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Phytoglycoprotein (38 kDa) Induces Cell Cycle (G_0/G_1) Arrest and Apoptosis in HepG2 Cells

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ABSTRACT

Styrax japonica Siebold et al Zuccarini (SJSZ) has been used to heal inflammation and bronchitis as folk medicine in Korea. Firstly, glycoprotein isolated from SJSZ (SJSZ glycoprotein) has a molecular weight with 38 kDa and consists of carbohydrate (57.64%) and protein (42.35%). In the composition of SJSZ glycoprotein, carbohydrate mostly consists of glucose (28.17%), galactose (21.85%), and mannose (2.62%) out of 52.64%, respectively. The protein consists of Trp (W, 7.01%), Pro (P, 6.72%), and Ile (I, 5.42%) out of 42.35% as three major amino acids, while total amount of other amino acids is 23.20%. The purpose of this study is to know whether the SJSZ glycoprotein (38 kDa) induces the cell cycle arrest and apoptosis in HepG2 cells. Cytotoxicity was evaluated using MTT and lactate dehydrogenase assay and amount of intracellular reactive oxygen species (iROS) and nitric oxide (NO) was measured using fluorescence microplate reader. Activities of cell cycle-related proteins [p53, p21, p27, Cyclin D1, and cyclin-dependent kinase (CDK)4] and apoptosis-related factors [iNOS, Bid, Bcl-2/bax, cytochrome *c*, caspase-9, caspase-3, and poly-(ADP-ribose) polymerase (PARP)] were assessed by Western blot and fluorescence-activated cell sorter (FACS) analysis. In the cell cycle-related proteins, SJSZ glycoprotein (50 μg/ml) significantly enhances the expression of p53, p21, and p27, whereas it suppressed the activity of cyclin D1/CDK4. In the apoptosis-related factors, SJSZ glycoprotein (50 μg/ml) stimulates to increase iROS, and NO, to activate iNOS, Bid, Bcl-2/bax, cytochrome *c*, caspase-9, caspase-3, and PARP. SJSZ glycoprotein (50 μg/ml) has potent effect to arrest cell cycle from G_0/G_1 to S and to induce apoptosis in HepG2 cells. J. Cell. Biochem. 112: 3129–3139, 2011.

KEY WORDS: SJSZ GLYCOPROTEIN (38 kDa); p27; p53; CYCLIND1/CDK4; CASPASE-3

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and particularly prevalent in Asia and Africa [Gomaa et al., 2000; Okuda, 2000]. It is a major health problem, accounting for approximately 6% of all human cancers and the third leading cause of mortality [Zender et al., 2008]. It is known to be a type of tumor highly resistant to available chemotherapeutic agents [Llovet et al., 1999]. The high prevalence and high death rate have spurred a search for novel strategies in the prevention and treatment of hepatic cancer.

Cancer is characterized by uncontrolled cell cycle progression and deregulation of apoptosis [Kaufmann and Earnshaw, 2000]. Therefore, induction of apoptotic cell death and inhibition of cell cycle progression is a promising emerging strategy for prevention and treatment of cancer [Hanahan and Weinberg, 2000]. Achieving cell cycle control is an important goal in the treatment of disease characterized by uncontrolled cell cycle progression, such as cancer. Many cytotoxic agents and DNA damaging agents have been reported to arrest a normal cell cycle in G_0/G_1, S, or G_2/M phase [Orren et al., 1997]. Activity of cyclin-dependent kinases (CDKs) and cyclin regulatory subunits are one of most important factors that are responsible for orderly progression in the cell cycle [Collins et al., 1997]. For instance, transition through the G_1 phase into the S phase in the cell cycle phase is required to bind and activate cyclin/CDK complex, especially cyclin D1/CDK4. Transcription factor (p53) is known to protect the cells from possible accumulation of damaged DNA, because it permits the halting of the cell cycle to allow for DNA repair in normal cells [Marchetti et al., 2004]. Also, p21 and p27, which are induced by p53, are member of cell cycle inhibitors (CKIs) and are able to inhibit, CDKs, therein arresting the transition from G_0/G_1 in to the S phase [Sherr and Roberts, 1995; Sohn et al., 2006].

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Apoptosis, or programmed cell death, is an essential mechanism to eliminate selectively unnecessary cells [Kerr et al., 1994]. It has two main signaling pathways involving extrinsic and intrinsic pathway. Especially, the mitochondria play a central role in apoptosis occurrence resulting from many chemical or chemotherapeutic agents [Robertson and Orrenius, 2002]. The mitochondria-dependent apoptotic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial protein, such as Bcl-2, bak, and cytochrome *c*. The Bcl-2 family is separated into two members that either inhibit apoptosis (anti-apoptosis proteins, Bcl-2) or promote apoptosis (pro-apoptosis proteins, Bax and Bid). Pro-apoptotic members of the Bcl-2 family induce apoptosis through the release of cytochrome *c* [Ott et al., 2009]. Once cytochrome *c* is the cytosol, cytochrome *c* together with apoptosis protease activating factor (Apaf)-1 activates caspase-9, and the latter then activates caspase-3 [Desagher and Martinou, 2000]. Poly-(ADP-ribose) polymerase (PARP) is a nuclear enzyme selectively activated by DNA strand damage to participate in DNA repair [Alano et al., 2010].

Natural products have played an important role in drug discovery. The use of herbal medicines as alternative medicines has become increasingly popular in Asia and throughout the world. In particular, the use of anti-cancer agents derived from natural products provides a novel opportunity to improve the existing standard of care for HCC and other cancer [Newman, 2008]. *Styrax japonica* Siebold et al Zuccarini (Styracaceae, SJSZ) has been used to heal sore throat, cough, odontalgia, and paralysis as folk medicine in Korea. Additionally, its pericarps are used at making soap and piscicide agent, because it contains egosaponin as one of major components [Kim, 1998; Yoshikawa et al., 2000]. It has been reported that triterpenoid which is isolated from stem and leaves of SJSZ has an activity of anti-inflammation and inhibits proliferation of HL-60 (human leukemic) cells. Recently, we isolated a glycoprotein with an approximate molecular mass of 38 kDa from SJSZ. The glycoprotein has a carbohydrate and protein content of 57.65% and 42.35%, respectively. However, nobody has studied anti-tumoric effect of glycoprotein from fruits of SJSZ in human liver cancer cell.

Therefore, aim of this study is whether SJSZ glycoprotein induces programmed cell death in HepG2 cells. To confirm apoptotic activity of SJSZ glycoprotein, intracellular ROS, activity of Bid and cytochrome *c*, and apoptosis-mediated proteins (caspase-3 and PARP) were evaluated. Moreover, to know cell cycle arrest activity of SJSZ glycoprotein, cyclin-dependent kinase inhibitors (p53, p21, and p27), cyclin D1/CDK4 complex and cell cycle were examined.

MATERIALS AND METHODS

CHEMICALS

All the plastic materials were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ). Penicillin G and streptomycin were obtained from Sigma (St Louis, MO). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). Other chemicals and reagents were of the highest analytical grade available.

