Elevated Expression of DNA Ligase I in Human Cancers

Daekyu Sun, Rheanna Urrabaz, Myanh Nguyen, Jennifer Marty, Stephanie Stringer, Edna Cruz, Leticia Medina-Gundrum, and Steve Weitman

Institute for Drug Development, Cancer Therapy & Research Center, 14960 Omicron Drive, San Antonio, Texas 78245-3217

ABSTRACT

Purpose: Human DNA ligase I plays an essential role in DNA replication, recombination, and repair. We catalyze the formation of phosphodiester bonds between adjacent 5'-phosphoryl and 3'-hydroxyl termini at single breaks in duplex DNA molecules. DNA ligase I is responsible for the majority of DNA ligase activity present in proliferating cells among four different forms of DNA ligases, designated DNA ligase I, II, III, and IV. In this study, we were interested in comparing DNA ligase I level in human tumors versus normal tissues and studying whether the inhibition of DNA ligase I could lead to tumor cell death.

Experimental Design: DNA ligase I level was measured by Western immunoblot assay in various human malignant tumor specimens and benign tissues obtained from patients, in peripheral blood lymphocytes obtained from healthy donors, and in human tumors grown in nude mice. We also have designed antisense oligonucleotides (ODNs) targeting the mRNA of DNA ligase I and tested whether DNA ligase I antisense ODNs could control tumor cell growth.

Results: The amount of DNA ligase I enzyme in malignant tumors was considerably higher than that in benign normal tissues and peripheral blood lymphocytes. The level of DNA ligase I in human tumors grown in nude mice was also very high, and the expression of DNA ligase I appears to be constitutive during in vivo tumor development. We have observed that DNA ligase I antisense ODN inhibited tumor cell growth in a dose-response manner, but nonspecific control ODNs had little effect on the growth of the same cell lines.

Conclusions: The presence of DNA ligase I at higher levels in human tumors than in benign normal tissues and normal peripheral lymphocytes suggests DNA ligase I plays more of a role in proliferating cells than in resting cells. These results, together with our finding that DNA ligase I antisense ODNs can suppress tumor cell proliferation, warrant the design and testing of human DNA ligase I inhibitors as new anticancer agents.

INTRODUCTION

Human DNA ligases are major components in DNA replication, recombination, and repair. They function by catalyzing the formation of phosphodiester bonds between adjacent 5'-phosphoryl and 3'-hydroxyl termini at single breaks in duplex DNA molecules (1–3). In DNA ligation reactions mediated by mammalian DNA ligases, ATP is used as a cofactor to form a covalent enzyme-adenylate complex, and 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 (4). In the final step of DNA ligation, unadenylated DNA ligase is involved in the generation of a phosphodiester bond by catalyzing the displacement of the AMP residue through attack by the adjacent 3'-hydroxyl group on the adenylated site. In mammalian cells, four distinct DNA ligases, named DNA ligase I, II, III, and IV, have been thus far identified and are different from each other in their catalytic, physical, and serological properties (1, 5–8). DNA ligase I is responsible for the majority of DNA ligase activity in proliferating cells, whereas most of the ligase activity in resting cells is attributable to other DNA ligases (2). DNA ligase III is regarded as having a role in meiotic recombination in mammalian cells, because it is highly expressed in testis (6). DNA ligase IV was most recently identified and might be responsible for the ligation step of V(D)J recombination in lymphoid cells and the repair of DNA double-strand breaks in mammalian cells (7, 8). DNA ligase II is believed to be a specific proteolytic product of DNA ligase III (3). There is sufficient evidence that indicates major involvement of DNA ligase I in DNA replication (9–11). 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 (9). Other studies suggest DNA ligase I is also involved in DNA repair (12–14). 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 (3).

