Enhancement of DNA Ligase I Level by Gemcitabine in Human Cancer Cells

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ABSTRACT

Purpose: DNA ligase I is an essential enzyme for completing DNA replication and DNA repair by ligating Okazaki fragments and by joining single-strand breaks formed either directly by DNA-damaging agents or indirectly by DNA repair enzymes, respectively. In this study, we examined whether the DNA ligase I level could be modulated in human tumor cell lines by treatment with gemcitabine (2',2'-difluoro-2'-deoxycytidine), which is a nucleoside analogue of cytidine with proven antitumor activity against a broad spectrum of human cancers in clinical studies.

Experimental Design: To determine the effect of gemcitabine on DNA ligase I expression, Western blot analysis was used to measure the DNA ligase I levels in MiaPaCa, NGP, and SK-N-BE cells treated with different concentrations of gemcitabine and harvested at different time intervals. Cell cycle analysis was also performed to determine the underlying mechanism of DNA ligase I level enhancement in response to gemcitabine. In addition, other agents that share the same mechanism of action with gemcitabine were used to elucidate further details.

Results: When different types of tumor cell lines, including MiaPaCa, NGP, and SK-N-BE, were treated with gemcitabine, the level of DNA ligase I increased severalfold despite significant cell growth inhibition. In contrast, other DNA ligases (III and IV) either remained unchanged or decreased with treatment. Cell cycle analysis showed that arrest in S-phase corresponded to an increase of DNA ligase I levels in gemcitabine treated cells. Other agents, such as 1β-D-arabinofuranosylcytosine and hydroxurea, which partly share mechanisms of action with gemcitabine by targeting DNA polymerases and ribonucleotide reductase, respectively, also caused an increase of DNA ligase I levels. However, 5-fluorouracil, which predominantly targets thymidylate synthase, did not cause an increase of DNA ligase I level.

Conclusions: Our results suggest that an arrest of DNA replication caused by gemcitabine treatment through incorporation of gemcitabine triphosphate into replicating DNA and inhibition of ribonucleotide reductase would trigger an increase in DNA ligase I levels in cancer cells. The elevated presence of DNA ligase I in S-phase-arrested cells leads us to speculate that DNA ligase I might have an important role in repairing DNA damage caused by stalled replication forks.

INTRODUCTION

Gemcitabine (2',2'-difluoro-2'-deoxycytidine) is a pyrimidine antimetabolite (1), has well-known activity in a variety of solid tumors, and is well tolerated in clinical trials (2–4). Gemcitabine is metabolized to gemcitabine diphosphate and gemcitabine triphosphate inside the cell by nucleoside kinases (4, 5). The diphosphate form of gemcitabine inhibits ribonucleotide reductase, which is responsible for creating the deoxyribonucleoside triphosphate building blocks of DNA (5, 6). A reduction in the concentration of deoxyribonucleotides results in the inhibition of DNA synthesis. In addition, reduction of the dCTP concentration inside the cell can enhance incorporation of gemcitabine triphosphate into the replicating strand of DNA (4, 5). Gemcitabine triphosphate can compete with dCTP in binding to replicating DNA polymerases and then be incorporated into DNA to prevent further elongation of the replicating strand (7). Therefore, gemcitabine can inhibit DNA synthesis either directly or indirectly through different pathways, which is believed to be the principal mechanism of cytotoxicity for this drug.

Human DNA ligases play essential roles in DNA replication, recombination, and repair by catalyzing the formation of phosphodiester bonds between adjacent 5'-phosphoryl and 3'-hydroxyl termini at single breaks in duplex DNA molecules (reviewed in Refs. 8, 9). The reaction is initiated by the formation of a covalent enzyme-adenylate complex. Mammalian DNA ligases use ATP as a cofactor, whereas bacterial DNA ligases use NAD to generate the adenyl group. In subsequent steps, the activated AMP residue of the DNA ligase-adenylate intermediate is transferred to the 5'-phosphate terminus of a single-strand break in double-stranded DNA to generate a covalent DNA-AMP complex with a 5'-5' phosphoanhydride bond. In the final step of DNA ligation, nonadenylated DNA ligase is involved in the generation of a phosphodiester bond by catalyzing the displacement of the AMP residue through an attack by the adjacent 3'-hydroxyl group on the adenylated site. In mammalian cells, four distinct DNA ligases, whose functions are not yet completely understood, have been identified. These enzymes, designated as DNA ligase I, II, III, and IV, can be distinguished on the basis of their catalytic, physical, and serological properties.
DNA ligase I represents the majority of the DNA ligase activity in proliferating cells, whereas most of the ligase activity in resting cells is attributable to other DNA ligases (8). DNA ligase III, which is highly expressed in testis, is considered to have a role in meiotic recombination in mammalian cells (11). DNA ligase IV was most recently identified and might be responsible for the ligation step in V(D)J recombination in lymphoid cells and the repair of DNA double-strand breaks in mammalian cells (13). The highest levels of this enzyme are found in the thymus and testis (14). At present, DNA ligase II is thought to be generated from DNA ligase III by a specific proteolytic processing mechanism (9). Several studies have indicated major involvement of DNA ligase I in DNA replication (15, 16). The strongest evidence of a role in DNA replication comes from the human 46 BR cell line, in which a mutation in the DNA ligase I gene correlates with a delay in the joining of the Okazaki fragments (17). Other studies have suggested that DNA ligase I might also be involved in base excision repair or nucleotide excision repair (18, 19). Taken together, these data suggest that DNA ligase I is involved in different aspects of DNA metabolism, probably depending on associations with different enzymatic complexes (9).

