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Normalizing Effect of SJSZ glycoprotein (38 kDa) on Proliferating Cell Nuclear Antigen and Interferon-γ in Diethylnitrosamine-Induced Mice Splenocytes

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ABSTRACT

One of the immunosuppressive responses when hepatocellular carcinoma (HCC) develops in mammals is defective proliferation in the spleen. The objective of this study was to investigate the protective effect of the *Styrax japonica* Siebold et al. *Zuccarini* (SJSZ) glycoprotein on the proliferation of splenocytes induced by diethlynitrosamine (DEN). To assess whether the SJSZ glycoprotein modulates splenocyte proliferation, Balb/c mice were injected intraperitoneally with DEN (50 mg/kg, BW) for 7 weeks. After 7 weeks, the mice were sacrificed, and spleens were isolated. We evaluated [^{3}H]-thymidine incorporation, extracellular signal-regulated kinase (ERK), cell cycle-related factors [p53, p21, p27, cyclin D1/cyclin dependent kinase (CDK) 4], proliferating cell nuclear antigen and interferon (IFN)-γ using radiation activity, immunoblot analysis, and the reverse transcription-polymerase chain reaction. The results revealed that the SJSZ glycoprotein (10 mg/kg, BW) increased [^{3}H]-thymidine incorporation, ERK phosphorylation, expression levels of cyclin D1/cyclin dependent kinase 4, and IFN-γ. However, the SJSZ glycoprotein decreased levels of p53, p21, and p27. Taken together, these results suggest that the SJSZ glycoprotein inhibited defective splenocyte proliferation induced by DEN. J. Cell. Biochem. 114: 808–815, 2013.

KEY WORDS: SJSZ GLYCOPROTEIN (38 kDa); SPLENOCYTE PROLIFERATION; IFN-γ; [^{3}H]-THYMIDINE INCORPORATION; PCNA

Hepatocellular carcinoma (HCC) ranks as the fifth most common malignant neoplasm in the world [Parkin et al., 2001]. However, HCC can also harm healthy cells and cause side effects such as immunosuppression, which limit their use in tumor treatment. *N*-nitrosodiethylamine (DEN) is a representative chemical in a family of carcinogenic *N*-nitroso compounds. DEN is a potent hepatocellular carcinogenic nitrosamine present in tobacco smoke, water, cheddar cheese, cured and fried meals, occupational settings, cosmetics, agriculture chemicals, and pharmaceutical agents [Li et al., 2005]. DEN has immunosuppressive effects and also roles as a carcinogen to induce HCC [Ceriello et al., 1994].

The spleen is the largest secondary immune organ in the body and plays an important role maintaining immune stasis [Mebius and Kraal, 2005]. Splenocyte proliferation is an essential factor in the immune response. The proliferation ability of splenic lymphocytes decreases in patients with HCC [Zhang et al., 2008]. Interferon (IFN)-γ is associated with many immunopotentiating effects such as T and B cell proliferation [Shaw et al., 1988]. Proliferating cell nuclear antigen (PCNA) and the G_0/G_1 cell cycle factor are very important signals during cell proliferation. PCNA is one of the principal indicative markers of cell proliferation and is a nuclear protein that plays a critical role in cell proliferation, DNA repair, and cell cycle control [Gramantieri et al., 2003]. The activity of cyclin-dependent kinases (CDKs) and cyclin regulatory subunits is one of the most important factors responsible for the orderly progression in the cell cycle [Collins et al., 1997]. For example, transition through G_1 phase into S phase requires the binding and activation of the cyclin/CDK complex, particularly cyclin D1/CDK4. p53 Protects cells from

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possible accumulation of damaged DNA, because it permits halting of the cell cycle to allow for DNA repair in normal cells [Marchetti et al., 2004]. Additionally, p21 and p27, which are induced by p53, are cell cycle kinase inhibitors (CKIs) and are able to inhibit CDKs, thereby arresting the transition from G_0/G_1 to the S phase [Sherr and Roberts, 1995; Sohn et al., 2006].

Immunomodulatory agents used with chemotherapy drugs appear to reduce myelosuppression and enhance the immune response. In fact, co-administration of immunomodulatory agents and chemotherapy drugs is used to improve immune potential in traditional Chinese medicine [Yuan et al., 2006]. One of the most fruitful approaches to modulate the immune system has been to identify natural compounds such as phytoglycoproteins, as natural compounds have the ability to suppress cancer invasion and metastasis. 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 types of cancer [Newman, 2008]. *Styrax japonica* Siebold et al. *Zuccarini* (*Styracaceae*, SJSZ) has been used to heal sore throats, cough, odontalgia, and paralysis in Korean folk medicine. Additionally, the pericarp is used to make soaps and pesticide agents, because it contains egosaponin as one of its major components [Kim, 1998]. Triterpenoid, which has been isolated from the stems and leaves of SJSZ, has anti-inflammatory activity and the ability to inhibit proliferation of HL-60 (human leukemic) cells [Kim et al., 2004]. We recently isolated a glycoprotein from SJSZ with an approximate molecular mass of 38 kDa. This glycoprotein has carbohydrate and protein contents of 57.64% and 42.35%, respectively [Lee and Lim, 2011ab]. However, no studies have investigated the immunomodulatory effect of the glycoproteins derived from the fruits of SJSZ on splenocyte proliferation in a murine hepatoma model.