PREPARATION OF GLYCOPROTEIN

The SJSZ glycoprotein was isolated and purified from SJSZ fruit, as follows. Briefly, fruits of SJSZ were harvested in July 2009 from Moodeung mountain in the Chonnam province, South Korea. They were identified by Dr. H.T. Lim (Chonnam National University). A voucher specimen (No. LKT0100722) was deposited at Molecular Biochemistry Laboratory, Chonnam National University, Gwangju, Korea. SJSZ (12 kg) was chopped into small pieces and soaked in ethanol (11 L, w/v) for 3 months in a dark basement. The ethanol extract was passed through Whatman filter paper (No. 2) to remove debris and concentrated using a rotary evaporator (B465; Buchi, Flawil, Switzerland). The concentrated solution was freeze-dried (SFDS06; Samwon, Seoul, Korea). The dried powder (2800 g) was dissolved again in distilled water. The solution was subjected to concanavalin A-sepharose 4B affinity chromatography (24–45 mm, Sigma, C9017) and eluted with 0.5 M methyl α-D-glucopyranoside containing 0.5 M NaCl at pH 7.4. The eluted solution was dialyzed with a dialysis membrane (Spectra/por, MWCO 8,000–12,000, CA) against 20 mM Tris–Cl (pH 7.4) at 4°C overnight and lyophilized. The yield of SJSZ glycoprotein from starting crude materials was 21.0 g (0.75%). To confirm glycoprotein, we performed SDS–PAGE of a sample of protein (25–50 mg/ml) containing 0.1% SDS, using a 15% polyacrylamide mini-gel and a Mini-PROTEIN II electrophoresis cell (Bio-Rad) at 110 V and 30 mA for 2.5 h. The SJSZ glycoprotein was confirmed by staining with Schiff's reagent [Neville and Glossmann, 1974], which is a specific staining reagent for the glycoprotein detected through a redox reaction. The purity of the SJSZ glycoprotein is approximately more than 98%. The sample of glycoprotein on the gel has revealed one band with 38 kDa and consists of carbohydrate content (52.64%) [Masuko et al., 2005] and protein content (47.36%) [Lowry et al., 1951].

COMPOSITION OF CARBOHYDRATE AND AMINO ACID IN SJSZ GLYCOPROTEIN

For composition of carbohydrate analysis, the hexose content of the purified sample was measured according to method of Chaplin [1994] slightly modified. A sample (25 pmol of a glycoprotein) was placed in a hydrolysis tube fitted with a Teflonlined screw cap. To the sample was added 50 μl of 2 M/L HCl-2 M/L trifluoroacetic acid (TFA) and heated at 100°C for 6 h. The neutral sugars (fucose, galactose, glucose, and mannose) and amino sugars (glucosamine and galactosamine) were separated and quantified on a Carbopac PA10 column (4.5 × 250 nm) with a Bio-LC ICS5000 (Dionnex Co., Sunnyvale, CA) using the Carbopac PAI cartridge (4.5 × 50 nm).

For composition of amino acid analysis, amino acid analysis of the isolated glycoprotein was performed using PICO.TAG amino acid system according to PICO.TAG operation manual (Waters, USA). Dialysed and dried SJSZ glycoprotein (50 μg) was hydrolyzed by 6 N HCI containing 5% thioglycollic acid [Matsubara and Sasaki, 1969] for 24 h at 105°C in the PICO.TAG workstation. Hydrolysed sample and standard amino acid mixture, standard A (0.005 ml) was taken in respective tubes (vials) and was dried completely. These were then derivatized [Ghosh et al., 1997] by phenyl isothiocyanate (PITC) solution (ethanol: triethyl acetate: water: PITC: 7:1:1:1 by volume) for 20 min at 25°C in a nitrogen atmosphere. The vials were then dried and the samples were reconstituted in a diluents solution

(Na_2HPO_4, 0.071% w/v in distilled water, pH 7.4; pH was adjusted by 10% H3PO4 containing 5% v/v acetonitrile. The samples were analyzed by HPLC at 38°C as per the PICO.TAC manual using a PICO.TAG C18 hydrophobic column (5 μm, 3.9 × 150 mm), waters, and detection at 254 nm. Amino acids present in the unknown sample were determined quantitatively by comparing the peak area (754 B data module print out) of amino acids present in standard A.

CELL CULTURE AND CYTOTOXICITY

HepG2 cells (human hepatoma cell line) were incubated in DMEM containing 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C and atmosphere containing 5% CO_2. The medium was renewed two times per week. The cells (1×10^6 cells/ml) were distributed into 35 mm culture dishes or 96-well flat bottom plates. The final volumes were 2 ml/dish on the 35 mm culture dishes and 100 μl/well on the 96-well flat bottom plates. Cell viability for the cytotoxicity of the SJSZ glycoprotein was determined by MTT assay, as reported previously [Mosmann, 1983]. Briefly, the cells were treated with various concentration of SJSZ glycoprotein for 12 h. After incubation, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) stock solution (5 mg/ml) was added into each well, and the plates were incubated at 37°C in 5% CO_2 atmosphere for 4 h. Acidic isopropanol was then added into 96-well multiplates, and the plates were read at 570 nm using a SpectraCount™(Packard Instrument Co Downers, IL). The cellular cytotoxicity was also determined by assessing the release of lactate dehydrogenase (LDH) into the medium according to the method of Bergmeyer and Bernt [1974]. Cells were treated with various concentration of SJSZ for 12 h. After incubation, the culture medium (35 μl) was mixed with reaction mixture containing 0.6 mM pyruvate in 48 mM potassium phosphate buffer (pH 7.5), and the final volume of the reaction mixture was brought up to 3.15 ml. The reaction was initiated by the addition of 0.18 mM β-NADH (reduced nicotinamide adenine dinucleotide) and LDH activity was measured as the rate of loss of β-NADH absorption at 340 nm for 2 min. The cells were treated with Triton X-100 (1%) for 30 min and centrifuged at 1,000*g* for 10 min. The resulting supernatants were added to the reaction mixtures and LDH assays were carried out according to the method of Bergmeyer and Bernt [1974]. In this experiment, Triton X-100 (1%) treatment used as internal control in LDH assay. LDH released into culture supernatant was calculated by comparison with Triton X-100 treatment (1%) and represented in relative percentage compared to the Triton X-100.

PROPIDIUM IODIDE STAINING OF APOPTOTIC CELLS

The programmed death of cells (apoptosis) was determined by the propidium iodide (PI) staining technique. The HepG2 cells were seeded on cover slips and placed in petriplates at a concentration of approximately 5×10^5 cell/plate. The plates were incubated in CO_2 incubator for 48 h to obtain sheet of cells on the coverslips. After incubation the media was removed and coverslips were gently washed with phosphate buffered saline (PBS). Before the addition of SJSZ glycoprotein, it was mixed with PBS and then slowly added to the plates until the coverslips were fully immersed. The plates were incubated for 12 h in CO_2 incubator. After incubation the coverslips were fixed with ethanol fixation and then stained with 100 μl of PI and kept undisturbed for few minutes. After that the apoptotic cells were identified by using inverted fluorescent microscope [Idziorek et al., 1995]. At least, three hundred cells were counted and the number of fragmented nuclei, increased cytoplasm, and condensed chromatin reliably indicated that apoptosis had occurred. The percentage of apoptotic cells was calculated using the following formula:

$$\text{Apoptosis } \% = (\text{total number of cells with apoptotic nuclei} / \text{total number of cells}) \times 100$$

Experiments were performed three times and each value represents the mean ± SE of triplicate experiments.

