Enhanced Cytotoxicity of Nucleoside Analogs by Overexpression of Mitochondrial Deoxyguanosine Kinase in Cancer Cell Lines*

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The cytotoxic anti-cancer purine nucleoside analogs 2-chloro-2'-deoxyadenosine (CdA), 9-β-D-arabinofuranosylguanine (araG), and 2',2'-difluoro-deoxyguanosine (dFdG) are phosphorylated by human mitochondrial deoxyguanosine kinase (dGK) in vitro. We overexpressed dGK as a fusion protein to the green fluorescent protein in the human pancreatic cancer cell lines PanC-1 and MIA PaCa-2 to determine the importance of dGK-mediated nucleoside analog phosphorylation. The transfected cells showed mitochondrial fluorescence patterns, and the mitochondrial locations of endogenous and overexpressed dGK were verified by Western blot analysis of cell extracts with polyclonal anti-dGK antibodies. The increase of dGK activity in the overexpressing cells was ~4-fold. These cell lines exhibited increased sensitivity to CdA, araG, and dFdG as compared with the untransfected parent cell lines. This is, to our knowledge, the first demonstration of a correlation between the activity of a mitochondrial deoxyribonucleoside kinase and the cytotoxicity of nucleoside analogs. Our data imply that the dGK activity is rate-limiting for the efficacy of nucleoside analogs in the cell lines investigated.

Nucleoside analogs are commonly used in antileukemic chemotherapy (1). Certain compounds are also active against solid tumors, although the efficiency against nonhematological malignancies is poor (2). The nucleoside analogs inhibit DNA synthesis after intracellular phosphorylation to their 5’-triphosphate form. The rate-limiting step in the pharmacological activation of the nucleoside analogs is the phosphorylation by a deoxyribonucleoside kinase. Differences in the expression levels and activities of the activating deoxyribonucleoside kinases in leukemic cells versus cells in solid tumors have been proposed as determinants of the differences in drug sensitivity. There are four human deoxyribonucleoside kinases that phosphorylate nucleoside analogs to their corresponding monophosphates: the S phase-specific thymidine kinase 1 and the cell cycle constitutively expressed deoxycytidine kinase (dCK),1 deoxynucleosine kinase (dGK), and thymidine kinase 2 (3).

Deficiency of dCK causes resistance to nucleoside analogs phosphorylated by this enzyme (4, 5). Recent work also demonstrates that sensitivity to nucleoside analogs phosphorylated by dCK increases by overexpression of dCK in tumor cell lines (6, 7). The presence of dCK is thus shown to be both a prerequisite for activity of nucleoside analogs, and its expression level is quantitatively correlated to the cytotoxicity of certain drugs. dCK is closely sequence-related to dGK, and the two enzymes have overlapping substrate specificity. Both enzymes phosphorylate the purine nucleosides 2'-deoxyadenosine and 2'-deoxyguanosine as well as the purine nucleoside analogs 2-chloro-2'-deoxyadenosine (CdA) and 9-β-D-arabinofuranosyl-guanine (araG) (8, 9). CdA is clinically used for treatment of indolent lymphoproliferative disorders (1). araG is presently undergoing clinical trials for treatment of acute T-cell leukemia (10). dCK is regarded as the major nucleoside kinase for the activation of CdA and araG due to its high expression in lymphocytes. However, studies with purified enzymes suggest that dGK may contribute to the activation of CdA and araG (9). The difluoro-substituted deoxyguanosine analog 2',2'-difluoro-deoxyguanosine (dFdG) is another cytotoxic purine analog with anti-leukemic activity (11). This analog is not a substrate for dCK, and dFdG is believed to be activated only by dGK. Although the cytotoxic nucleoside analogs discussed above are demonstrated to be substrates for dGK, the contribution of this enzyme for pharmacological effects of nucleoside analogs is not clear. A difference between dCK and dGK is their subcellular location. dCK was until recently regarded as a cytosolic enzyme, but it is shown to be imported into the cell nucleus, whereas dGK is located in the mitochondria (12). The initial phosphorylation of purine nucleoside analogs is thus performed within the mitochondria when a compound is activated by dGK or in the cell nucleus when activated by dCK. The aim of the present study was to investigate if overexpression of mitochondrial dGK would enhance the cytotoxicity of nucleoside analogs phosphorylated by the enzyme. Pancreatic cancer cell lines were chosen as a model of solid tumors with low sensitivity toward the purine nucleoside analogs. In conclusion, we have shown that overexpression of mitochondrial dGK in these cells increases the sensitivity to the purine nucleoside analogs araG, dFdG and CdA.

