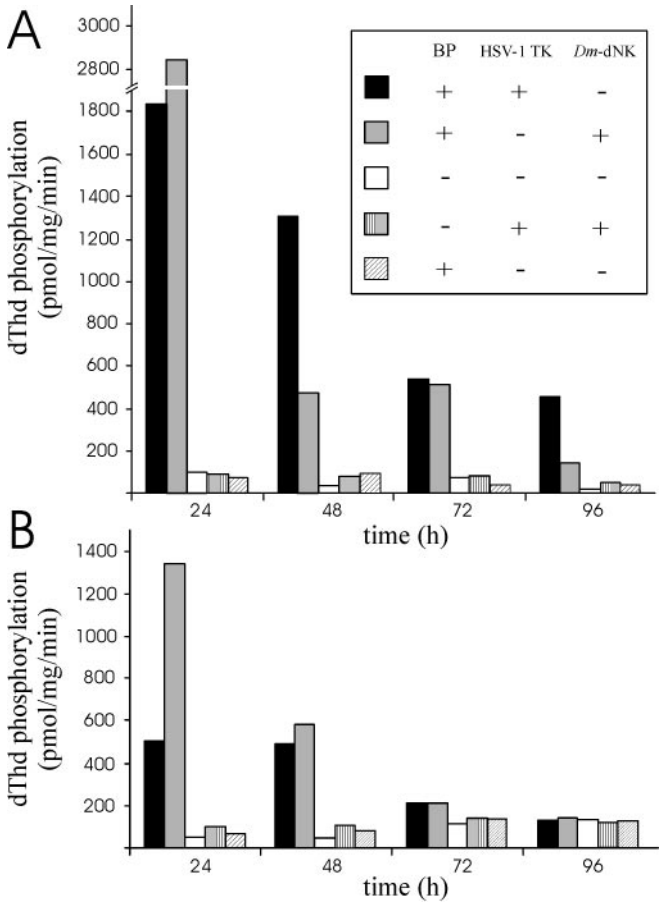

Fig. 3. Nucleoside kinase activity in cells incubated with the lipid enzyme complexes. dThd phosphorylation in crude cell extracts of osteosarcoma cells (*A*) or CHO cells (*B*) incubated with HSV-1 TK or *Dm*-dNK-containing lipid protein complexes for 24, 48, 72, or 96 h. Cells incubated with either HSV-1 TK, *Dm*-dNK, or BioPorter lipids alone were used as controls.

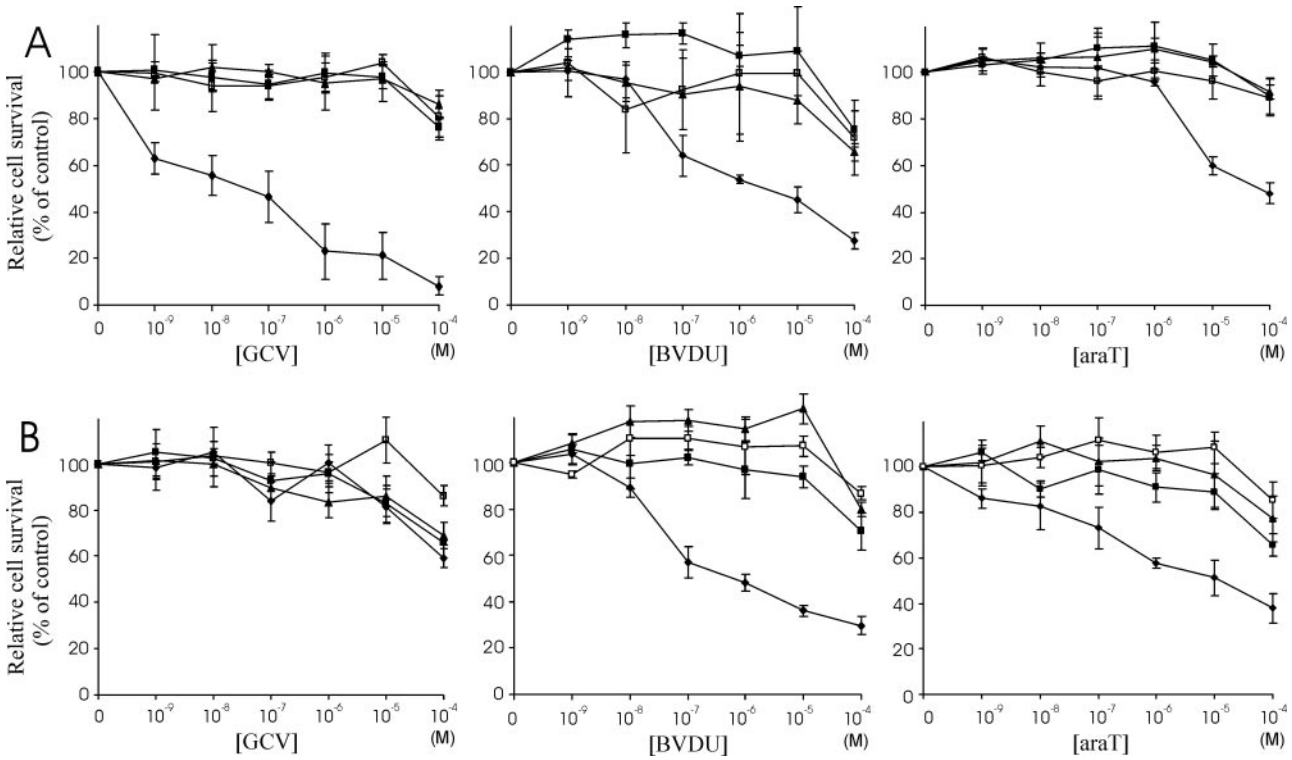

Fig. 4. Sensitivity of osteosarcoma cells to the nucleoside analogues GCV, BVDU, or araT incubated with either HSV-1 TK (*A*) or *Dm*-dNK (*B*). The cells were preincubated 24 h with enzyme/BioPorter complexes (♦) or incubated with either BioPorter lipids alone (▲), enzyme only (□), or none of the reagents (■). Cell survival (mean ± SD) was determined after 3-day incubation with the nucleoside analogues.