PREPARATION OF CELL EXTRACTS

Preparation of cell extract was basically as described elsewhere [Lee and Lim, 2006]. Briefly, the cells were treated with SJSZ glycoprotein (10, 25, and 50 μg/ml) for the indicated times. Then cells were washed in 1 ml of ice-cold PBS after the medium was removed, and scraped into 300 μl of ice-cold buffer A (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40, 25% glycerol) containing a phosphatase inhibitor cocktail. For lysis, the cells were freeze-thawed, shaken for 30 min at 4°C, and centrifuged at 14,000*g* for 30 min. The supernatant was designated as a whole extract for Western blotting of iNOS, Bid, Bcl-2/bax, caspase-9, caspase-3, PARP, p53, p21, and p27.

To prepare nuclear extracts for immunoblotting of cyclin D1 and CDK4 after cell activation for the times indicated, cells were resuspended in 400 μl of ice-cold hypotonic buffer B (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40), left on 15 min and then centrifuged at 3,000*g* for 5 min at 4°C. Pelleted nuclei were gently resuspended in 150 μl of NE buffer C (20 mM HEPES, pH 7.5, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40, 25% glycerol) containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany) and centrifuged at 12,000*g* for 15 min at 4°C.

To prepare mitochondria and cytosolic extracts for Western blotting of cytochrome *c*, after cell activation for the times indicated, cells were rinsed twice with PBS after replacement of the medium. Cell pellets were re-suspended in 300 μg/ml of buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 250 mM sucrose) containing a protease inhibitor cocktail. The cells were homogenized by 10 strokes in a Dounce homogeniter (B. Braun, Melsungen, Germany). To collect nuclei and debris, the homogenates were centrifuged twice at 750*g* for 5 min at 4°C. The supernatants were centrifuged at 10,000*g* for 15 min at 4°C, to collect mitochondria-enriched heavy membrane pellets. The resulting supernatants were centrifuged at 100,000*g* for 1 h at 4°C and the final supernatants are referred to as cytosolic fractions. The amount of protein was measured by the method of Lowry [Lowry et al., 1951], and the cellular proteins were stored at −70°C prior to use.

IMMUNOBLOT ANALYSIS

Cellular proteins were separated in a 10% polyacrylamide mini-gel at 100 V for 2 h at room temperature using a mini-protein II electrophoresis cell (Bio-Rad). After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Millipore, Bedford, MA). The membranes were incubated for 1 h at room temperature in TBS-T solution (10 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.1% (v/v) Tween-20) containing 5% non-fat dry milk. The membranes were subsequently incubated for 2 h at room temperature with primary antibodies (p53, p21, p27, cyclin D1, CDK4, iNOS, Bid, Bcl-2/bax, cytochrome *c*, caspase-9, caspase-3, PARP, and α-tubulin) in TBS-T solution. After washing three times with TBS-T, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG (1:10,000; Cell Signaling Technology, MA) in TBS-T solution. The resulting protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK). The results of immunoblot assay were calculated as relative intensity using Scion imaging software (Scion Image Beta 4.02, Frederick, MD).

FLUORESCENCE-ACTIVATED CELL SORTER (FACS) ANALYSIS

To find out the reason which SJSZ glycoprotein kills HepG2 cells, number of cell in G_0/G_1 and S phase was determined after staining with PI using FACS analysis [Sreeja and Sreeja, 2009]. Briefly, the cells were treated with SJSZ glycoprotein (10, 25, and 50 μg/ml) for 12 h. The cells were then dissociated in trypsin/EDTA, pelleted by centrifugation, and resuspended at approximately 1×10^5 cells/ml in PBS containing 0.1% BSA. Subsequently, the cells were fixed in 70% ice-cold ethanol, followed by incubation in freshly prepared nuclei staining buffer for 30 min at 37°C. Cell cycle histograms were generated after analyzing the PI-stained cells by FACS (Beckman Coulter, CA). At least 3×10^4 events were recorded for each sample. The samples were analyzed using CXP software (Beckman Coulter, Fullerton, CA).

MEASUREMENT OF INTRACELLULAR ROS

The intracellular ROS were measured using a non-fluorescent dye, 2′,7′-dichlorodihydrofluorescein (H_2DCF-DA), which is a membrane permeable fluorigenic tracer that is oxidized by various species of ROS. The dye is deacetylated by intracellular esterases to non-fluorescent 2′,7′-dichlorohydrofluorescein (DCFH), which is oxidized to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) by ROS. The cells were pre-incubated with 10 μM H_2DCF-DA for 30 min at 37°C, and then washed twice with PBS to remove the extracellular H_2DCF-DA. After that, the cells were treated with SJSZ glycoprotein (10, 25 and 50 μg/ml) for 3 h. Finally, the intensity of fluorescence was measured at an excitation of 485 nm and an emission of 530 nm using a fluorescence microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Corporation, Sunnyvale, CA). The values were calculated as the relative intensity of DCF fluorescence, compared with the control.

MEASUREMENT OF NITRIC OXIDE

The amount of nitrite, a stable oxidized product of nitric oxide (NO), was measured in cell culture media using the Griess method [Green et al., 1982]. In brief, after treatment for 3 h, cells were centrifuged at 1,000*g* for 10 min and supernatants were collected for determination of NO production. One hundred microliters of cell culture supernatant was combined with 100 μl of Griess reagent [mixture of equal volume of 1% sulfanilamide in 2.5% H_3PO_4 and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in dH_2O] in 96-well plates and the mixture was reacted for 10 min at room temperature. The absorbance was measured at 540 nm with a MicroReader (Hyperion, Inc., USA) and the nitrite concentration was assessed using a standard curve of $NaNO_2$.

STATISTICAL ANALYSIS

All experiments were carried out in triplicate experiments separately. All data were represented as means ± SE. One-way analysis of variance and Duncan test were carried out to determine significant differences of multiple comparisons (Statistical Package of the Social Sciences program, version 11.0).

RESULTS

SJSZ GLYCOPROTEIN

As shown in Figure 1 A, the gel was stained with Schiff's reagent for the glycoprotein isolated from SJSZ fruit in accordance with the method of Neville and Glossmann [1974]. The results showed that the SJSZ glycoprotein was detected as a band with a molecular weight of 18% SDS–PAGE (Fig. 1). In addition, the total carbohydrate and protein moieties of the SJSZ glycoprotein were 52.64% and 47.35%, respectively. As shown in Figure 1 B,C, the carbohydrate mostly consists of glucose (28.17%), galactose (21.85%), and mannose (2.62%) out of 52.64%, respectively. the protein consists of Trp (W, 7.01%), Pro (P, 6.72%), and Ile (I, 5.42%) out of 42.35% as three major amino acids, while total amount of other amino acids is 23.20%.

EFFECT OF SJSZ GLYCOPROTEIN ON CYTOTOXICITY IN HEPG2 CELLS

To determine whether SJSZ glycoprotein has cytotoxicity effect, it carried out MTT and LDH assay in SJSZ glycoprotein-treated HepG2 cells. As shown in Figure 2A, when HepG2 cells were exposed to various concentrations of SJSZ glycoprotein and for suitable incubation time, the cellular cytotoxicities were significantly increased. In dose-dependent manner, the viability values were 98.16, 76.46, and 45.26% at 10, 20 and 50 μg/ml SJSZ glycoprotein for 12 h, respectively (Fig. 2A). In time-dependent manner, the viability values were 61.25, 49.18, and 39.85% for 2, 4 and 12 h at 50 μg/ml SJSZ glycoprotein, respectively (Fig. 2B). As shown in Figure 2C,D, SJSZ glycoprotein increased cytotoxicity in LDH assay in dose- and time-dependently. When the cells were treated with 50 μg/ml of SJSZ glycoprotein for 12 h, the results of cytotoxicity indicated IC_{50} compared to the control in the MTT assay and LDH assay.

