Apoptosis induction by glycoprotein isolated from *Laminaria japonica* is associated with down-regulation of telomerase activity and prostaglandin E_2 synthesis in AGS human gastric cancer cells

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Abstract. Glycoprotein isolated from *Laminaria japonica* (LJGP) is known to exhibit significant cytotoxic activity against human cancer cells; however, the mechanisms of its cytoxicity are poorly understood. In this study, we investigated further possible mechanisms by which LJGP exerts its anti-cancer action in cultured human gastric carcinoma AGS cells. LJGP treatment of AGS cells resulted in inhibition of growth and induction of apoptosis in a time- and concentration-dependent manner, as determined by MTT assay, fluorescence microscopy, and flow cytometry analysis. The increase in apoptosis was associated with up-regulation of pro-apoptotic Bax expression, down-regulation of anti-apoptotic Bcl-2 and IAP family members, and activation of caspase-3 and -9. LJGP treatment markedly down-regulated the activity of telomerase and expression of human telomerase reverse transcriptase, a main determinant of telomerase enzymatic activity, with inhibition of Sp1 and c-Myc expression in a concentration-dependent manner. Furthermore, LJGP treatment also caused a progressive decrease in the expression levels of cyclooxygenase (COX)-2 without significant changes in the levels of COX-1, which was correlated with a decrease in prostaglandin E_2 synthesis. These results provide important new insights into the possible molecular mechanisms of the anti-cancer activity of LJGP.

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Introduction

Apoptosis plays a pivotal role in the normal development and pathology of a wide variety of tissues and is characterized by several unique features, including cell shrinkage, chromatin condensation, DNA fragmentation, cell surface expression of phosphatidylserine, and membrane blebbing (1,2). In general, depending on the cell type or trigger, apoptosis can be initiated in two ways: by an extrinsic (death receptor-mediated) pathway or by an intrinsic (mitochondrial-mediated) pathway (3-5). However, most cancer cells block apoptosis, which allows survival of malignant cells despite genetic and morphologic transformations. Therefore, induction of apoptotic cell death is an important mechanism in the anti-cancer properties of many drugs.

It has become increasingly clear that the process of neoplasia is characterized by activation of telomerase, which adds telomeric repeats to the ends of replicating chromosomes, telomeres, which are essential units that prevent the loss of genetic information (6,7). Normal somatic cells have little or no telomerase activity for synthesis of new telomeres and telomeric DNAs show progressive shortening with each cell division, to cause irreversible cell growth arrest and cellular senescence. However, cancer cells and spontaneously immortalized cells have mechanisms that compensate for telomere shortening, most commonly through activation of telomerase, allowing stable maintenance of telomeres and indefinite growth (8,9). These observations suggest that telomerase reactivation is a rate-limiting step in cellular immortality and carcinogenesis, and telomerase repression can act as a tumor-suppressive mechanism.

The link between the arachidonic acid (AA) pathway and cancer has also been the major challenge of the current study (10,11). Cyclooxygenase (COX)-1 and COX-2 are two key enzymes with involvement in AA metabolism; the former is the constitutive isoform, while the latter is the rate-limiting enzyme. Unlike the former, COX-2 catalyzes AA metabolism to form prostaglandin E_2 (PGE_2) and has been characterized as an immediate-early gene associated with neoplastic transformation, cell growth, angiogenesis, invasiveness, and metastasis (12,13). Moreover, compelling evidence from genetic and clinical studies indicates that COX-2 up-regulation is a key step in carcinogenesis and that there is a clear positive correlation between COX-2 expression and inhibition of cell proliferation (14,15). Thus, inhibition of COX-2 expression and blockade of the PGs cascade with chemotherapy agents have been proposed for cancer treatment trials.

Findings from recent studies have indicated that marine organisms are proving to be a novel and rich source of bioactive compounds. Among them, seaweeds have been used as an important dietary component in Asian communities. Previous results have shown that extracts of various edible seaweeds have protective factors against some types of cancer and that they exhibit suppressive effects against chemically induced tumorigenesis through suppression at the initiation and promotion phases (16-19). Of these, *Laminaria japonica*, a brown alga, is the most important economic seaweed cultured in the temperate seaside areas of the northwest Pacific, including Korea, Japan, and China. Because *L. japonica* has high nutritional value as a source of minerals, vitamins, and non-caloric dietary fiber, it has been widely used as a health food as well as a traditional Oriental herbal medicine for over a thousand years (20,21). Findings from previous studies have indicated that heteropolysaccharides, such as fucoidans, extracted from *L. japonica*, have diverse pharmacological properties, including antioxidant, anticoagulant and anti-cancer activities, and that they improve lipid metabolism (22-26). Although *L. japonica* is composed of 60.9% carbohydrate and 10.3% protein (27), the protein moieties present in this seaweed have not been thoroughly investigated. Go *et al* (28) recently reported that a novel glycoprotein (named as LJPG) isolated from *L. japonica* stimulated normal gastrointestinal cell growth by activation of the epidermal growth factor receptor (EGFR) signaling pathway. More recently, they also demonstrated that LJGP inhibited colon cancer HT-29 cell proliferation by inducing cell cycle arrest and apoptosis (29); however, the molecular mechanisms of its anti-proliferative action on malignant cell growth are not completely known.