EXPERIMENTAL PROCEDURES

Culture and Transfection of Cell Lines—PanC-1 and MIA PaCa-2 human pancreatic adenocarcinoma cell lines were obtained from the American Type Culture Collection. The PanC-1 cells were cultured in RPMI 1640 medium, and the MIA PaCa-2 cells were cultured in Dulbecco’s modified Eagle’s medium. The cell culture medium was supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. We used the pEGFP-N1 plasmid vector (CLONTECH) to express the human dGK cDNA as a fusion protein to the green fluorescent protein (GFP) in human cells (12). The pEGFP-N1 plasmid encodes the red-shifted S65T mutant of GFP (13) and a neomycin resistance gene for selection of stably transfected cells. The GFP expression plasmid was transfected into the human cell lines using LipofectAMINE (Life Tech-
nologies). 2 µg of plasmid DNA and 5 µl of liposomes dissolved in Opti-MEM medium (Life Technologies) were used for each transfection according to the Life Technologies protocol. For transient transfection, the cells were washed with phosphate-buffered saline 48 h after transfection. GFP fluorescence was observed in the living cells with a Nikon Optiphot microscope equipped with a Nikon B1-A fluorescein isothiocyanate filter cube (470–490-nm excitation filter, 510-nm emission filter) and a 40× Nikon Fluor 40/3.0 oil immersion objective lens. Cells were photographed with Kodak Ektachrome ASA 400 film. For stable transfection, the cells were subcultured 1:5 3 days after transfection, and 1.2 mg/ml Geneticin (Life Technologies) was added to the cell culture medium. The cells were cultured in the presence of Geneticin for 2 weeks. Geneticin-resistant cell colonies with green fluorescence were isolated and continuously cultured. In these cultures, >90% of the cells exhibited green fluorescence.

Preparation of Mitochondrial and Cytosolic Cell Extracts—We prepared crude protein extracts of the mitochondrial and cytosolic/nuclear subcellular fractions (14). 2 × 10^6 cells were treated with trypsin and harvested by centrifugation at 1,000 × g for 5 min. The cells were washed three times with 2.5 mM Tris-HCl, pH 7.5, 0.7 mM NaHPO₄, 0.13 M NaCl, 5 mM KCl. The cell pellet was resuspended in 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM CaCl₂ and incubated on ice for 15 min. The cells were homogenized until <5% of the cells were intact as determined by light microscopy. Mannitol-sucrose buffer (210 mM mannitol, 10 mM sucrose, 5 mM Tris-HCl, pH 7.5, 5 mM Na₂EDTA, pH 8.0) was added, and the cell extract was cleared by centrifugation at 1,200 × g for 15 min at 4 °C. The supernatant was recentlyrifuged 20,000 × g for 10 min at 4 °C. The supernatant, which represents the cytosolic fraction, was stored at −70 °C until needed. The pellet was resuspended in manitol-sucrose buffer and layered on top of a 1.0–1.5 M sucrose gradient. The samples were centrifuged at 40,000 × g for 15 min at 4 °C. The mitochondria, retrieved from the gradient interface, were washed with manitol-sucrose buffer. The purified mitochondria were lysed in 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 1% sodium dodecyl sulfate, and 0.5% deoxycholate. The purity of the cytosolic and mitochondrial protein extracts was verified with mitochondrial citrate synthase and cytosolic lactate dehydrogenase enzyme assays. The protein concentrations of the cell extracts were determined with the Bio-Rad protein assay.