APOPTOSIS-INDUCING EFFECT OF SJSZ GLYCOPROTEIN

As shown in Table I, inducing effect of SJSZ glycoprotein on apoptosis in nuclear staining with PI was observed. For instance, when the HepG2 cells were treated with 10, 25, 50, 100,

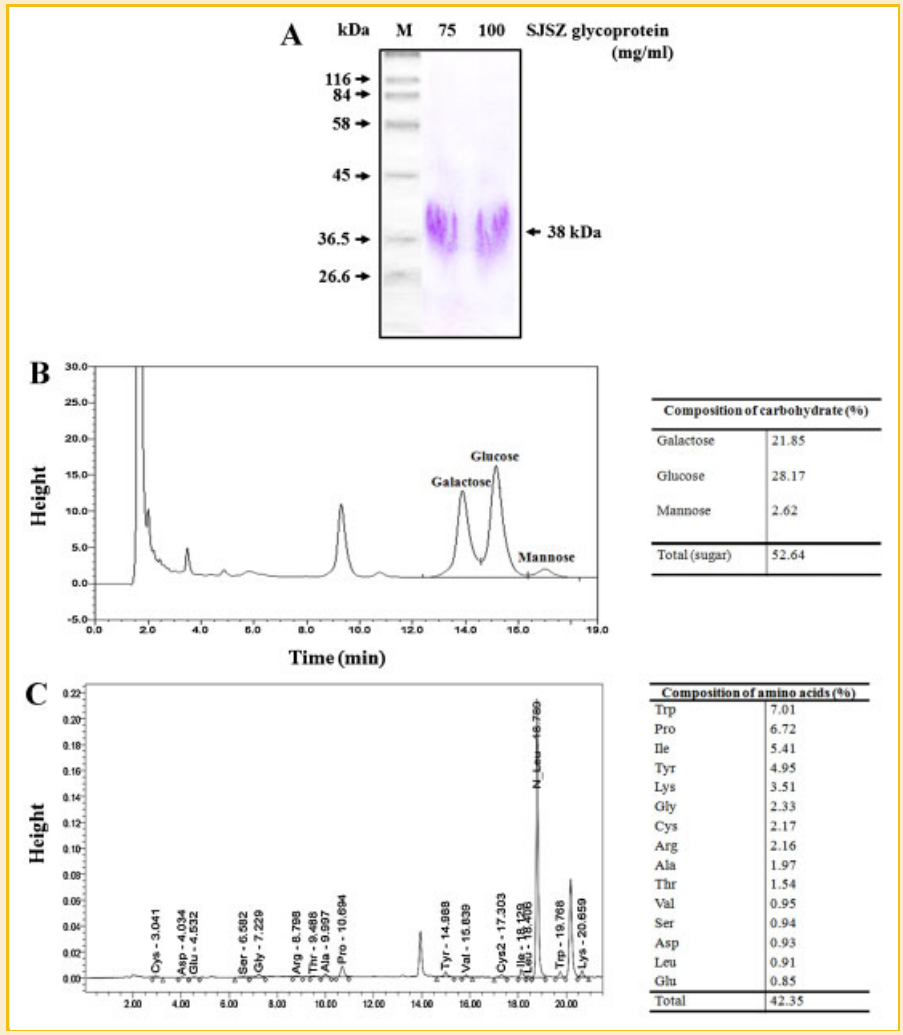

Fig. 1. Characterization of SJSZ glycoprotein. Electrophoresis was performed using 18% acrylamide gel containing 0.1% SDS. The gels were stained with Schiff's reagent for glycoprotein staining in accordance with the method of Neville and Glossmann [1974]. Lane 1, pre-stained molecular weight as marker; lane 2, 75 mg/ml; and lane 3, 100 mg/ml SJSZ glycoprotein (A). Composition of carbohydrate (B) and amino acids (C) in the whole molecular of SJSZ glycoprotein. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

and 150 μ/ml SJSZ glycoprotein, 20%, 46%, 58%, 76%, and 95% of cells apopotic, respectively, compared to the control cells.

EFFECT OF SJSZ GLYCOPROTEIN ON EXPRESSION OF P53, P27, AND P21 IN HEPG2 CELLS

The results obtained in this set of experiments (Fig. 3) suggest that SJSZ glycoprotein is able to induce the expression of p53, p21, and p27 in HepG2 cells. For example, the expression of p53, p21, and p27 was increased by 2.76-, 2.76-, and 3.10-fold, respectively, following exposure to SJSZ glycoprotein (50 μg/ml), compared to the control.

EFFECT OF SJSZ GLYCOPROTEIN ON EXPRESSION OF CYCLIN D1 AND CDK4 IN HEPG2 CELLS

The results obtained in this set of experiments (Fig. 4) suggest that SJSZ glycoprotein is able to induce the expression of cyclin D1 and CDK4 in HepG2 cells. For example, the expression of cyclin D1 and CDK4 was decreased by 0.10- and 0.12-fold, respectively, following exposure to SJSZ glycoprotein (50 μg/ml), compared to the control.

EFFECT OF SJSZ GLYCOPROTEIN IN CELL CYCLE ARREST IN HEPG2 CELLS

To investigate whether SJSZ glycoprotein has induction of effect on the molecular mechanisms underlying cell cycle arrest, we evaluated cell cycle analysis using FACS analysis. When cells treated with SJSZ glycoprotein, its G_0/G_1 phase gradually increased and S phase and G_2/M phase decreased compared to the control, dose-dependently. For example, in $G_0/G1$ phase, it increased 53.19 and 58.95% in 25 and 50 μg/ml compared to the control, whereas in S phase, it decreased 32.19 and 34.93% in 25 and 50 μg/ml compared to the control.