There is much evidence that proliferating cells contain a substantially higher level of DNA ligase I than other nondividing or resting cells, whereas such a correlation does not exist for DNA ligase II and III (15–18). However, no attempt has been made to determine the levels of this enzyme in various human tumor cells compared with those in normal tissues. As will be discussed in this report, we have measured human DNA ligase I levels in tumor specimens obtained from patients, human tumor cell lines, benign tissues, and peripheral blood lympho-
cytes. Using DNA ligase I antisense ODNs\(^3\) targeted against human DNA ligase I mRNA, we also tested whether DNA ligase I inhibition could lead to inhibition of tumor cell growth. Our results in this study provide evidence that the levels of DNA ligase I are considerably up-regulated in human tumor versus normal tissues, and the presence of DNA ligase I is essential for survival of tumor cells.

**MATERIALS AND METHODS**

**Cell Lines.** Human cancer cell lines SKN-MC (neuroblastoma), HT-29 (colon), Bon (pancreas), SKOV3 (ovarian), MCF-7 (breast), and MiaPaCa (pancreas) were obtained from the American Type Culture Collection. The MiaPaCa cell line was maintained in RPMI 1640 with L-glutamine and L-glutamine supplemented with 10% fetal bovine serum. The MCF-7 and HT-29 cell lines were maintained in RPMI 1640 with L-glutamine and Earle’s salts supplemented with 1.5 grams/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.01 μg/ml bovine insulin, and 15% fetal bovine serum. Cells were grown at 37°C and 5% CO\(_2\) in a humidified atmosphere.

**Preparation of Cell Lysates (S-100).** Cell lysates were routinely prepared as below. In brief, cell suspensions prepared from tissue samples and cultured human cells were washed once in PBS; resuspended in ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl\(_2\), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, 1 mM DTT, and 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, along with 10% glycerol (10°/h cells/20 μl of buffer); incubated for 30 min on ice; and centrifuged for 30 min in a microcentrifuge at 13,000 rpm at 4°C. The resulting supernatant (cell lysates) was carefully transferred into a new tube. The protein concentration in cell lysates was determined using a commercially available protein assay kit (Bio-Rad Laboratories) and normalized by adjusting the volume of cell lysate with cold lysis buffer. Cell lysates were stored in −80°C freezer until they were used.

**Western Blot Analysis.** The expression of DNA ligases I and III in cell lysates was determined by Western blot assay. Samples containing equal amounts of protein from cell lysates were boiled in SDS sample buffer, electrophoresed on 7.5 or 10% polyacrylamide gel, and transferred to polyvinylidene di-fluoride membrane (Bio-Rad). After blocking with 5% defatted milk powder, the membranes were incubated 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 signal was detected using an enhanced chemilumeninescence Western blotting system (Amersham Life Science).

\(^3\)The abbreviations used are: ODN, oligonucleotide; TdT, terminal deoxynucleotidyltransferase.

**Fractionation of DNA Ligase I and TdT from Cell Lysates.** The cell lysates (2 ml) were prepared from 10\(^6\) cells of peripheral blood lymphocytes (Human Tumor Cloning Lab of Cancer Therapy & Research Center, San Antonio, TX) and Molt-4 leukemia cells (National Cell Culture Institute, Minneapolis, MN), as described above, and were applied to heparin agarose column (5-ml bed volume) equilibrated with buffer containing Tris-HCl (pH 7.8), 50 mM NaCl, 2 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride (buffer A). After the column was washed extensively with buffer A, proteins were eluted with 12 ml of NaCl gradient (0.1–1 M) in the same buffer, and 200-μl aliquots of elutes were collected in each fraction. To examine the presence of DNA ligase I, 10-μl aliquots from every third fraction were used for Western immunoblot assay, and 1-μl aliquots from the same fraction were used for biochemical assay of TdT.

**Assay for TdT.** TdT activity was determined using a standard primer extension assay, except for the use of 5’-biotinylated (TTAGGG)\(_3\) instead of the primers with random sequences. We have learned that the telomeric primer is an equally effective substrate for TdT, but less exonucleolytic degradation was observed compared with the primers with random sequences. In brief, reaction mixtures (20 μl) contained 1-μl aliquots from the fraction, 0.2 μM potassium cacodylate (pH 7.5), 1.5 mM MgCl\(_2\), 1 mM 2-mercaptoethanol, 1 μM biotinylated primer, and 0.1 μM [α-\(^32\)P]-dGTP (800 Ci/mmol) and were incubated at 37°C for 1 h. TdT reaction products were then immobilized on streptavidin-coated Dynabeads and washed extensively to eliminate excess [α-\(^32\)P]-dGTP. TdT reaction products were separated from the magnetic beads by protein denaturation with 5 M guanidine-HCl at 90°C for 20 min. After ethanol precipitation of these products, analysis was performed by 8% denaturing PAGE.