In this study, we investigated whether DNA ligase I levels could be modulated by the anticancer drug gemcitabine in different human tumor cell lines. As reported, gemcitabine treatment resulted in a significant amount of cell growth inhibition of different tumor cell lines (e.g., MiaPaCa, NGP, and SK-N-BE cells). Interestingly, DNA ligase I activity was elevated with drug treatment in most of the tumor cell lines, whereas other DNA ligases remained unchanged or slightly decreased. Because a positive correlation exists between cell proliferation and DNA ligase I expression (20–22), our observations suggest that human DNA ligase I might have other unknown critical roles in the repair of DNA damages resulting from the arrest of DNA replication caused by nucleoside analogues including gemcitabine.

**MATERIALS AND METHODS**

**Chemicals and Cell Lines.** Gemcitabine was purchased from Eli Lilly Company (Indianapolis, IN) and ara-C, 5-FU, and hydroxyurea were from Sigma-Aldrich. Cell culture media and supplements were purchased from Cellgro (Herndon, VA). The human pancreatic carcinoma cell line MiaPaCa and human neuroblastoma cell lines NGP and SK-N-BE were obtained from American Type Culture Collection (Rockville, MD).

**Cell Culture and Drug Treatment.** The MiaPaCa cell line was maintained in DMEM with L-glutamine and 5.4 g/L glucose without sodium pyruvate supplemented with 10% fetal bovine serum. The NGP and SK-N-BE cell lines were maintained in RPMI 1640 with L-glutamine supplemented with 10% fetal bovine serum. Cells were grown at 37°C and 5% CO₂ in a humidified atmosphere. For drug treatment of cell lines, exponentially growing cells were plated at ~10⁵ cells/ml in T-175 cm² flasks, and drugs were added to the medium and incubated for the desired times. After drug treatment, the medium in the culture flask was aspirated, and the remaining monolayer cells were thoroughly washed once with PBS. The washed cells were trypsinized and harvested for cell number counting and subsequently for the preparation of cell lysates. The cell number was counted by either Coulter counter or hemocytometer. The cell viability was determined by trypan blue dye exclusion test.

**Preparation of Cell Lysates (S-100).** Cultured human cells were washed once in PBS, resuspended in ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 1 mM DTT, 0.5% 3-(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, and 10% glycerol (10⁶ cells/20 µl of buffer). The cell suspension was incubated for 30 min on ice and was centrifuged for 30 min in a microcentrifuge at 13,000 rpm at 4°C. The resulting supernatant (cell lysate) was carefully transferred into a new tube. The protein concentration in the cell lysate was determined using a commercially available protein assay kit (Bio-Rad Laboratories, Hercules, CA), and was normalized by adjusting the volume of cell lysate with cold lysis buffer. Cell lysates were stored in a −80°C freezer until they were used.

**Western Analysis of DNA Ligases.** Protein samples were incubated at 95°C for 5 min in SDS sample buffer and electrophoresed on 7.5 or 10% polyacrylamide gels. The proteins were then electroblotted to polyvinylidene difluoride membrane, and the membrane was blocked with 10 mM Tris and 150 mM NaCl plus 0.1% Tween containing 5% defatted milk powder. The membrane was incubated for 1 h at room temperature with monoclonal antibodies against either human DNA ligase I or III (Gentex, San Antonio, TX), washed, and probed with antimouse IgG antibody conjugated with horseradish peroxidase (Amersham Life Science). The rabbit polyclonal antibody specific for DNA ligase IV was used for probing DNA ligase IV. The signal was detected using an enhanced chemiluminescence Western blotting system (Amersham Life Science).