Enzyme Assays—dGK activity in crude cell extracts was determined as described (9, 15). The assays were performed in 50 mM Tris-HCl pH 7.6, 5 mM MgCl₂, 5 mM ATP, 2 mM dihtiothreitol, 15 mM NaF, 100 mM KCl, 0.5 mg/ml bovine serum albumin, 500 µM deoxyctydine, 8 µM unlabeled CdA, 2 µM [β-3H]CdA (20 Ci/mmol; Moravek Biochemicals Inc.). 10 µg of protein from the cell extracts in a total volume of 35 µl. 10 µl of the reaction mixtures were spotted on Whatman DE-81 filters after 10-, 20-, and 30-min incubation at 37 °C. The filters were washed three times in 50 mM ammonium formate. The filter-bound nucleoside analog monophosphate product of the reaction was eluted from the filter with 0.5 M KCl, and the radioactivity was determined by scintillation counting.

Production and Purification of Polyonal Anti-dGK Antibodies—Human dGK was expressed in the TB1 Escherichia coli strain as a fusion protein with the maltose-binding protein and was purified by amylose resin affinity chromatography (8). 1 mg of fusion protein in phosphate-buffered saline was subcutaneously injected into a rabbit three times with approximately 1 month between each injection. 2 weeks after the last injection, 40 ml of blood was retrieved and allowed to clot. The serum was collected and stored at −20 °C.

40 µg of recombinant human dGK and dCK were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by electroblotting. The nitrocellulose membranes were blocked with 1% bovine serum albumin in TBS buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween). The membranes were incubated for 1 h at room temperature with the affinity-purified dGK antibody and washed three times with TBS buffer. A secondary alkaline phosphatase conjugate anti-rabbit IgG antibody diluted 1:5000 (Sigma) was applied for 1 h, after which the membranes were again washed in TBS buffer. The alkaline phosphatase immobilized on the membrane was visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma).

Cell Proliferation Assays—The stably transfected cells were plated in 96-well microtiter plates at a density of ~10^4 cells/well. After 24 h, dFdG (Lilly), araG (a gift from Prof. J. Balzarini, Leuven, Belgium), CdA (Sigma), or araC (Sigma) was added to the indicated concentrations. Cell survival was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Boehringer Mannheim) 4 days after the nucleoside analogs were added. The number of viable cells was also determined by manual cell counting after trypan blue exclusion. Each experiment was performed in triplicate. Statistical analysis was performed using Student’s paired t test.

RESULTS

Expression of dGK-GFP in Pancreatic Adenocarcinoma Cells—dGK was expressed as a fusion protein with GFP to visualize dGK in living cells. The dGK-GFP plasmid (Fig. 1A) was transiently transfected in the pancreatic cancer cell lines Panc-1 and MIA PaCa-2. The cells transfected with the dGK-GFP plasmid showed irregular fluorescence around a nonfluorescent nucleus (Fig. 1B), similar to the pattern described for a mitochondrial marker (data not shown). In order to study nucleoside analog cytotoxicity in the pancreatic cancer cells, we selected stably transfected cells that expressed either GFP or dGK-GFP. The selection resulted in homogeneous fluorescent cell cultures with ~90% transfected cells. The stably transfected dGK-GFP-expressing cells showed weaker fluorescence as compared with the transiently transfected cells, whereas the expression of GFP resulted in strong fluorescence in both transiently and stably transfected cells (data not shown).

Detection of the dGK Protein in the Mitochondria of Transfected Cells—We decided to verify that both the wild-type cel-
The increase in dGK activity was transfected cells compared with the untransfected cells (Fig. 4). CdA phosphorylation (9, 15). The assay demonstrated that unlabeled deoxycytidine was added to inhibit dCK-catalyzed cytotoxic nucleoside analogs. The sensitivities of untransfected, Cells—

We used a cell proliferation assay with the transfected

ysis of the cell extracts.

were not able to detect any dGK protein by Western blot anal-

likely that the low cytosolic activity is due to dGK, since we

phosphorylation detected in the cytosolic fractions is probably

bands were observed in both dGK-GFP- and GFP-transfected

fraction of the dGK-GFP-transfected cells. Additional weaker

band at 55 kDa predicted molecular mass of the dGK-GFP fusion pro-

This band corresponded to the 28 kDa in the mitochondrial frac-

Total cell extract showed the 28-kDa dGK band. The band was present

mitochondrial (filled bars) protein extracts of Panc-1 cells determined as

Cytotoxicity of Nucleoside Analogs in dGK-overexpressing Cells—We used a cell proliferation assay with the transfected and untransfected pancreatic cancer cell lines to investigate if overexpression of dGK affected the cells’ sensitivity to the cytotoxic nucleoside analogs. The sensitivities of untransfected, GFP-transfected, and dGK-GFP-transfected PanC-1 and MIA PaCa-2 cells were determined for CdA, araG, dFdG, and araC