In the course of our screening program for bioactive marine natural products from seaweeds, the aim of this work was to investigate the effect of LJPG on growth inhibition and apoptosis in the human gastric carcinoma AGS cell line in parallel with reduction of human telomerase reverse transcriptase (hTERT) expression, a main determinant of telomerase activity, which was associated with inhibition of COX-2 expression and release of PGE_2.

Materials and methods

Cell culture, LJGP treatment, and cell proliferation assay. Human gastric carcinoma AGS cells were purchased from the American Type Culture Collection (Rockville, MD), and maintained at 37˚C in a humidified condition of 95% air and 5% CO_2 in DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. LJGP was prepared as previously described (29) and dissolved in dimethyl sulfoxide as a stock solution at 10 mg/ml concentration, and stored in aliquots at -20˚C. Measurement of cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme, as previously described (30).

Nuclear staining with DAPI. Cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS for 10 min at room temperature. Fixed cells were washed with PBS, and stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature. Cells were washed two more times with PBS and analyzed using a fluorescence microscope (Carl Zeiss, Germany).

Flow cytometric analysis. Following treatment with LJGP, cells were trypsinized, washed with PBS, and fixed in 75% ethanol at 4˚C for 30 min. Prior to analysis, cells were again washed with PBS, suspended in cold propidium iodide (PI, Sigma) solution, and incubated at room temperature in the dark for 30 min. A FACScan flow cytometry system (Becton-Dickinson, San Jose, CA) was used for performance of flow cytometry analyses.

Gel electrophoresis and Western blot analysis. For preparation of total proteins, cells were gently lysed for 30 min with lysis buffer (20 mM sucrose, 1 mM EDTA, 20 μM Tris-Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). Supernatants were collected and protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). For Western blot analysis, an equal amount of protein was subjected to electrophoresis on SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by electroblotting. Blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibodies, and visualized by enhanced chemiluminescence (ECL) Western blotting detection reagents (SuperSignal, Thermo scientific, Rockford, IL) according to the recommended procedure (31).

Assay of caspase activity. Enzymatic activity of caspases induced by LJGP was assayed using a colorimetric assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Cells were incubated in the absence and presence of LJGP for the indicated times. Cells were harvested and lysed in a lysis buffer for 30 min on an ice bath. Lysed cells were centrifuged at 14,000 rpm for 20 min, and equal amounts of protein (100 μg per 50 μl) were incubated with 50 μl of a reaction buffer and 5 μl of the colorimetric tetrapeptides, Asp-Glu-Val-Asp (DEVD)-*p*-nitroaniline (pNA) for caspase-3, Ile-Glu-Thr-Asp (IETD)-pNA for caspase-8, and

Leu-Glu-His-Asp (LEHD)-pNA for caspase-9, respectively, at 37˚C for 2 h in the dark. Caspase activity was determined by measurement of changes in absorbance at 405 nm using an ELISA reader (32).

Telomerase activity assay. Telomerase activity was measured using a PCR-based telomeric repeat amplification protocol (TRAP) enzyme-linked immunosorbent assay (ELISA) kit (Boehringer-Mannheim, Mannheim, Germany) according to the manufacturer's instuctions. In brief, cells were treated with LJGP, harvested, and ~1x10^6 cells were lysed in 200 μl lysis reagent and incubated on ice for 30 min. For the TRAP reaction, 2 μl of cell extract (containing 2 μg protein) was added to 25 μl of reaction mixture with the appropriate amount of sterile water to make a final volume of 50 ml. PCR was performed in a Mastercycler as follows: primer elongation (30 min, 25˚C), telomerase inactivation (5 min, 94˚C), and product amplification by the repeat of 30 cycles (94˚C for 30 sec, 50˚C for 30 sec, 72˚C for 90 sec). Hybridization and the ELISA reaction were performed according to the manufacturer's instructions.

PGE_2 EIA analysis. Following treatment with LJGP, the medium was removed and PGE_2 release by cells was measured. For measurement of PGE_2 accumulation, enzyme immunoassay (EIA) was performed using a commercial kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's protocol. PGE_2 production was normalized with respect to the number of viable cells present in the particular culture.