GCV when incubated with HSV-1 TK lipid–protein complex but not when incubated with *Dm*-dNK lipid–protein complexes. This difference is consistent with that GCV is phosphorylated by HSV-1 TK but not by *Dm*-dNK. We were not able to induce complete killing of all of the cells in the cultures. For most of the combinations of nucleoside kinase and nucleoside analogues, ≈20–40% of the cells were still viable even at the highest investigated nucleoside analogue concentration. The most efficient cell killing was achieved when combining HSV-1 TK lipid–protein complexes with GCV treatment. In these experiments, >95% cell death was induced for the osteosarcoma cells.

We used a bystander assay to determine whether a bystander cell killing occurred in the osteosarcoma cells incubated with HSV-1 TK lipid–protein complexes. Different amounts of HSV-1 TK-loaded cells were mixed with untreated cells, and the mixed cell population incubated with 100 μM GCV. Protein-loaded cells (10%) resulted in 47 ± 11% (mean ± SD) cell death, and 25% protein-loaded cells resulted in 68 ± 8% cell death compared with 92 ± 4% cell death when 100% protein-loaded cells were used in the assay. Untreated cells were unaffected by the GCV concentration used. The killing of a higher portion of cells than those incubated with the HSV-1 TK lipid–protein complexes indicated that a bystander effect occurred.

DISCUSSION

We have used lipid–protein complexes to deliver recombinant HSV-1 TK and *Dm*-dNK nucleoside kinases to cell lines and shown that the delivered enzymes retained enzymatic activity. The cells treated with the lipid–protein complexes also exhibited increased sensitivity to nucleoside analogues phosphorylated by the enzymes. Imaging studies on fluorescently labeled enzymes showed that the protein–lipid complexes were internalized by endocytosis and accumulated in intracellular vesicles. However, fluorescence was also detected in the cytosol and nucleus, suggesting that the proteins were released from the endosomes. It is likely that the mechanism of protein import is similar to the mechanism of DNA import by liposome-mediated cell transfection. The lipid–DNA complexes bind to the cell surface, are internalized into cells by endocytosis, and are released to the cytosol by disruption of the endosomes (16). Disruption of endosomes is a relatively infrequent event, and only a few of the endosomes release their contents to the cytosol. Accordingly, most of the endocytosed protein remains in the endosomal compartment, which is consistent with the data in the present study on internalization of the lipid–protein complexes.

Quantification of the enzyme activity in the cells suggests that ≈1–3% of the protein added to cells retained enzymatic activity after 24 h, whereas only ≈0.2% of the enzyme activity retained after 96 h. The retained activity does not represent the amount of functionally active protein delivered to the cytosol or nucleus, because of the accumulation of the proteins in endosomes. Therefore, we do not presently know the amount of protein delivered to the cytosol or nucleus, but the amount delivered was sufficient to increase the sensitivity to the nucleoside analogues. However, we were not able to induce complete cell killing. These results are consistent with delivery of HSV-1 TK and *Dm*-dNK to ≈60% of the cells as estimated by image analysis. Nucleoside kinases used as suicide genes frequently induce cell killing in untransduced neighboring cells mediated by transfer of phosphorylated nucleoside analogues to adjacent cells, a phenomenon known as bystander cell killing. We observed killing of 60–95% of the cells in culture with the highest investigated concentration of nucleoside analogue. These findings suggested that some of the cells killed by the nucleoside analogues did not contain enzyme but were killed by a mechanism similar to the bystander effect. We also provided evidence that bystander cell killing occurred in osteosarcoma cells incubated with HSV-1 TK lipid–protein complexes and

treated with GCV. Pyrimidine nucleoside analogues, such as BVDU and araT, are reported to induce poor bystander killing compared with purine nucleoside analogues, such as GCV (17). In our study, we observed the highest level of cell killing in the cells incubated with HSV-1 TK and GCV. A superior bystander effect of GCV compared with BVDU or araT can accordingly explain this difference in cell killing.

The concept of direct delivery of suicide gene proteins to cells is a novel strategy that should be further evaluated in cancer models as an alternative to suicide gene therapy. Suicide protein therapy does not require introduction of foreign genetic material into the target cells. In addition, the protein is active directly once it has been imported across the cell membrane, whereas DNA delivered to cells by different vectors needs to be transported to the cell nucleus and uncoated from the vector molecules to become transcriptionally active (10). We have in the present study shown that the amount of protein delivered to the cell lines is sufficient to increase nucleoside analogue sensitivity, but additional studies are required to determine the amount of functional protein delivered to the cytosol and nucleus compared with the amount expressed from traditional gene therapy vectors. A high concentration of intracellular active enzyme will be important both for the sensitivity of the individual cells to nucleoside analogues as well as for the amount of phosphorylated nucleoside analogues available for transfer to adjacent cells and the subsequent induction of bystander cell killing. It is possible that the relative short half-life of the nucleoside kinases delivered to cells in the lipid–protein complexes may be a problem for achieving a high intracellular concentration of phosphorylated nucleoside analogues. Similar to DNA-based gene therapy methods, suicide protein therapy is also limited today by the lack of efficient methods for selective targeting of cells and efficient delivery *in vivo*. These problems need to be addressed before lipid-mediated delivery of nucleoside kinases can reach clinical application.

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