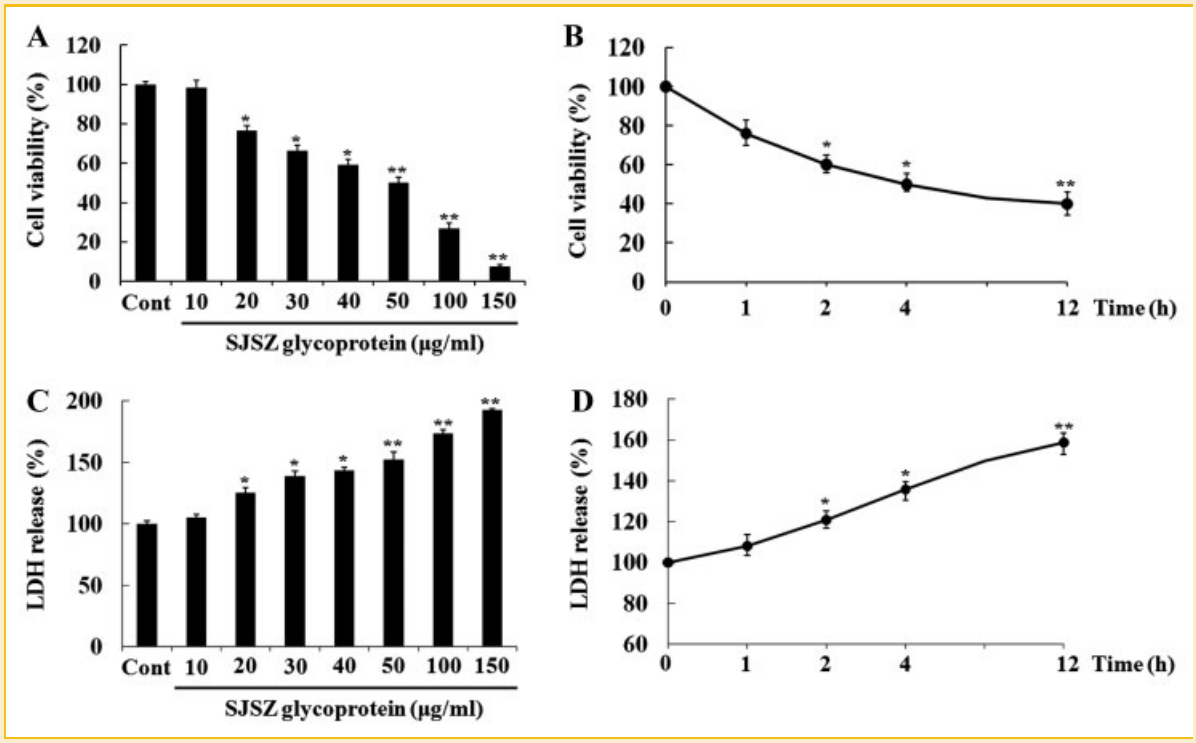

Fig. 2. Effect of SJSZ glycoprotein on cytotoxicity in HepG2 cells. HepG2 cells were treated with SJSZ glycoprotein (various concentrations, 12 h) in MTT assay (A,B) and LDH assay (C,D). All data represent the means ± SE from triplicate separately. Significant difference between control and treatment of SJSZ glycoprotein, $^{*}P < 0.05$ and $^{**}P < 0.01$.

EFFECT OF SJSZ GLYCOPROTEIN ON PRODUCTION OF INTRACELLULAR ROS, NO, AND EXPRESSION OF INOS IN HEPG2 CELLS

As shown in Figure 6A,B, when the HepG2 cells were treated with SJSZ glycoprotein for 3 h, the relative amounts of intracellular ROS and NO increased compared to the control, dose-dependently. For example, the values of intracellular ROS and NO were increased by 1.56-, and 2.61-fold at SJSZ glycoprotein (50 μg/ml), compared to the control. The results obtained in this set of experiments (Fig. 6C) suggest that SJSZ glycoprotein is able to induce the expression of iNOS in HepG2 cells. For example, the expression of iNOS was increased by 2.06-, 4.52-, and 5.42-fold at 10, 25, and 50 μg/ml SJSZ glycoprotein, compared to the control.

TABLE I. Apoptosis-Inducing Effect of SJSZ Glycoprotein on HepG2 cells (nuclei staining)

Treatment	Number of apoptotic cells (%)
Control	0 + 5 (0)
SJSZ glycoprotein (10 μg/ml)	60 ± 7 (20)
SJSZ glycoprotein (25 μg/ml)	138 ± 5* (46)
SJSZ glycoprotein (50 μg/ml)	176 ± 3* (58)
SJSZ glycoprotein (100 μg/ml)	228 ± 6* (76)
SJSZ glycoprotein (150 μg/ml)	287 ± 2* (95)

Serum-starved HepG2 cells (5×10^5 cells/ml) were treated with various concentrations (10, 25, 50, 100, and 150 μg/ml) of SJSZ glycoprotein for 12 h. The apoptosis-inducing effect of SJSZ glycoprotein were evaluated by PI staining. The values presented are means ± SE (n = 3).
$^{*}P < 0.05$, significant difference between SJSZ glycoprotein treatments.

EFFECT OF SJSZ GLYCOPROTEIN ON APOPTOSIS-RELATED PROTEIN (INOS, BID, BCL-2/BAX, CYTOCHROME C, CASPASE-9, CASPASE-3, AND PARP) IN HEPG2 CELLS

To determine whether SJSZ glycoprotein induces activation of Bid in HepG2 cells, the activity of Bid was determined using Western blot analysis. As shown in Figure 7A, when the HepG2 cells were treated with SJSZ glycoprotein (10, 25, and 50 μg/ml) for 2 h, the relative activity of Bid was decreased dose-dependently, compared to the control. Indeed, the relative band intensities of Bid were significantly decreased by 0.91-, 0.84-, and 0.83-fold at treatment 10, 25, and 50 μg/ml SJSZ glycoprotein compared to the control, respectively. However, tBid was shown opposite tendency. The relative activity of Bcl-2 was decreased dose-dependently, compared to the control. Indeed, the relative band intensities of Bid were significantly decreased by 0.83-, 0.30-, and 0.23-fold at treatment 10, 25, and 50 μg/ml SJSZ glycoprotein compared to the control, respectively. However, Bax was shown opposite tendency.

As shown in Figure 7B, when the HepG2 cells were treated with SJSZ glycoprotein (10, 25, and 50 μg/ml), the activity of cytochrome *c* was gradually decreased in mitochondria fraction at 2 h, while the activity of cytochrome *c* was gradually increased in cytosolic fraction, compared to the control. That is, the relative band densities of cytochrome *c* in mitochondria fraction were diminished by 0.49-, 0.12-, and 0.12-fold at 10, 25, and 50 μg/ml of SJSZ glycoprotein, respectively. In the cytosolic fraction, activity of cytochrome *c* was shown opposite tendency.

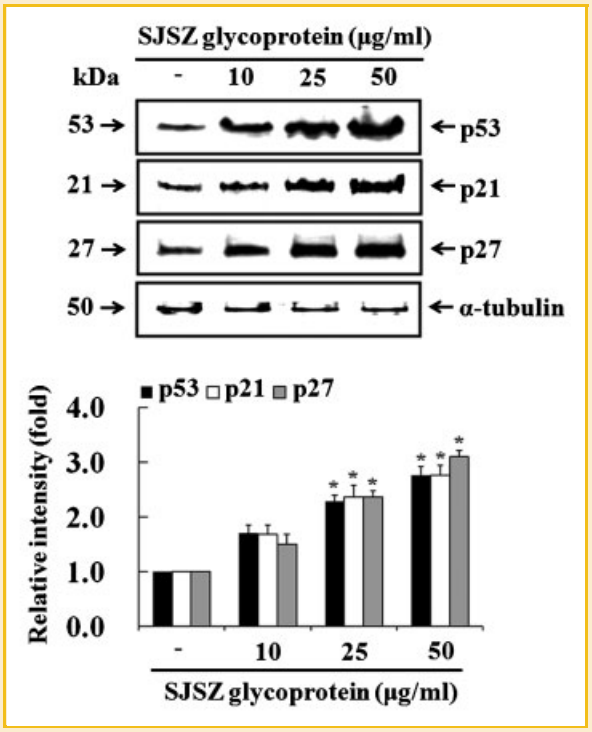

Fig. 3. Effect of SJSZ glycoprotein in activity of CKI proteins (p53, p21, and p27) HepG2 cells were treated with SJSZ glycoprotein (10, 25, and 50 μg/ml) for 4 h. The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02, MD). All data represent the means ± SE from triplicate separately. Significant difference between control and treatment of SJSZ glycoprotein, $^{*}P < 0.05$. Lane 1, control; lane 2, 10 μg/ml SJSZ glycoprotein; lane 3, 25 μg/ml SJSZ glycoprotein; lane 4, 50 μg/ml SJSZ glycoprotein. α-tubulin was used as an internal control.