Statistical analysis. All data are presented as the mean ± SD. Significant differences among the groups were determined using the unpaired Student's t-test. A $p<0.05$ was accepted as an indication of statistical significance. All the figures shown in this report were obtained from at least three independent experiments.

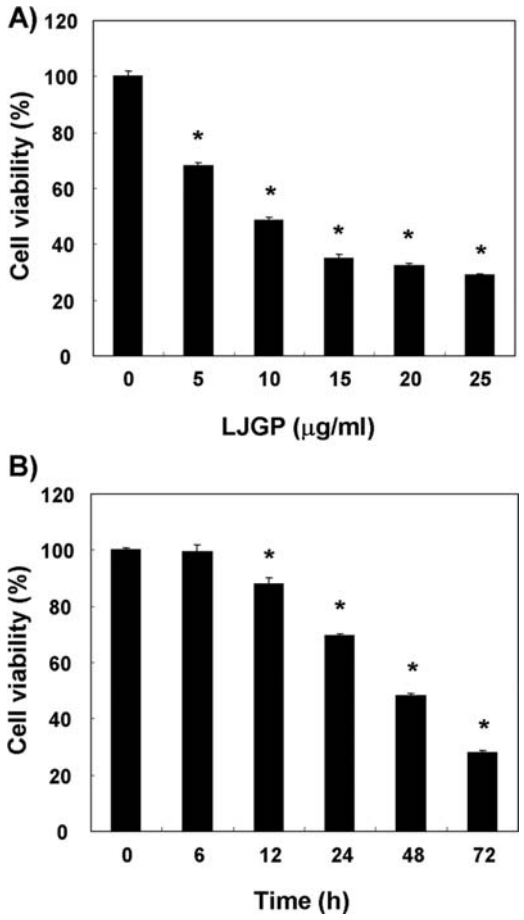

Figure 1. Inhibition of cell growth by LJGP treatment in human gastric carcinoma AGS cells. Cells were plated at 1x10^3 cells per 60-mm plate, and incubated for 24 h. Cells were treated with varied concentrations of LJGP for 72 h (A) or with 25 μg/ml of LJGP for the indicated times (B). Cell viability was measured by the metabolic-dye-based MTT assay. Data represent relative mean values ± SE of three independent experiments. The significance was determined by the Student's t-test (*$p<0.05$, compared with control).

Results

Growth inhibition and apoptosis induction by LJGP. To evaluate the effects of LJGP on proliferation of AGS cells, we initially assessed the effect on growth of AGS cells. As shown in Fig. 1, cell growth was significantly inhibited by LJGP in a concentration- and time-dependent manner. Analysis by light microscopy showed distinct morphological change in LJGP-treated cells. LJGP-treated cells were variable in size, with protuberances that resulted in more of a spindle shape, membrane shrinkage, and cell rounding up (Fig. 2A). In order to determine whether growth inhibition by LJGP was associated with apoptosis induction, cells treated with LJGP were examined after DAPI staining. Control cells displayed intact nuclear structure, while cells treated with LJGP showed chromosomal condensation and formation of apoptotic bodies in a time-dependent fashion, indicating LJGP induced apoptotic cell death (Fig. 2B). We next analyzed the amount of sub-G1 DNA, which contained less DNA than G1 cells, in order to quantify the degree of dead cells. Flow cytometric analysis indicated that LJGP treatment resulted in markedly increased accumulation of sub-G1 phase in a concentration- and time-dependent manner (Fig. 2C and D). Taken together, these results demonstrated that growth inhibition observed in response to LJGP is associated with induction of apoptosis.

Effects of LJGP on expression of Bcl-2 and IAP family proteins and caspase activation. To investigate the apoptotic cascades involved by LJPG, we evaluated the levels of Bcl-2 and inhibitor of apoptosis protein (IAP) family proteins in LJGP-treated AGS cells. Western immunoblot analysis indicated that treatment of cells with LJPG up-regulated expression of pro-apoptotic Bax, but down-regulated expression of anti-apoptotic Bcl-2, suggesting that LJGP alters the Bax:Bcl-2 ratio in AGS cells. To gain further insight into the mechanism by which LJPG induces apoptosis, we examined the effects of LJPG on caspase protein levels and their activity. As shown in Fig. 4A, LJPG-induced apoptosis of AGS cells was associated with decreased expression of the pro-caspase-3 and -9 proteins; however, levels of caspase-8 remained unchanged. To quantify the activities of caspases, cell lysates containing equal amounts of total protein from cells treated with LJPG were assayed for *in vitro* caspase activity using fluorogenic substrates. As indicated in Fig. 4B, treatment with LJPG for the indicated times significantly increased the