**Flow Cytometry.** MiaPaCa cells were treated with 15 nM gemcitabine, and the monolayer cells were harvested after 24, 48, and 72 h of drug treatment. Cell pellets were fixed by incubation in 0.5% paraformaldehyde (EM grade) in PBS. For flow cytometric analysis, cell pellets were gently resuspended in 1 ml of hypotonic PI solution (50 µg/ml PI in a hypotonic sodium citrate solution containing 0.3% NP40 and 1.0 mg/ml RNase A) at 1.0 × 10⁶ cells/ml, vortexed, and stained for 30 min at room temperature in the dark. Before flow cytometric measurements, samples were filtered through a 37 µm nylon mesh into 12 × 75 mm tubes and stored at 4°C until analysis within 24 h. All samples were analyzed with an EPICS ELITE flow cytometer (Coulter Cytometry, Miami, FL) using a 15 mW argon ion laser operated at 6 A of power at 488 nm. Photomultiplier tube voltage was adjusted for each control sample to position the G₀-G₁ peak to channel 240 on a 1024 channel presentation. Histograms were analyzed for cell cycle compartments using MultiCycle-PLUS Version 4.0.

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3 The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; 5-FU, 5-fluorouracil; PI, propidium iodide; dNTP, deoxynucleotide triphosphate.
RESULTS

Effect of Gemcitabine on DNA Ligase I Expression in MiaPaCa Cells. To study the effect of gemcitabine on DNA ligase I expression, MiaPaCa cells were treated with different concentrations of gemcitabine and harvested at different day intervals. Cell growth was determined by counting cells after harvesting the monolayer cells. More than 90% of harvested cells were viable based on the trypan blue dye exclusion test (data not shown). As shown in Fig. 1, this drug exposure causes significant growth inhibition in a dose-dependent manner at 15–240 nM gemcitabine. Total protein lysates were prepared from harvested cells, and the levels of DNA ligases I, III, and IV in cell lysates were measured using Western blot analysis. The effects of various gemcitabine concentrations on the expression of DNA ligases are shown in Fig. 2A. Exposure of cells to gemcitabine produced an ~2–3-fold increase in DNA ligase I levels within 1 day, and high levels of DNA ligase I remained until day 2 or 3. In contrast, no significant differences in DNA ligase III and IV levels were observed between gemcitabine-treated and untreated control cells at concentrations <30 nM (Fig. 2, B and C). At gemcitabine concentrations >60 nM, the expression of DNA ligase III decreased, indicating that the expression of DNA ligase III might be related to cell proliferation. To further determine the kinetics of DNA ligase I expression, MiaPaCa cells were exposed to 15 nM gemcitabine, and the cells were harvested at 2, 4, 12, 24, and 48 h for measurement of DNA ligase I levels. The elevated DNA ligase I level in drug-treated cells was clearly visible 4 h after treatment. DNA ligase I reached a maximum level within 24 h and remained at this level until 48 h, whereas the ligase I level remained unchanged in control cells (Fig. 3).

Effect of Gemcitabine on Cell Cycle Distribution. Cell cycle analysis was carried out to determine whether the increases of DNA ligase I levels in response to gemcitabine resulted from an alteration in cell cycle distribution. MiaPaCa cells treated with the vehicle or 15 nM gemcitabine were har-

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**Fig. 1**  Dose-dependent inhibition of the proliferation of MiaPaCa cells by gemcitabine. The cells were treated with 0, 15, 30, 60, 120, and 240 nM gemcitabine for 4 days. Each day, cells were harvested and counted to determine cell growth.

**Fig. 2**  DNA ligase I expression in gemcitabine-treated MiaPaCa cells. The cells were treated with gemcitabine at the concentrations indicated at the top and harvested at each day until day 4. The cell lysates were prepared from harvested cells to examine DNA ligase I levels by Western immunoblot analysis. A, DNA ligase I level; B, DNA ligase III level; C, DNA ligase IV level; D, actin level.