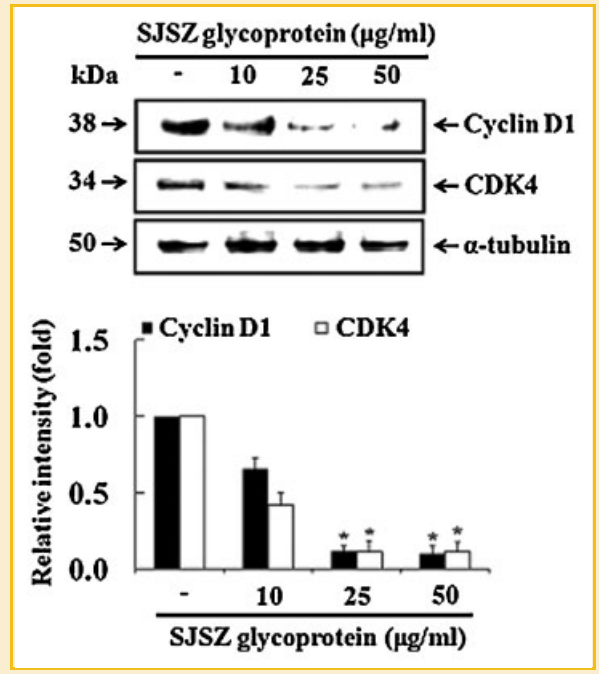

Fig. 4. Effect of SJSZ glycoprotein on expression of cyclin D1 and CDK4 in HepG2 cells HepG2 cells were treated with SJSZ glycoprotein (10, 25, and 50 μg/ml) for 4 h. The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02, MD). All data represent the means ± SE from triplicate separately. Significant difference between control and treatment of SJSZ glycoprotein, $^{*}P < 0.05$. Lane 1, control; lane 2, 10 μg/ml SJSZ glycoprotein; lane 3, 25 μg/ml SJSZ glycoprotein; lane 4, 50 μg/ml SJSZ glycoprotein. α-tubulin was used as an internal control.

When the cells were exposed to SJSZ glycoprotein (10, 25, and 50 μg/ml) for 4 h, caspase-3 and PARP proteins in whole-cell extract fractions were cleaved in a dose-dependent manner (Fig. 7C). After treatment of 50 μg/ml of SJSZ glycoprotein for 4 h, the relative intensities of pro-forms were significantly decreased by 0.07-, 0.05-, and 0.12-fold at pro-caspase-9, pro-caseapse-3 and PARP compared to the control, respectively. However, when HepG2 cells treated with SJSZ glycoprotein (10, 25, and 50 μg/ml), cleaved forms of apoptotic proteins were strongly detected and relative intensities of apoptotic proteins were significantly increased dose-dependently, compared to the control.

DISCUSSION

Evasion of apoptosis is a major cancer hallmark and major target for therapeutic strategies [Abbott et al., 2006]. Recently evidence has suggested that apoptosis should be an underlying mechanism by which various anticancer and chemopreventive agents exert anticancer effects, especially from natural compounds [Sandur et al., 2006; Park et al., 2008]. As shown in Figure 1, it showed just one band with 38 kDa as molecular weight on the gel. The band indicated in the broadly and smear form for typical glycoprotein at the staining with Schiff's reagent. In SJSZ glycoprotein, the main carbohydrate compositions in SJSZ protein seem to be glucose and galactose, but not mannose, while the composition of amino acid consists of hydrophobic amino acid dominantly.

Firstly, we determined cytotoxicity of the SJSZ glycoprotein in the HepG2 cells using MTT and LDH assay. The results of cytotoxicity of SJSZ glycoprotein indicated that IC_{50} was 50 μg/ml and treated for 12 h in Figure 2. Moreover, such apoptotic activity of SJSZ glycoprotein was shown in nuclei stained with PI. Generally, the number of apoptotic cells was significantly increased after the addition of SJSZ glycoprotein (Table I). From the result, we speculated that SJSZ glycoprotein has a potential activity of apoptosis in HepG2 cells. In present study, although we have not determined the interaction between SJSZ glycoprotein and death ligands/death receptors, we evaluated that SJSZ glycoprotein activates cell cycle arrest and mitochondria-mediated apoptosis pathway.

Accumulating evidences indicated that chemotherapeutic agents induce tumor regression through activation of cell cycle arrest and/or apoptosis. Regulation of proteins that mediate critical events of the cell cycle may be a useful anti-tumor target [Stewart et al., 2003]. It is well known that there are the cell cycle-related proteins for arresting G_0/G_1 and S phase such as p53, p21, p27, cyclines, and

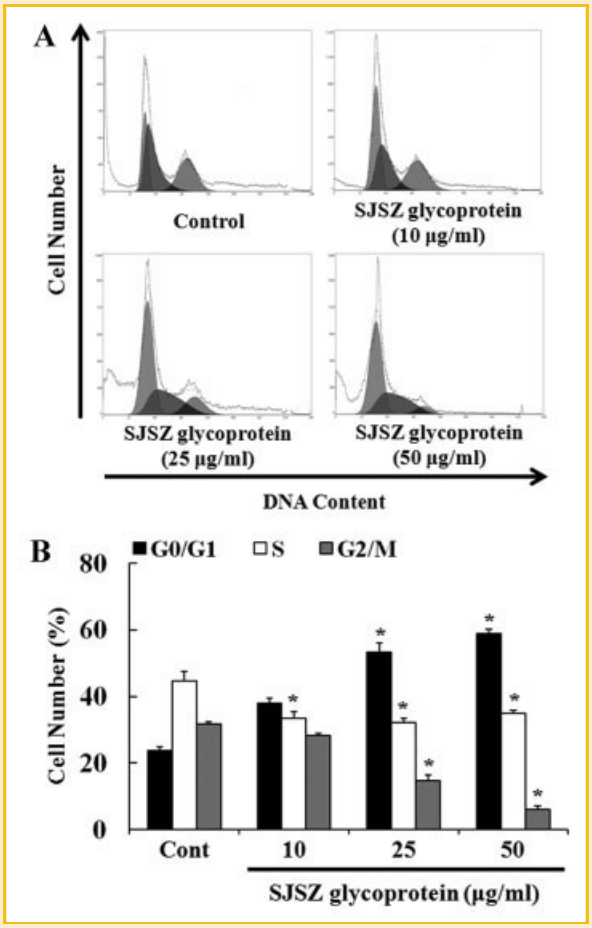

Fig. 5. Effect of SJSZ glycoprotein in cell cycle arrest in HepG2 cells. After HepG2 cells were harvested afterward treatment with SJSZ glycoprotein for indicated time, cell cycle analysis was carried out using flow cytometry (FACS) staining with PI. Distribution histogram of nuclear DNA detected by fluocytometry was indicated according to cellular cycle phase (A). The bar graph represents the percentage of cell number in the G0/G1 or S phase (B). All data represent the means ± SE from triplicate separately. Significant difference between control and the SJSZ glycoprotein treatment, $^{*}P < 0.05$.