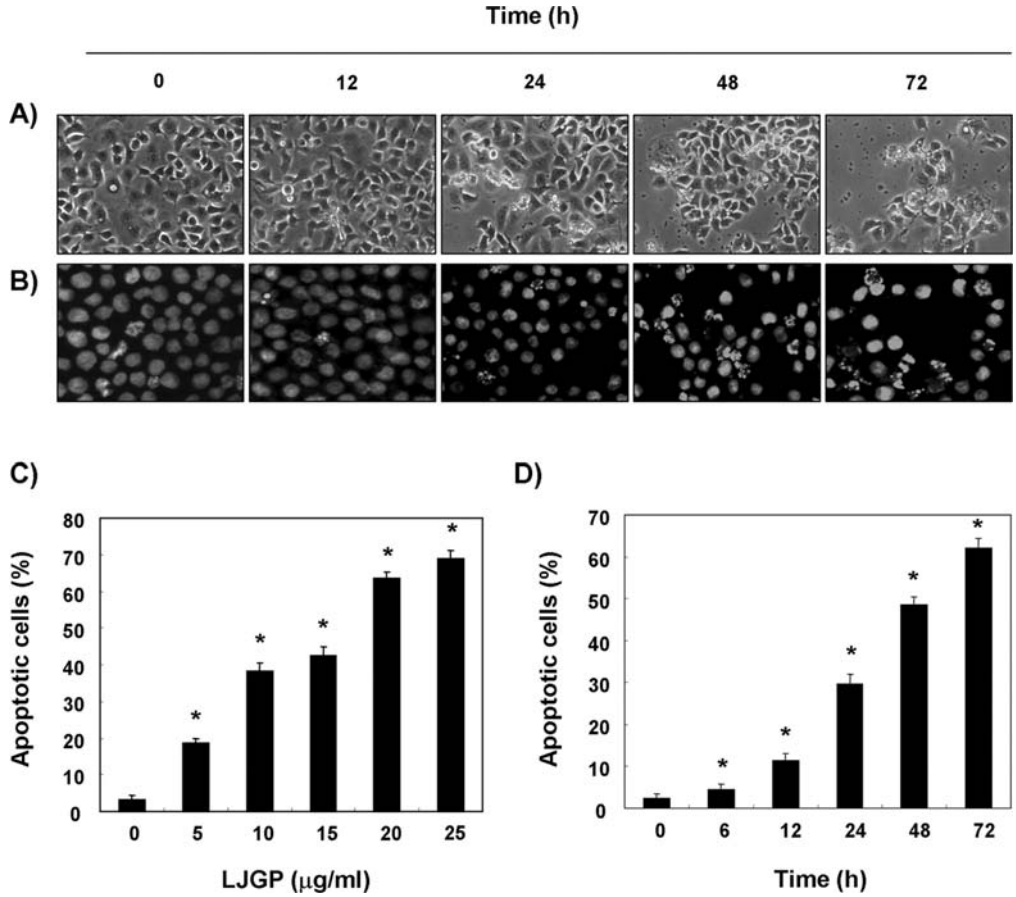

Figure 2. Induction of apoptosis by LJGP in AGS cells. (A) Cells were plated at $1x10^3$ cells per 60-mm plate, and incubated for 24 h. Cells were incubated with 25 μg/ml of LJGP for the indicated times. At the each time-point, cells were sampled and photographed under light microscopy (original magnification x200). (B) Cells grown under the same conditions as (A) were fixed and stained with DAPI. Following a 10-min incubation period at room temperature, stained nuclei were observed under a fluorescence microscope using a blue filter (original magnification x400). (C and D) To quantify the degree of apoptotic cells, cells were treated for 72 h with the indicated concentrations of LJGP for 72 h (C) or 25 μg/ml of LJGP for the indicated times (D). The cells were then collected and stained with PI for flow cytometry analysis. The fraction of apoptotic sub-G1 cells is indicated. Data represent relative mean values ± SE of three independent experiments. The significance was determined by the Student's t-test ($^*p<0.05$, compared with control).