**Design of Human DNA Ligase I Antisense ODNs.** Antisense DNA ligase I, sense DNA ligase I, and mixed sequence ODNs were synthesized commercially by standard methods (Genosys, Woodlands, TX). The sequence of a 15-mer antisense DNA ligase I ODN was 5’-GAGGCTCTGGACCGA-3’ (19). The corresponding sense sequence and mixed sequence ODN (5’-AGTCGATCGTCACGT-3’) were used as controls. To increase a half-life without affecting specificity of ODNs, three consecutive phosphodiester backbones from both 3’- and 5’-ends of ODNs were substituted with a phosphorothioate backbone, in which nonbridging oxygen is substituted with sulfur. For in vitro transfection, antisense ODNs were formulated into DNA/Liposome mixtures by combining N-[2,3-dioleoyloxy]-propyl]-N,N,N-tri-methylammoniummethyl sulfate with antisense ODNs at a ratio of 5:1 as described by the manufacturer (Boehringer Mannheim). Cells were harvested after 72-h treatment, and DNA ligase I level was measured by Western immuno blot. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to measure the growth inhibitory effect of ODNs on in vitro cell cultures, which is based on the ability of viable cells to metabolize the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye to a purple formazan (20). After solubilizing the formazan, the absorbance at 595 nm is measured using a plate reader. We have used this method for estimating the in vitro growth inhibitory activity of ODNs in tumor cell lines.
the presence of DNA ligase I, along with cell lysates from five
eral blood lymphocytes from healthy donors and examined for
Cell lysates were prepared from six samples of periph-
cytes.

RESULTS
Western immunoblot analysis of DNA ligase I in human cancer
Fig. 1
A, cell lines and normal bone marrow cells. A, DNA ligase I level in
pediatric cell lines SKN-AS, SKN-BE, SKN-MS, CHP212, and NGP
(Lanes 1–6, respectively) and in peripheral blood lymphocytes obtained
from six different healthy donors (Lanes 7–12). Loading was normal-
ized for the same amount of protein in each lane, and ~60 µg of total
protein were loaded in the gel.

DNA Ligase Expression in in Vivo Tumors Grown in
Nude Mice. Groups of 30 nude mice were injected s.c. with
SKN-MC cells, and the formation of tumors was monitored.
When tumors were ~60, 100, 500, 1000, 1500, and 2000 mg, 5
animals were sacrificed, and tumor tissues were harvested for
the measurement of DNA ligase I level. Each group of 2 mice
was also inoculated s.c. with HT-29, Bon, SKOV3, MCF-7, and
MiaPaCa cells, and tumors were harvested from animals when
tumors reached ~200–500 mg. The harvested tumor tissues
were snap frozen with liquid nitrogen and stored at −80°C until
processed. Tissue samples were mechanically disassociated with
scissors and forced through a number 100 stainless steel mesh.
Cell suspension was collected and centrifuged to remove culture
media. Cell pellets were washed with PBS buffer once and used
for the preparation of cell lysates.

Tissue Samples from Patients. After obtaining in-
formed consent in accordance with federal and institutional
guidelines, specimens were collected from patients undergoing
tissue and/or fluid procurement procedures as part of their
diagnostic work-up or as part of their routine therapeutic care.
Specimens included malignant effusions, ascites, and bone mar-
row aspirates containing tumor cells, as well as solid tumors.
Solid tumors or malignant lymph nodes were placed in McCoy’s
5A Medium (Mediatech-cellgro, Herndon, VA) containing 10%
heat-inactivated newborn calf serum and 1% penicillin/strepto-
mycin. These solid tumors were mechanically disassociated with
scissors and forced through a number 100 stainless steel mesh.
Cell suspension was collected and centrifuged to remove culture
media. Cell suspension was collected and centrifuged to remove culture
media. Cell pellets were washed with PBS buffer once and used
for the preparation of cell lysates.