**Fig. 3**  Time kinetics of DNA ligase I expression in MiaPaCa cells in response to gemcitabine treatment. MiaPaCa cells were treated with 15 ng/ml gemcitabine and harvested at 2, 4, 12, 24, and 48 h. The cell lysates were prepared from harvested cells to measure DNA ligase I (Lig I) levels.
Effects of ara-C, Hydroxyurea, and 5-FU. Because gemcitabine is a multifaceted drug whose metabolites can inhibit ribonucleotide reductase or thymidylate synthetase, or be incorporated into growing DNA during DNA synthesis, we tested other agents that share a mechanism of action with gemcitabine but have more selectivity than gemcitabine. We first tested ara-C for its effect on DNA ligase I expression. ara-C is a well-known deoxycytidine analogue that inhibits chain elongation after being incorporated into the C sites of growing DNA strands, but ara-C does not inhibit ribonucleotide reductase or thymidylate synthetase, which distinguishes ara-C from gemcitabine (5–7). As shown in Fig. 5A, exposure of cells to 5 μM ara-C also increased the DNA ligase I level 3–5-fold, suggesting that the incorporation of gemcitabine triphosphate into DNA might be partly responsible for the observed increased expression of DNA ligase I. We next tested the effect of hydroxyurea, which blocks replication by both inhibiting the activity of ribonucleotide reductase and lowering dNTP concentrations without incorporation into DNA, on DNA ligase I expression. As shown in Fig. 5B, hydroxyurea also induced an ~3-fold increase of DNA ligase I expression. We then tested 5-FU, which mainly targets thymidylate synthetase and inhibits DNA synthesis as well as other agents. In contrast with other agents, 5-FU decreased the expression of DNA ligase I in a dose-dependent manner (Fig. 5C). These results suggest that increased expression of DNA ligase I in response to gemcitabine might be caused by both incorporation into DNA and inhibition of ribonucleotide reductase activity by gemcitabine metabolites, but not by inhibition of thymidylate synthetase. Because hydroxyurea and ara-C are known to stall the replication fork during the S-phase of the cell cycle, DNA ligase I induction could be triggered by replication fork arrest.

Effect of Gemcitabine on DNA Ligase I Expression in Human Pediatric Tumor Cell Lines. To determine whether gemcitabine could induce DNA ligase I expression in other types of human tumor cells, including NGP and SK-N-BE, these cell lines were treated with the indicated concentrations of gemcitabine (Fig. 6). As shown in Fig. 6, a 2–5 fold increase in DNA ligase I levels was observed in neuroblastoma cell lines treated with gemcitabine compared with untreated control cells, whereas DNA ligase III and IV expression remained unchanged or decreased. Although the antitumor efficacy of gemcitabine has not been reported in pediatric tumor models, comparable cytotoxic effects of gemcitabine on pediatric tumor cell lines were observed, in comparison with adult tumor cell lines. Because pediatric neuroblastomas showed the same pattern of response to gemcitabine, i.e., inhibition of cell growth and elevation of DNA ligase I levels, future trials using gemcitabine on pediatric tumors might be warranted.

DISCUSSION

Because DNA ligase I is an important component in the DNA damage repair pathway (9, 18, 19), we have been exploring the possibility of modulation of DNA ligase I expression by anticancer drugs that damage DNA directly or indirectly. Interestingly, the present study shows that gemcitabine, an anticancer drug derived from nucleoside analogues, could increase the level of DNA ligase I in various human tumor cells either without affecting or with decreased levels of other DNA ligases (III and IV). The same effect was also observed in MiaPaCa cells treated with other anticancer agents, including hydroxyurea and ara-C, which share mechanisms of action with gemcitabine. Previous studies regarding the regulatory mechanism of DNA ligase I expression suggested that a positive correlation exists between DNA ligase I expression and cell proliferation (20–22). For example, a significant increase in DNA ligase I gene expression was observed in HL-60 and NIH-3T3 cells when cell proliferation was induced from a resting state, whereas DNA ligase I level decreased in the same cell lines after differentiation (21). In human hematopoietic cells, the expression and activity of DNA ligase I were drastically reduced after induction of pre-B-cell differentiation, whereas those of DNA ligase III and IV remained virtually constant (22). It is also known that DNA ligases might be activated for the repair of DNA adducts caused by anticancer drugs (23–26). In one previous study, tumor cells treated with cisplatin increased their capacity to repair Pt-DNA adducts by increasing DNA polymerase β and total DNA ligase activities, which are considered to be important components of the DNA repair complex (25). However, there were no previous reports regarding the modulation of DNA ligase I expression in human tumor cells in response to treatment with nucleoside anticancer drugs, including gemcitabine.