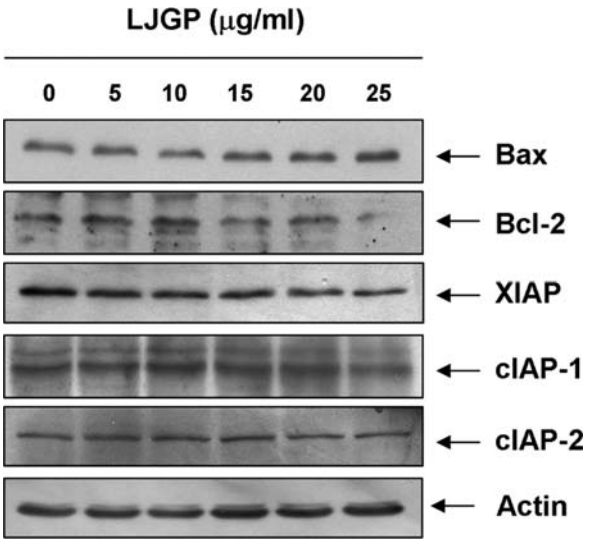

Figure 3. Effects of LJGP on levels of Bcl-2 and IAP family proteins in AGS cells. Following a 72-h incubation period with various concentrations of LJGP, cells were lysed, and cellular proteins were then separated by 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control.

activities of caspase-3 and -9 in a concentration-dependent manner, but not caspase-8. Furthermore, LJPG induced a concomitant degradation of poly (ADP-ribose) polymerase (PARP) protein and accumulation of the 85-kDa protein, which is a substrate protein of caspase-3 (33). We also examined the effects of LJPG on anti-apoptotic IAP family proteins, which bind to caspases, leading to inactivation of caspases (34). As shown in Fig. 3, LJPG progressively inhibited XIAP and cIAP-1, whereas cIAP-2 levels remained virtually unchanged in response to LJGP.

Down-regulation of hTERT and inhibition of telomerase activity by LJGP. Using TRAP-ELISA, we next attempted to determine whether the apoptotic effects of LJPG are associated with inhibition of telomerase activity. As shown in Fig. 5A, marked reduction of telomerase activity in AGS cells was observed with LJPG treatment in a concentration-dependent manner. Of the components comprising telomerase, hTERT is a critical determinant of the enzyme activity of telomerase (35); we examined changes in hTERT expression on treatment with LJPG. We found that hTERT expression showed a marked decreased as a result of treatment with LJPG, suggesting that repression of telomerase activity by LJPG was associated with down-regulation of hTERT expression.

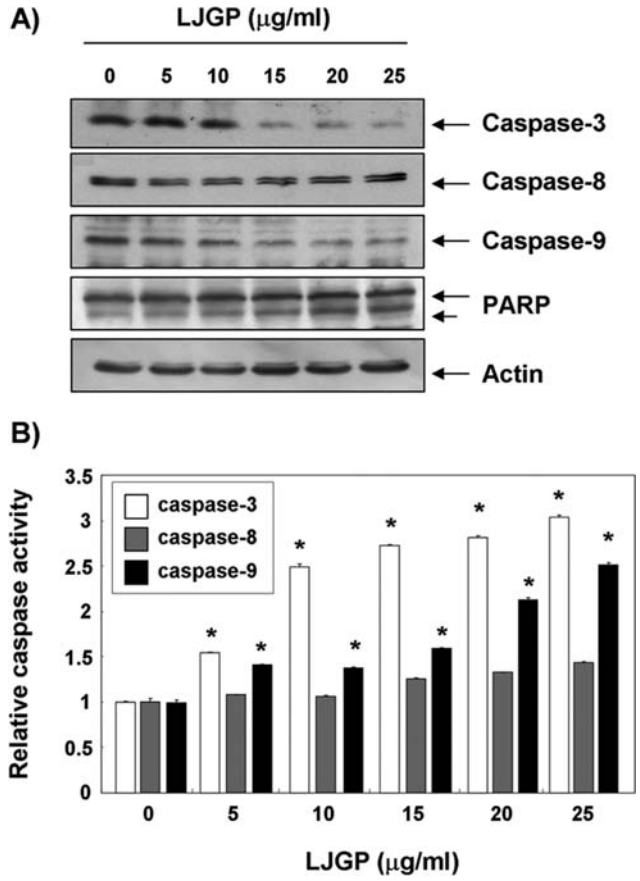

Figure 4. Activation of caspase-3 and -9, and degradation of PARP by LJGP in AGS cells. (A) Cells grown under the same conditions as in Fig. 3 were lysed, and cellular proteins were then separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control. (B) Following a 72-h incubation period with the indicated concentrations of LJGP, cells were lysed and aliquots (50 μg protein) were assayed for *in vitro* caspase-3, -8, and -9 activity using DEVD-pNA, IETD-pNA, and LEHD-pNA as substrates, respectively, at 37˚C for 1 h. Released fluorescent products were measured. Data represent relative mean values ± SE of three independent experiments. The significance was determined by the Student's t-test (*p<0.05, compared with control).