Expression of DNA Ligase I in Peripheral Lympho-
cytes. Cell lysates were prepared from six samples of peripheral
blood lymphocytes from healthy donors and examined for
the presence of DNA ligase I, along with cell lysates from five

neuroblastoma cell lines (SKN-AS, SKN-BE, SKN-MS,
CHP212, and NGP), as positive controls. As shown in Fig. 1, the
five neuroblastoma cell lines had a high level of DNA ligase I,
whereas cell lysates from normal lymphocytes had no detectable
amounts of DNA ligase I in Western immunoblot assay. To
confirm this finding, the cell lysates were prepared from ~10^6
cells of peripheral blood lymphocytes (collected from healthy
donors) and Molt-4 leukemia cells. Both cell lysates were ap-
plied to a heparin agarose column to separate DNA ligase I from
other proteins in cell lysates. Western immunoblot assay de-
scribed in Fig. 2A shows that there is a remarkable difference
of DNA ligase I between normal lymphocytes and Molt-4 leuke-
mia cells in chromatographic fractions obtained from cell lys-
ates. The presence of TdT activity was also determined, be-
cause TdT is known as a prevalent marker for both lymphocytic
leukemia and normal lymphocytes. As expected, TdT activity
can be detectable in chromatographic fractions from both cell
lysates, despite a significant elevation of Molt-4 cells over
lymphocytes (Fig. 2B). Taken together, the data indicate that
DNA ligase I level is significantly down-regulated in normal
lymphocytes compared with other tumor cell lines, such as
neuroblastoma and leukemia.

DNA Ligase I Level in Human Tumors Versus Normal
Tissues. Next, we determined whether DNA ligase I level is
elevated in human tumors versus normal tissues. We examined
29 different human tumor specimens (six breast, two lung, three

Fig. 2
A

B

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In Vivo in Nude Mice. Because tumor specimens from patients contain a significant portion of normal tissues and dead necrotic tumor cells, the level of DNA ligase I present in cell lysates from tumor specimens may underestimate the amount of DNA ligase I present in actively dividing cancer cells. Therefore, selected human tumor cell lines were inoculated s.c. into nude mice and were allowed to grow until tumors reached ~200–500 mg/mouse. The mice were then sacrificed, and human tumors were separated for additional processing to prepare cell lysates. DNA ligase I level in cell lysates prepared from tumors grown in nude mice was examined by Western immunoblot. As shown in Fig. 4A, truncated form (M, 85,000) of wild-type DNA ligase I was detected, presumably because of the proteolytic degradation of DNA ligase I during processing tissue samples to prepare cell extracts (3). Next, we examined the expression of DNA ligase I in SKN-MC human cell lines during in vivo tumor development to investigate the kinetics of DNA ligase I expression during tumor development. As shown in Fig. 4B, DNA ligase I expression appears to be constitutive and increased until tumors reached ~2 grams in size. All together, these results support the active role of DNA ligase I in rapidly growing human tumors.

Inhibition of DNA Ligase I Expression and Cell Growth in MCF-7 Cells by DNA Ligase I Antisense ODN. Because the level of DNA ligase I is considerably up-regulated in human tumors versus normal tissues, we tested whether DNA ligase I inhibition could lead to inhibition of tumor cell growth. In this study, we designed a DNA ligase I antisense ODN that was targeted against human DNA ligase I mRNA. The antisense ODN against DNA ligase I inhibited the expression of DNA ligase I in MCF-7 cells without affecting DNA ligase III expression, whereas corresponding sense ODN and mixed sequence ODN had little effects on DNA ligases I and III expression (Fig. 5A). As anticipated, DNA ligase I inhibition could lead to inhibition of tumor cell growth, because the growth of MCF-7 cells was suppressed in a dose-dependent manner by DNA ligase I antisense ODN with a range from 0.5 to 5 μM (Fig. 5B). The number of untreated cells and cells treated with either sense ODN or mixed sequence ODN was almost equal.
The concentration of ODNs was 1 and antisense ODNs on DNA ligase I and III expression in MCF-7 cells.