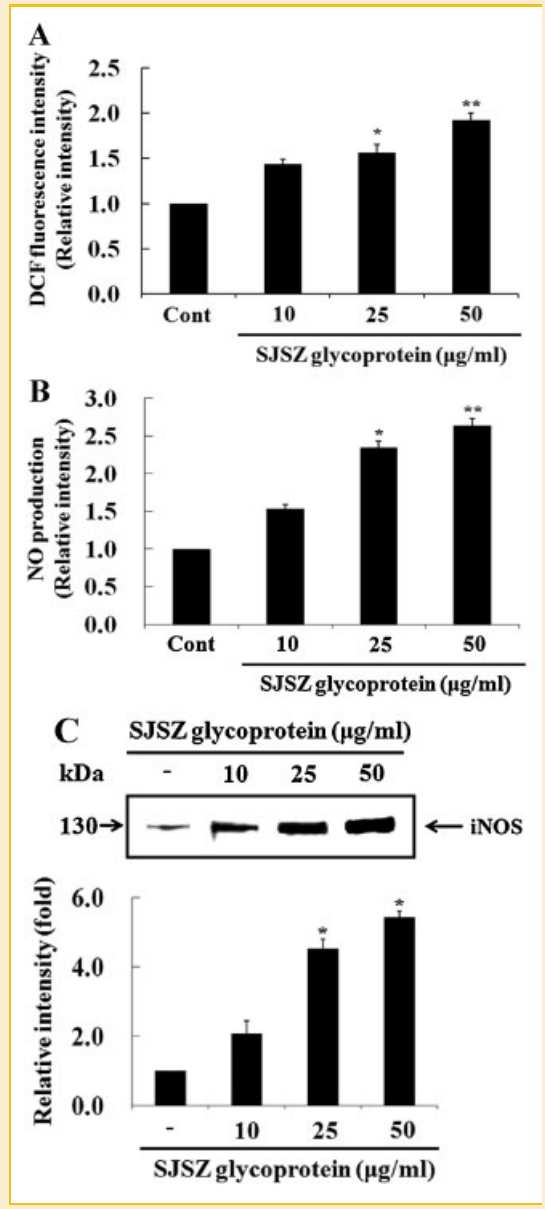

Fig. 6. Effect of SJSZ glycoprotein on production of intracellular ROS, NO and expression of iNOS in HepG2 cells. HepG2 cells were treated with SJSZ glycoprotein (10, 25, and 50 μg/ml) for 3 h. The results were represented as relative intensity of fluorescence based on monitoring of dichlorodihydrofluorescein (DCF) using a fluorescence microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Corporation, Sunnyvale, CA) (A). Cells were harvested by centrifugation. The supernatants were used for measurements of NO production as described in the Materials and Methods Section (B). Detection of iNOS was conducted by Western blotting. The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02, MD) (C). Lane 1, control; lane 2, 10 μg/ml SJSZ glycoprotein; lane 3, 25 μg/ml SJSZ glycoprotein; lane 4, 50 μg/ml SJSZ glycoprotein. All data represent the means ± SE from triplicate separately. Significant difference between control and treatment of SJSZ glycoprotein, $^{*}P < 0.05$ and $^{**}P < 0.01$.

CDKs [May and May, 1999]. The tumor suppressor gene p53 is considered as a key element in controlling the balance between cell growth and death [Aylon and Oren, 2007]. As shown in Figure 3, the p53-mediated cell cycle arrest in SJSZ glycoprotein-treated HepG2 cells. Arrest of the p53-dependent cell cycle is achieved with p21 expression following exposure of cells to arsenic as well [Nuntharatanapong et al., 2005].

One more important regulator in controlling cell-cycle protein is CKI. Among CKI, p21 and p27 are important regulators of cycline-CDK complexes. They can inhibit the kinase activities of several cyclin-CDK complexes, such as cyclin D1-CDK4. Also, it arrests cell proliferation at the G_0/G_1 and S transition [Ortega et al., 2002]. Some drugs express their anti-cell cycle arrest potential by up-regulating p21 and p27 expression in several cells [Marra et al., 2000]. The results obtained in the present experiments suggest that SJSZ glycoprotein induces expression of p21 and p27 (Fig. 3). These results suggest that SJSZ glycoprotein-induced G_1 arrest by enhancing of p21 and p27 expression dependent on p53.

Mammalian cell cycle progression is tightly regulated by cyclins, CDKs, CDK inhibitors, and many other cellular factors. CDK-cyclin

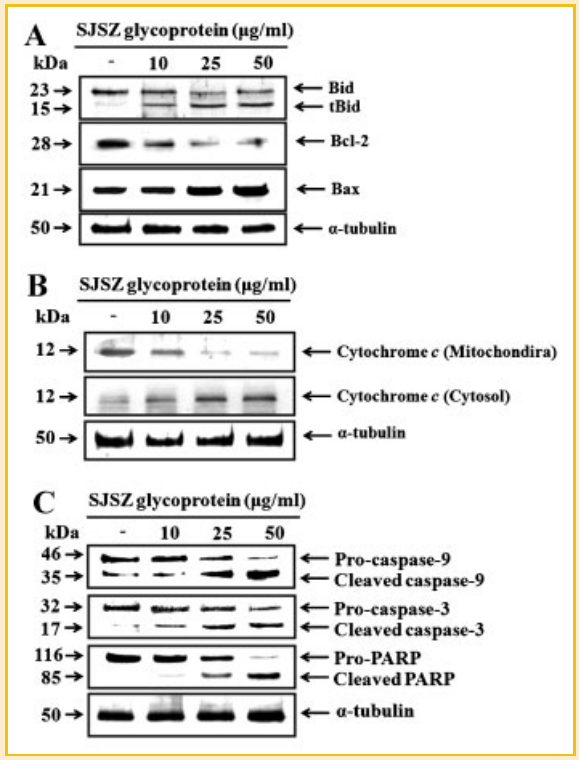

Fig. 7. Effect of SJSZ glycoprotein on apoptosis related proteins (iNOS, Bcl-2/Bax, Bid, cytochorome c, caspase-9, caspase-3 and PARP) in HepG2 cells. HepG2 cells were treated with SJSZ glycoprotein (10, 25, and 50 μg/ml) for 2 h (A,B). HepG2 cells were treated with SJSZ glycoprotein (10, 25, and 50 μg/ml) for 4 h (C). The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02, MD). All data represent the means ± SE from triplicate separately. Lane 1, control; lane 2, 10 μg/ml SJSZ glycoprotein; lane 3, 25 μg/ml SJSZ glycoprotein; lane 4, 50 μg/ml SJSZ glycoprotein. α-tubulin was used as an internal control.

complexes are negatively controlled by the CKIs namely p27 and p21 [Besson et al., 2008]. Cyclin D1 is a major regulator of progression of cells through the cell cycle; it is rate limiting for the G_1/S cell-cycle checkpoint. It has been reported that chronic cyclin D1 over-expression in transgenic mice is associated with rapidly progressing development of hepatocellular adenomas and carcinomas [Deane et al., 2001]. Cyclin D1 and its catalytic partner CDK4 are known to play important roles in the G_1/S checkpoint of the cell cycle. As shown in Figure 4, it indicates that SJSZ caused down-regulation of cyclin D1 expression, which was well correlated with decrease in expression level of CDK4. It has been reported that synthesis of both cyclin D1 and CDK4 is necessary and rate limiting for G_1 progression [Baldin et al., 1993].