(5). The untransfected control cells were affected only to a small extent by the nucleoside analogs within the tested concentration range. There was no difference in sensitivity between the wild type cell lines and the GFP-transfected cells. In contrast, overexpression of dGK-GFP resulted in an increased sensitivity to all of the dGK-activated nucleoside analogs. The increase in sensitivity was estimated to be ~18-fold for dFdG, ~16-fold for araG, and ~5-fold for CdA, expressed as estimated IC50 values. Similar results of increased sensitivity to these nucleoside analogs were found for the transfected MIA PaCa-2 cells (data not shown). No difference in the cytotoxicity for araC was observed in any of the cells studied as expected, since araC is not a substrate for dGK.

DISCUSSION

We have investigated the role of mitochondrial dGK for the cytotoxicity of purine nucleoside analogs phosphorylated by this enzyme. In summary, we have shown that the cytotoxicity of araG, dFdG, and CdA is enhanced by overexpression of dGK in the two investigated pancreatic adenocarcinoma cell lines. Our data strongly suggest that the level of dGK activity in cells is a rate-limiting factor for the activation of these therapeutic nucleoside analogs. Western blot analysis of subcellular fractionated protein extracts showed that both the endogenous cellular dGK and the artificial fusion protein of dGK-GFP were exclusively located in the mitochondrial fraction and we were not able to detect any dGK protein in the cytosolic fraction. Our conclusion is that dGK is exclusively located in the mitochondria. This report is thereby, to our knowledge, the first demonstration that phosphorylation of nucleoside analogs by a mitochondrial deoxyribonucleoside kinase contributes to the

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FIG. 2. Western blot analysis of cross-reactivity of the anti-dGK antibodies to dCK and thymidine kinase 2. Equal amounts of pure recombinant human dGK, dCK, and thymidine kinase 2 in fusion with glutathione S-transferase were electrophoresed and blotted to a nitrocellulose membrane. The affinity-purified anti-dGK antibodies bind only to the 54-kDa fusion protein of dGK and glutathione S-transferase.

FIG. 3. Western blot analysis of dGK expression in the Pan-C1 cell line with the purified anti-dGK antibodies. Wild type (wt) total cell extract showed the 28-kDa dGK band. The band was present in the mitochondrial, but not the cytosolic, extracts of PanC-1 cells transfected with GFP. Cells transfected with the dGK-GFP plasmid showed an additional band at ~60 kDa that corresponded to the predicted molecular mass of the dGK-GFP fusion protein. The dGK-GFP band was present only in the mitochondrial extract. cyt, cytosolic extract; mit, mitochondrial extract; tot, total cell extract.

FIG. 4. dGK activity in cytosolic (empty bars) and mitochondrial (filled bars) protein extracts of Panc-1 cells determined as CdA phosphorylation in the presence of deoxycytidine. wt indicates extracts of untransfected cells. GFP and dGK-GFP are extracts of cells transfected with plasmids that express either GFP or dGK-GFP.

FIG. 5. Western blot analysis of dGK expression in the Pan-C1 cell line with the purified anti-dGK antibodies. Wild type (wt) total cell extract showed the 28-kDa dGK band. The band was present in the mitochondrial, but not the cytosolic, extracts of PanC-1 cells transfected with GFP. Cells transfected with the dGK-GFP plasmid showed an additional band at ~60 kDa that corresponded to the predicted molecular mass of the dGK-GFP fusion protein. The dGK-GFP band was present only in the mitochondrial extract. cyt, cytosolic extract; mit, mitochondrial extract; tot, total cell extract.
cytotoxicity of these compounds.