Lane 2
Lane 3
methyl sulfate alone (Lane 1), mixed sequence (Lane 2), sense (Lane 3), and antisense ODNs on DNA ligase I and III expression in MCF-7 cells.

The concentration of ODNs was 1 µM. B, dose-dependent growth inhibition of MCF-7 cells by DNA ligase I antisense ODN in comparison with mixed sequence and sense ODNs. Data point represents average of two separate experiments. Bars, SD.

These results provide strong evidence that the level of DNA ligase I is considerably up-regulated in human tumor versus normal tissues, and the presence of DNA ligase I is essential for survival of tumor cells.

DISCUSSION

DNA ligase I is an important component in both the DNA replication and DNA damage repair pathways (9–14). It is also known that DNA ligase I expression is modulated on cell differentiation and proliferation (15–18). Previously, it was reported that DNA ligase I level increased significantly in HL-60 and NIH-3T3 cells when cells were induced to proliferate, whereas DNA ligase I level decreased in the same cell lines after differentiation (16). In murine hematopoietic cells, the expression and activity of DNA ligase I were drastically reduced on induction of pre-B-cell differentiation, whereas those of DNA ligase III and IV remained virtually constant (18). Altogether, these studies provide proof of a strong correlation between DNA ligase I activity and cellular proliferation.

Despite the major role of DNA ligase I in rapidly proliferating cells, we are not aware of any attempt to compare the amount of this enzyme in various human tumors (versus normal tissues). Therefore, we have examined the level of human DNA ligase I in cell lysates prepared from tumor specimens of primary and metastatic tumors taken from patients in comparison with normal tissues. We also compared DNA ligase I level in cell lysates prepared from human tumor cell lines grown in flasks, normal bone marrow, and human tumor cell lines grown in nude mice. The overall results of our experiments clearly show that DNA ligase I is highly elevated in human tumors. In contrast, DNA ligase I enzyme was rarely detected in any of the normal tissues or normal bone marrow cells we examined. The DNA ligase I antisense ODN targeting mRNA of human DNA ligase I showed strong cytotoxicity against human breast carcinoma MCF-7 cells through down-regulation of DNA ligase I expression with little change in DNA ligase III expression. In contrast, a matching sense ODN and control mixed ODN did not show any cytotoxic effect on the same cell line and induced no change in the expression of DNA ligases I and III. In addition, the level of DNA ligase I in human tumors grown in nude mice was also very high, and the expression of DNA ligase I appeared to be constitutive during in vivo tumor development. This information provides us with substantial evidence that DNA ligase I is potentially a very intriguing and unique target for the design of new anticancer agents, and there is a strong possibility of achieving selective inhibition against rapidly proliferating tumor cells.

Most conventional therapies used for the treatment of human cancers are known to cause major side effects, such as bone marrow toxicity, because they are not very selective and damage normal tissues. Many improvements in the survival of cancer patients could be made through new therapies, which are much safer to cancer patients than conventional therapy. Of particular importance, DNA ligase I was found at a much higher level in both tumor cell lines and tumor specimens obtained from patients than in benign tissues and normal human lymphocytes. Although actively dividing normal fibroblasts were known to express DNA ligase I, resting hematopoietic stem cells derived from bone marrow are almost DNA ligase I negative, and its activity is only up-regulated after phytohemagglutinin stimulation (16). Therefore, the high proliferative index of most cancer cells compared with the more sporadic cycles of normal stem-cell populations will make an anti-DNA ligase I strategy clinically acceptable. Therefore, new therapy based on small molecules that interfere with DNA ligase I are expected to be much safer for cancer patients than conventional chemotherapy.

Future design and identification of specific human ligase I inhibitors should help to clarify the biological roles of this enzyme in tumor cells and test the antitumor activity of DNA ligase I-interfering compounds.

REFERENCES