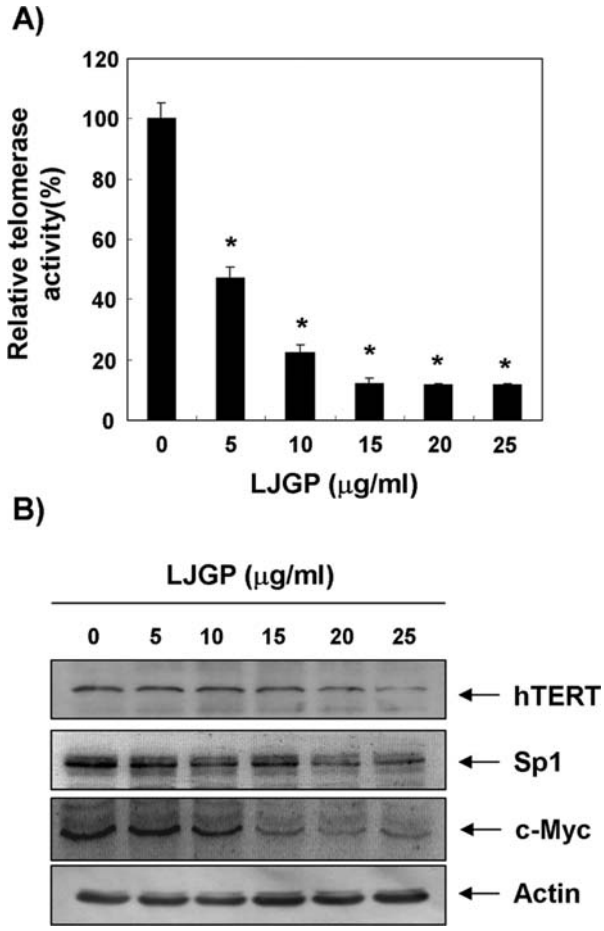

Figure 5. Inhibition of telomerase activity by LJGP treatment in AGS cells. (A) Following a 72-h period of incubation with LJGP, telomerase activity of AGS cells was measured using a TRAP-ELISA kit, as described in Materials and methods. Data represent relative mean values ± SE of three independent experiments. The significance was determined by the Student's t-test (*p<0.05, compared with control). (B) Cells grown under the same conditions as in (A) were collected, lysed, and cellular proteins were then separated by 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were probed with antibodies against hTERT, Sp1, and c-Myc. Proteins were visualized using an ECL detection system. Actin was used as an internal control.

Because Sp1 and c-Myc are important regulators of hTERT gene expression at the transcriptional levels (36), we also attempted to determine whether LJPG treatment could affect the levels of Sp1 and c-Myc expression. As indicated in Fig. 5B, LJPG treatment decreased Sp1 as well as c-Myc proteins in a concentration-dependent fashion (Fig. 5B), indicating that down-regulation of hTERT was connected with inhibition of Sp1 and c-Myc expression.

Inhibition of COX-2 expression and PGE_2 production by LJGP. We next attempted to determine whether LJPG-induced apoptotic effects of AGS cells were also connected with reduced expression of COXs and production of PGE_2. Western blot analyses showed a significant decrease in COX-2 protein expression over time after LJPG treatment in a concentration-dependent manner; however, LJPG was ineffective against COX-1 expression (Fig. 6A). To confirm that reduced PGE_2 production was associated with catalytic inactivation of the COX-2 isoform, AGS cells were cultured in the presence of LJPG, and PGE_2 levels were measured using EIA. As shown in Fig. 6B, synthesis of PGE_2 was concentration-dependent, and its production decreased significantly after LJGP treatment, which was well correlated with down-regulation of COX-2 expression.

Discussion

Although findings from a recent study have demonstrated that LJGP, a glycoprotein extracted from *L. japonica*, can suppress the growth of cultured human cancer cells *in vitro* (29), the signaling pathway by which this occurs has not been elucidated. The aim of the present study was to determine the capacity of LJGP to induce apoptosis, and to identify other biochemical mechanisms in a human gastric carcinoma AGS cell line. The present results clearly demonstrate that LJGP inhibits AGS cell growth by induction of apoptotic cell death, which appears to account for its anti-proliferating activity. Induction of apoptosis by LJGP was confirmed by