We believe that there are two separate deoxyribonucleotide pool compartments in a cell: the cytosolic-nuclear and the mitochondrial. The physical basis for the exchange of deoxyribonucleotides between the nucleus and the cytosol is that the nuclear pore complexes allow passive transport of small molecules across the nuclear membrane (17). The inner mitochondrial membrane is impermeable to hydrophilic compounds such as deoxyribonucleotides, and transport of deoxyribo nucleotides across the membrane would require a carrier protein. Indirect evidence that phosphorylated nucleoside analogs can be imported into the mitochondria comes from experiments showing that nucleoside analogs phosphorylated in the nuclear-cytoplasmic compartment inhibit mitochondrial DNA replication (18). Berk and Clayton (19) demonstrated that deoxycytidine is incorporated into mitochondrial, but not nuclear, DNA in thymidine kinase 1-deficient cells. Their data suggest that deoxycytidine phosphorylated by mitochondrial thymidine kinase 2 is not exported from the mitochondria to the cytosol. In agreement with the model that deoxyribonucleotides are not exported from the mitochondria, it has been shown that depletion of the cytosolic-nuclear deoxyribonucleotide pool is not accompanied by a similar decrease in mitochondrial deoxyribonucleotide levels (20). It is therefore likely that deoxyribonucleotides phosphorylated by dGK remain in the mitochondria.

The pharmacological targets of cytotoxic anti-cancer nucleoside analogs are believed to be nuclear DNA replication and repair. However, several pyrimidine nucleoside analogs cause cytotoxic effects by interference with mitochondrial DNA replication (21). The mitochondrial toxicity described for these compounds is delayed and occurs after several weeks of drug exposure due to decreased transcription of genes encoded by the mitochondrial genome. Mitochondrial DNA damage has not been described for purine nucleoside analogs, and there is no evidence that nucleoside analogs phosphorylated by dGK affect mitochondrial function or damage mitochondrial DNA. We therefore do not know if purine nucleoside analogs phosphorylated by dGK are able to interfere with mitochondrial DNA replication in a similar way as described for the pyrimidine compounds. The purine nucleoside analogs used in this study are cytotoxic to the dGK-overexpressing cells after 4 days of drug exposure. These data rather suggest that the nucleoside analogs phosphorylated in the mitochondrial matrix are transported to the nucleus and interfere with nuclear DNA replication. This model would explain the early toxicity seen in the rapidly growing pancreatic cancer cell lines that are highly dependent on rapid nuclear DNA replication. However, there is still no evidence that deoxyribonucleotides or phosphorylated nucleoside analogs are exported from the mitochondria to the cytosol and nucleus as discussed above.

The overlapping substrate specificity between dCK and dGK in regard to purine nucleoside analog phosphorylation makes it difficult to speculate about the contribution of dGK activation of nucleoside analogs in different tissues. There is strong evidence from cell culture experiments that dCK deficiency is associated with acquired resistance to deoxyadenosine, deoxyguanosine, and CdA (5). However, it is not known if these cells retain dGK activity, and it is possible that the cells have lost both dCK and dGK activity. Indirect evidence for such an event is that a mouse model of purine nucleoside phosphorylase deficiency that accumulates high levels of deoxyguanosine shows signs of deficient dGK activity (22). The production of specific anti-dGK antibodies reported in this paper should facilitate future studies on the correlation of dCK and dGK expression and activity in purine nucleoside analog-resistant cells.

We used pancreatic adenocarcinoma cell lines to determine the cytotoxicity of the nucleoside analogs with and without overexpression of dGK. Pancreatic adenocarcinoma responds poorly to available chemotherapy or radiotherapy, and better modalities to treat this malignant tumor are needed. In the future, gene therapy against pancreatic cancer may be possible if the current problems with efficient and tissue-directed gene transfer are solved. Expression of a suicide gene, such as dGK, in combination with nucleoside analog chemotherapy may be an option. There is so far no evidence that the human deoxyribonucleoside kinases, dGK or dCK, would be superior suicide genes as compared with the more carefully studied herpes simplex virus type-1 thymidine kinase. However, combinations of different nucleoside kinases and nucleoside analogs should be compared to find an optimal combination and thereby provide the best conditions to design future nucleoside kinase gene therapy trials.

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