It is known that cell cycle dysregulation is a hallmark of tumor cells. In the present study evaluated that cell cycle arrest using the FACS analysis, when HepG2 cells were exposed to SJSZ glycoprotein, the number of cells in the G_0/G_1 phase was higher than the number in other cell phases. This suggests that SJSZ glycoprotein arrests the cell cycle in the G_0/G_1 phase (Fig. 5). Thus, it means that cell cycle well not worked in transition of G_1 and G_2 phase. Therefore, it can induce to arrest cell cycle in HepG2 cells. It notes that it was shown as if treatment of 50 μg/ml of SJSZ is more decreased G_0/G_1 arrest ratio in Figure 5A by FACS analysis, when comparing with 25 μg/ml. However, statistical data (Fig. 5B) indicated G_0/G_1 arrest ratio was increased when dose was increased. The reason why the G_0/G_1 arrest ratio at treatment with 50 μg/ml of SJSZ seems to be more decreased compared to it at 25 μg/ml of SJSZ by FACS analysis is follows: In Figure 5A, the *Y*-axis by FACS analysis at 25 μg/ml of SJSZ was graded from 0 to 1,080 as number of cells, while it at 50 μg/ml of SJSZ was graded from 0 to 1,680. Therefore, the ratio between 25 μg/ml and 50 μg/ml of SJSZ seems to be optically different. In fact, the results of G_0/G_1 arrest ratio were calculated in integral from G_0/G_1 area obtained from FACS analysis. After that, the bar graph (Fig. 5B) is drawn with integral of G_0/G_1 area and represented. For instance, integral from G_0/G_1 area is 53.797% at 25 μg/ml of SJSZ, while it is 58.954% at 50 μg/ml of SJSZ. Such a propensity, in Figure 5A,B of S phase was calculated and represented just like G_0/G_1 arrest ratio. Also, the results about S phase must be understood similarly in the case of G_0/G_1 arrest ratio.

Apoptosis has considered one of critical mechanism for cancer chemoprevention [Johnson et al., 2010]. ROS are constantly generated and eliminated in the biological system, and play important roles in a variety of normal biochemical functions and abnormal pathological processes [Brunelle and Chandel, 2002]. The intracellular ROS concentration could generate the DNA damage, and induce cell apoptosis [Miwa et al., 2000]. As shown in Figure 6, the result showed that SJSZ glycoprotein induced intracellular ROS in HepG2 cells. It has been reported that ROS promotes outer membrane permeabilization and from mitochondria to cytosol translocation of cytochromc *c*, which triggers caspase-dependent cytosolic signaling events [Circu and Aw, 2010]. In general, ROS are frequently associated with cytotoxicity, often being described as damaging, harmful or toxic in the cells through oxidation. This passive attack of cellular components by ROS triggers cell death (initiating stimulus for apoptosis) as a result of cumulative oxidative damage [Carmody and Cotter, 2001]. NO is known to induce apoptosis via several pathways including the activation of a caspase cascade [Kim et al., 2000], the release mitochondrial cytochrome *c* [Brown and Borutaite, 1999], and the regulation of apoptosis. It has reported that NO can rapidly react with ROS to produce more reactive oxidant (peroxynitrite, $ONOO^-$), which causes cellular damage which means cell death. Furthermore, subtle changes in the rate of production of reactive nitrogen species such as NO may critically impact cellular homeostasis, consequently initiating a variety of cellular signaling processes including apoptosis [Griscavage et al., 1995]. By the way, because NO inhibits ATP synthesis, it would suppress ATP-dependent metabolisms which is occurred in the mitochondria. Also, when NO generation increased, the expression of bcl-2, which is well known to exhibit antiapoptotic activity by stabilizing their membrane potential, decreased markedly with time [Marin et al., 1996; Murphy et al., 1996].

Among the anti-apoptotic members, Bcl-2 is negative regulators of cell death, preventing cells from undergoing apoptosis induced by various stimuli in a wide variety of cell types [Korsmeyer, 1992], whereas others, such as Bax and Bid promote or accelerate cell death. In mammals, Bcl-2 regulation of apoptosis involves its mitochondrial membrane association. Bid is part of the bcl-2 family

of proteins, which includes both pro-apoptotic proteins (for example bid, bax, bak, bcl-XS, bad, bim). It is cleaved into a p15 form of tBid and translocated into the mitochondria, where it activates the oligomerization of Bak or Bax, release cytochrome *c*, and induces apoptosis [Yin, 2006]. Bid is cleaved into tBid in SJSZ glycoprotein-treated HepG2 cells. Also, Bax is increased, whereas Bcl-2 is decreased by SJSZ glycoprotein in HepG2 cells. Moreover, cytochrome *c* is released from mitochondria to cytosol by SJSZ glycoprotein (Fig. 7). Previous studies suggest that Bid expression is closely associated with the sensitivity of HCC cells to chemotherapeutic drugs [Miao et al., 2004]. It is reported that Bid-deficient livers show significantly less cell proliferation and is able to decrease the apoptotic protein levels (pro-casepase-9 and pro-caspase-3) [Bai et al., 2005].

In mammalian cells, caspase cascade has been shown to be involved in chemical- and agent-induced apoptosis [Huigsloot et al., 2001]. Interaction of cytochrome *c* with a cytosolic apoptosis protease activating factor, Apaf-1, induces recruitment of pro-caseapse-9 into a high-molecular-weight complex, termed the apoptosome, which gives rise to activated caspase-9 and caspase-3. Especially, caspase-3, one of the main executioners of apoptosis, cleaves several important intracellular molecules, leading to the morphological and biochemical changes associated with apoptosis. PARP is substrate for activated caspase-3 [Li and Darzynkiewicz, 2000]. Our results in this study showed that SJSZ glycoprotein has apoptotic effects by stimulating the activity of caspase-3 in HepG2 cells (Fig. 7C). Moreover, we have also shown additional evidence of down-stream caspase activation evaluating the PARP cleavage in SJSZ glycoprotein-treated HepG2 cells (Fig. 7C). It suggested that caspase and PARP-induced apoptosis has potential mechanism of SJSZ glycoprotein induced cell death. Our results suggest that SJSZ glycoprotein activates mitochondria-mediated apoptosis pathway by stimulating the activation of Bid, release of mitochondria cytochrome *c* and the cleavages of caspase-9, caspase-3, and PARP protein in HepG2 cells. Also, it has possessed to induce cell cycle arrest in G_1/S transition.

In conclusion, in the cell cycle-related proteins, SJSZ glycoprotein (50 μg/ml) significantly enhances the expression of p53, p21, and p27, whereas it suppressed activity of cyclin D1/CDK4. Also, SJSZ glycoprotein (50 μg/ml) stimulates to increase apoptosis-related factors intracellular reactive oxygen species (iROS, to activate Bid, cytochrome *c*, caspase-3, and PARP). Taken together, SJSZ glycoprotein (50 μg/ml) induced apoptosis and arrested cell cycle from G_0/G_1 to S in HepG2 cells. However, it still remains problem to elucidate the precise mechanisms of apoptosis in vivo.

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