Because DNA ligase I is responsible for major DNA ligase activity present in proliferating cells, increases in DNA ligase I levels under the growth-arrested condition produced by gemcitabine treatment led us to speculate that DNA ligase I might have critical functions related to the repair of certain kinds of DNA damage. The dying or dead cells harvested from the medium

Table 1  Cell cycle distribution of MiaPaCa cells treated with 15 ng/ml gemcitabine, compared with control cells

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Control (G0-G1, S, G2-M)</th>
<th>Gemcitabine (G0-G1, S, G2-M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>48.3 37.1 14.6</td>
<td>11.2 81.3 7.5</td>
</tr>
<tr>
<td>48</td>
<td>49.7 35.2 15.1</td>
<td>45.9 33.3 20.8</td>
</tr>
<tr>
<td>72</td>
<td>64.4 26.4 9.2</td>
<td>47.6 39.1 13.4</td>
</tr>
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suspension failed to induce DNA ligase I expression in response to gemcitabine treatment (data not shown). Furthermore, the harvested monolayer cells, which had elevated DNA ligase I, showed >90% viability and did not show any signs of apoptotic death based on flow cytometric analysis throughout treatments. Therefore, DNA ligase I might be involved in the process of improving the survival of tumor cells from the challenge of anticancer drug such as gemcitabine. Gemcitabine is a multifaceted drug that can inhibit ribonucleotide reductase or thymidylate synthetase or be incorporated into growing DNA. To determine the mechanism of action of gemcitabine responsible for DNA ligase I induction, we compared the effects of other

Fig. 4 Flow cytometric analysis of MiaPaCa cells treated with the vehicle or gemcitabine. The cells were exposed to 15 ng/ml gemcitabine or grown in drug-free medium. The monolayer cells were harvested after 24, 48, and 72 h of drug treatment for DNA analysis. The cells were stained with PI and measured by fluorescence-activated cell sorting (A–F). A–C, cell cycle distribution of untreated control cells harvested at 24, 48, and 72 h, respectively. D–F, cell cycle distribution of gemcitabine-treated MiaPaCa cells harvested at 24, 48, and 72 h, respectively. G, DNA ligase I (Lig I) levels in untreated or gemcitabine-treated cell lysates. The cell lysates were prepared from the same cells and used for determination of DNA ligase I level (B).
anticancer agents, including hydroxyurea, ara-C, and 5-FU, that share mechanisms of action with gemcitabine but are more selective. Interestingly, both hydroxyurea and ara-C, but not 5-FU, had the same effect on DNA ligase I expression as gemcitabine. Because both hydroxyurea and ara-C are known to arrest DNA replication forks, elevated ligase I would be a result of replication fork stalling caused by gemcitabine. In agreement with previous studies, cell cycle analysis of gemcitabine-treated cells indicates an initial arrest at the G₁–S-phase boundary 24 h after treatment, which is indicative of an arrest of replication fork progression during S-phase (29).

Early S-phase arrest can be attributable to the inhibition of DNA elongation as a result of incorporation of gemcitabine triphosphate into growing DNA strands or a decrease in dNTP pools through the inhibition of ribonucleotide reductase by gemcitabine metabolites (29). The increases in DNA ligase I levels caused by gemcitabine in human cancer cells might simply be a result of cell cycle arrest in S-phase if DNA ligase I is cell cycle regulated (high in S-phase). However, it is evident from other studies that expression of DNA ligase I is not regulated by cell cycle (28), but by cell proliferation status. Furthermore, 5-FU, which fails to induce DNA ligase I expression, is known to arrest the cell cycle at S-phase, similar to other agents, including gemcitabine, ara-C, and hydroxyurea. It has previously been reported that ara-C caused DNA strand breaks through inhibition of DNA synthesis on previously synthesized (mature) DNA as well as replicating (nascent) DNA (29). Hydroxyurea, which causes arrest of replication fork by reducing the dNTP pool, is known to cause the same strand breakage (30). In contrast to ara-C and hydroxyurea, 5-FU was reported to induce DNA breakage only on nascent DNA, possibly because of inhibition of DNA synthesis, but not mature DNA (31). Therefore, elevated DNA ligase I might be involved in the repair of breaks in mature DNA caused by the arrest of replication forks. Because gemcitabine is already used in clinics and possesses a wide spectrum of antitumor activity against various kinds of tumors, enhancement of DNA ligase I level in response to gemcitabine treatment in tumor cells might be used as a reliable indicator of responsiveness to the drug.

In conclusion, our study first provides strong evidence that...
exposure of tumor cells to antitumor drugs such as gemcitabine and ara-c, which directly or indirectly damage DNA, results in an increase of DNA ligase I levels. Our ongoing study is directed toward defining the molecular pathway that leads to the enhancement of DNA ligase I levels as a result of antitumor agent exposure as well as determining whether increased DNA ligase I levels could play a role in rescuing cells from exposure to antitumor drugs.

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REFERENCES

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