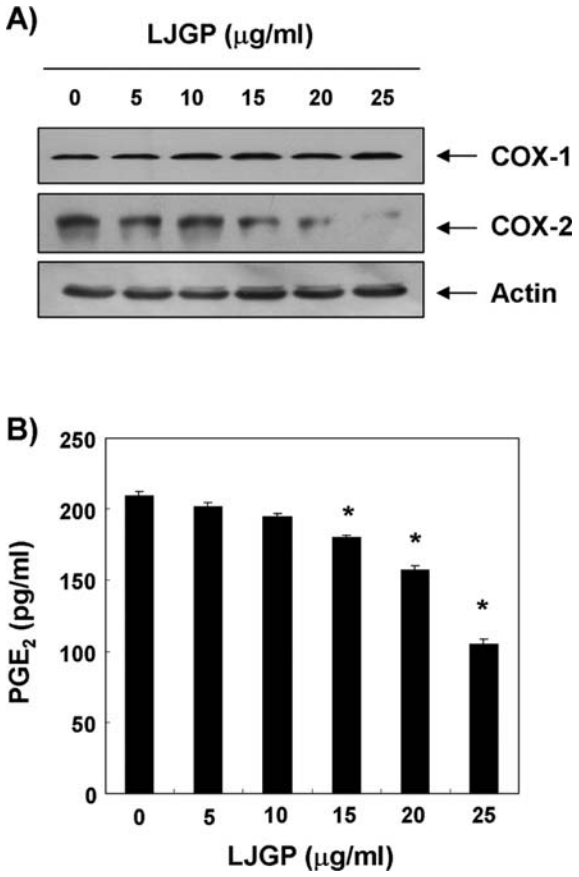

Figure 6. Down-regulation of COX-2 expression and inhibition of PGE_2 production by LJGP in AGS cells. (A) Following a 72-h incubation period with various concentrations of LJGP, cells were lysed, and cellular proteins were then separated by 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were probed with anti-COX-1 and anti-COX-2 antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control. (B) Cells grown under the same conditions as in (A) were collected and PGE_2 accumulation in the medium was determined using an EIA kit, as described in Materials and methods. Data represent relative mean values ± SE of three independent experiments. The significance was determined by the Student's t-test ($^*p<0.05$, compared with control).

characteristic morphological changes, chromatin condensation, and flow cytometry analysis (Fig. 2).

Apoptosis is a process of gene-mediated programmed cell death that can be triggered by various stimuli for elimination of unwanted cells in various biological systems; it is the key mechanism of chemotherapeutic agents (1,2). Apoptosis can be initiated via two canonical pathways: the intrinsic and the extrinsic pathway, which both ultimately activate the same effector caspases and apoptosis effector molecules. Several gene products have been demonstrated to be critical in regulation of apoptosis, and one of the major gene groups is the Bcl-2 family. Anti-apoptotic Bcl-2 proteins have been reported to protect against multiple signals that lead to cell death, whereas pro-apoptotic Bax proteins induce apoptosis. Bcl-2 inhibits *cytochrome c* translocation from the mitochodria to the cytoplasm, thereby blocking the caspase activation step of the mitochondrial/intrinsic apoptotic process (3,5). Thus, it has been suggested that the ratio between the levels of Bax and Bcl-2 determines whether or not a cell will respond to an apoptotic signal (37). The present data revealed a concentration-dependent increase of Bax expression in LJGP-treated AGS cells; however, the levels of Bcl-2 decreased, resulting in an increase in the ratio of Bax/Bcl-2 (Fig. 3).

Catalytic activity of the caspase family of proteins and levels of IAP family proteins were investigated in order to gain further mechanical insights into LJGP-induced apoptosis of AGS cells. Caspases are the primary executors of the apoptotic process in both the extrinsic and intrinsic apoptosis pathways. They belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive pro-forms or zymogens, for which signaling is initiated and propagated by proteolytic autocatalysis, as well as cleavage of downstream caspases and substrates (38,39). The death receptor pathway is initiated by binding of ligands to death receptors on the cell surface, which then activates caspase-8, followed by cleavage of Bid (tBid), which further activates a series of caspase cascades, resulting in apoptotic cell death (5). However, changes in mitochondrial integrity in response to a broad range of physical and chemical stimuli can trigger the intrinsic pathway of apoptosis (3). Once in the cytosol, *cytochrome c* can activate caspase-9, which in turn cleaves and activates the key executioner, caspase-3, which is either partially or totally responsible for proteolytic cleavage of many key proteins, including PARP, which is important for cell viability, but also serves as a marker of apoptosis when cleaved (33). IAP family proteins reportedly block apoptosis due to their function as direct inhibitors by binding to and inhibiting several caspases (34). Our data demonstrated that LJPG decreased the levels of IAP family proteins, such as XIAP and cIAP-1, and increased the enzymatic activity of the intrinsic caspase cascade, such as caspase-9, while the activity of the extrinsic caspase cascade, such as caspase-8, remained unchanged (Figs. 3 and 4). Further studies have shown that exposure of AGS cells to LJPG caused proteolytic cleavage and activation of caspase-3, a main executioner of apoptosis (Fig. 4) and the concomitant degradation of PARP, which plays a central role in the biological processing of apoptosis (33). Therefore, our results, which showed modulated levels of Bcl-2 and IAP family proteins, activation of caspase-3 and -9, and degradation of PARP suggest that a change in the intrinsic pathway may have contributed, at least in part, to LJPG-induced apoptosis of AGS cells.