Therefore, we evaluated [^{3}H]-thymidine incorporation, phosphorylation of ERK, activities of cell cycle-related factors [p53, p21, p27, cyclin D1/CDK 4], PCNA and IFN-γ using radiation activity, immunoblot analysis and RT-PCR to determine whether the SJSZ glycoprotein modulates splenocyte proliferation in DEN-induced mice.

MATERIALS AND METHODS

CHEMICALS

All plastic materials were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ). Penicillin G and streptomycin were obtained from Sigma (St Louis, MO). Cyclin D1, CDK4, PCNA, and α-tubulin antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA). p53, p21, and p27 Antibodies were purchased from Cell Signaling Technology (Danvers, MA). All other chemicals and reagents were of the highest analytical grade available.

PREPARATION OF THE SJSZ GLYCOPROTEIN

SJSZ fruits were harvested in July 2009 from Moodeung mountain in Chonnam province, South Korea, and the glycoprotein from SJSZ was isolated and purified as described previously [Lee and Lim, 2011ab]. The final amount of the SJSZ glycoprotein was 21.0 g (0.75%) from the initial SJSZ sample. We confirmed that the SJSZ glycoprotein has a molecular weight of 38 kDa using 18% PAGE analyses. The glycoprotein is comprised of 52.64% carbohydrate and 47.36% protein. After verifying the high purity (>98%) of the glycoprotein, we used it as the sample in this study.

EXPERIMENTAL DESIGN

Male mice (Balb/c), aged 3 weeks, were purchased from Daehan Lab (Animal Research Center Co., Ltd., DaeJeon, Korea). Animals were maintained in a clean room at a temperature of $23 \pm 2^{\circ}$C with a 12 h light/dark cycle (an illumination intensity of 150–300 lux; lights on 700 h, lights off 1,900 h). The relative humidity was $55 \pm 15\%$, and the air ventilation frequency was 15–20 times/h. Animals were housed according to Guiding Principles in the use of animals in Toxicology, adopted by the Society of Toxicology in 1989 at the experimental animal room of the college of veterinary medicine, Chonnam National University (CNU). This protocol was approved by the animal care and use committee of the college of veterinary medicine, CNU. All mice were fed a commercial diet and water ad libitum, and acclimated for at least 1 week prior to the experiments. Body weight and food intake of each group were recorded once weekly. Mice were divided into the following five groups:

- Group 1: control (n = 6).
- Group 2: 50 mg/kg DEN (n = 6).
- Group 3: 50 mg/kg DEN + 5 mg/kg SJSZ glycoprotein (n = 6).
- Group 4: 50 mg/kg DEN + 10 mg/kg SJSZ glycoprotein (n = 6).
- Group 5: 10 mg/kg SJSZ glycoprotein (n = 6).

Mice in Group 1 were fed a commercial diet and water *ad libitum*. Mice in Group 2 received an intraperitoneal injection of 0.1 ml of saline containing 50 mg/kg DEN five times per week for 6 weeks. Mice in groups 3 and 4 were treated with the SJSZ glycoprotein (5 and 10 mg/kg) daily for 7 weeks and co-treated with 50 mg/kg DEN five times per week for 6 weeks. Mice in Group 5 were administered 10 mg/kg SJSZ glycoprotein orally alone daily for 7 weeks.

CELL CULTURE

Spleen was collected from Balb/c mice under aseptic conditions in Hank's balanced salt solution. The collected spleen was minced using passing a fine steel mesh (82 mesh) to obtain a homogeneous cell suspension, and erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation, the precipitated cells were washed with PBS and re-suspended in RPMI 1,640 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 atmosphere, The cells (1×10^6 cells/ml) were dispensed into 6-well or 96-well flat bottom plates.

[^{3}H]-THYMIDINE INCORPORATION

Experiments assessing [^{3}H]-thymidine incorporation were conducted as described by Gabelman and Emerman [1992]. After the indicated incubation period, 1 μCi of [methyl-^{3}H] thymidine was added to the cultures, and the incubation continued for 5 h at 37°C. The cells were washed twice with PBS, fixed for 15 min in 10% TCA at 23°C, and then washed twice in 5% TCA. The acid-insoluble material was dissolved for 12 h in 2 N NaOH at 23°C. Aliquots were

removed to assess radioactivity using a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA). All experiments were conducted in triplicate, and the values were converted from absolute counts to a fold of the control to compare the results between experiments.