Because telomerase activation is strongly suppressed in normal human somatic cells, but reactivated in immortal cells, upregulation of telomerase activity is crucial to the process of oncogenesis. These observations suggest that inhibition of telomerase activity may be a potential therapeutic modality for treatment of human cancers. The telomerase complex is composed of three major components, hTERT, telomerase RNA (TR), and telomerase-associated proteins (TEP1) (6,7). Recent studies have suggested that expression of the telomerase catalytic subunit gene hTERT mainly regulates expression of human telomerase enzymatic activity with posttranslational alterations and hTERT localization into the nucleus (35,36). The 200- to 400-bp region proximal to the transcription initiation site of the hTERT promoter is responsible for most of its transcriptional activity. Its binding sites include two typical E-boxes and several GC-boxes for the transcription factors c-Myc and Sp1, respectively (40,41).

c-Myc directly binds with the E-box and induces hTERT transcription and subsequent cell proliferation. The core promoter, which is necessary for hTERT expression, also contains Sp1/Sp3 binding sites. Sp1 works in conjunction with c-Myc to activate transcription of hTERT (36). In addition, overexpression of Bcl-2 in human cancer cells has been reported to result in increased telomerase activity and resistance to apoptosis (42) and overexpression of Bcl-2 could protect cells against apoptosis by telomerase inhibitors (43), indicating a link between Bcl-2 expression and telomerase activity in human cancer cells. Thus, it has been suggested that telomerase is a site of action before caspase is activated and mitochondria become dysfunctional. The present results showed that the increased apoptosis of AGS cells caused by LJPG was accompanied by significant inhibition of telomerase activity and hTERT expression (Fig. 5). Moreover, LJPG treatment was found to attenuate the levels of c-Myc and Sp1 expression. Although further studies are required in order to confirm the role of the transcriptional factors c-Myc and Sp1 in LJPG-induced suppression of telomerase activity, the present data suggest that LJPG treatment attenuates the activity of c-Myc and Sp1 to their recognition sites on the hTERT promoter, resulting in decreased hTERT transcription.

Recent data have suggested that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of cancer and that inhibition of carcinogenesis by NSAIDs is mediated through modulation of PG production from the substrate AA by rate-limiting enzymes known as COXs. PGs are lipid mediators with involvement in many normal physiological processes, and have been implicated in many pathological processes, including inflammation, edema, fever, hyperalgesia, cancer, and Alzheimer's disease (10,11). Conversion of AAs to PGs is catalyzed by two isoenzymes, COX-1 and COX-2. COX-1, which is regarded as the constitutively expressed form, is believed to serve in housekeeping functions; its expression does not fluctuate in response to stimuli. However, COX-2 is expressed at very low basal levels and is rapidly induced by different products, including tumor promoters, growth factor mitogens, reactive oxygen intermediates, and inflammatory cytokines (44,45). In a number of experimental studies, overexpression of COX-2 is sufficient to cause tumorigenesis and inhibition of the COX-2 pathway, resulting in a reduction in tumor incidence and progression (14,15). Although the exclusive role of COX-2 in tumor development and progression has yet not been fully elucidated, these observations suggest that COX-2 up-regulation is a key step in carcinogenesis, and inhibition of COX-2 activity promises to be an effective approach in prevention and treatment of cancer. Thus, we further investigated the question of whether LJPG-induced pro-apoptotic effects of AGS cells were associated with inhibition of COX-2 expression and activity. As shown in Fig. 6, LJPG treatment down-regulated expression of COX-2 in a concentration-dependent manner; however, the levels of COX-1 remained unaltered. Furthermore, LJPG also inhibited production of PGE_2, suggesting that inhibition of COX-2 expression and PGE_2 production is consistent with results showing that LJPG inhibited growth and induced apoptosis.

In the present study, we have suggested that the mechanism of LJPG, a glycoprotein Isolated from *L. japonica*, induced inhibition of human gastric carcinoma AGS cell growth with apoptosis induction through an increase in the Bax/Bcl-2 ratio, activation of caspase-3 and -9, and concomitantly caused a loss of telomerase activity and PGE_2 release by decreasing the expression of hTERT and COX-2, respectively. These novel phenomena have not been previously described and provide important new insights into the possible biological effects of LJPG. Although further studies are needed, the present data suggest that down-regulation of hTERT and COX-2 expression may be an attractive surrogate biomarker for assessment of the anti-tumor activity of LJPG.

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