PREPARATION OF CELL EXTRACTS

Cell extracts were prepared as described previously [Oh and Lim, 2008]. The cells were washed in 1 ml ice-cold PBS after the medium was removed, and scraped into 300 μl of ice-cold buffer C (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, and 25% glycerol) containing a phosphatase inhibitor cocktail. The cells were freeze-thawed, shaken for 30 min at 4°C, and centrifuged at 14,000*g* for 30 min for lysis. The supernatant was designated as the whole extract for immunoblotting of ERK, p53, p21, and p27.

To prepare nuclear extracts for immunoblotting of cyclin D1/CDK4 and PCNA after cell activation for the times indicated, cells were resuspended in 400 μl ice-cold hypotonic buffer D (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 ice for 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 E (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, and 25% glycerol) containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany) and centrifuged at 12,000*g* for 15 min at 4°C. The amount of protein was measured by the Lowry method [Lowry et al., 1951], and cellular proteins were stored at −70°C prior to use.

IMMUNOBLOT ANALYSIS

Cellular proteins were separated on a 10% polyacrylamide mini-gel at 100 V for 2 h at room temperature using a mini-protein II electrophoresis cell (Bio-Rad, Hercules, CA). After electrophoresis, the proteins were transferred to 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) Tween20) 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, PCNA, 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, Danvers, MA) in TBS-T solution. The resulting protein bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK). The immunoblot assay results were calculated as relative intensity using Scion imaging software (Scion Image Beta 4.02, Frederick, MD).

REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was isolated from splenocytes using the TRIzol® Reagent according to the manufacture's protocol (Invitrogen Life Technologies, Carlsbad, CA). Complementary DNA (cDNA) was obtained from total RNA using the SuperScript First synthesis system RT-PCR kit (Invitrogen Corp.). Quantitative real-time polymerase chain reaction (qPCR) amplifications were performed in triplicate using the SYBR Green I assay and were carried out using the Strategene M3000 Sequence Detection System (Stratagene). Specific primers were used for IFN-γ (sense: 5′-GCA GAG CCA AAT TGT CTC CT-3′, antisense: 5′-ATG CTC TTC GAC CTC GAA AC-3′) and GAPDH (sense: 5′-TGT GGA TCT GAC ATG CCG CC-3′, antisense: 5′-AAC CAC CCT GTT GCT GTA GC-3′). The reaction were carried out in a 96-well plate in 20 μl reactions containing 2× SYBR Green Mater mix (Invitrogen)-2 pmol each of sense and antisense primer, and the conditions were as follows: 95°C for 10 min, followed by 95°C for 10 s, 58°C for 50 s and 72°C for 20 s, for 45 cycles. For each assay, a standard curve was determined concurrently with the examined samples. For standard curve determination, a six dilution series of standard samples (1/5, 1/25, 1/125, 1/625, and 1/3,125) of the selected standard cDNA for each gene was utilized. Finally, relative quantitative values of each sample were determined with 1/25 diluted cDNA and were normalized with those of the GAPDH genes. Gene expression was quantified using a modified version of the $2^{-\Delta\Delta ct}$ method as previously described [Livak and Schmittgen, 2001].

CELL CYCLE ANALYSIS

The primary cultured splenocytes were harvested. Cell pellets were washed with cold PBS and fixed in ice-cold 70% ethanol to determine cell cycle distribution. The cells fixed in 70% ethanol were washed with PBS and resuspended in 0.1% sodium citrate, 0.1% Triton X-100 and 50 mg/ml propidium iodide (PI) for 30 min at room temperature in the presence of 10 mg/ml ribonuclease A (Sigma–Aldrich, St. Louis, MO). At least 10,000 events were collected in each histogram. Cell cycle analysis was performed using Cell-Quest and WinMDI version 2.9 (The Scripps Research Institute, San Diego, CA).

STATISTICAL ANALYSIS

All experiments were carried out in triplicate (n = 3), separately. All data are mean ± standard error. A one-way analysis of variance and Duncan's test were carried out to determine differences between groups using SPPSS version 11.0 software (SPSS Inc., Chicago, IL).

RESULTS

DETERMINE PROPER DOSE AND TIME OF DEN AND SJSZ GLYCOPROTEIN IN PRIMARY CULTURED SPLENOCYTES

To determine proper dose and time of DEN and SJSZ glycoprotein, it carried out [^{3}H]-thymidine incorporation in primary cultured splenocytes. As shown Figure 1A, when splenocytes were exposed to various concentrations of DEN at 7 weeks, the [^{3}H]-thymidine incorporation were significantly decreased. In dose-dependent manner, the values were 0.94-, 0.78-, and 0.81-fold at 25, 50, and 75 mg/kg DEN at 7 weeks, respectively (Fig. 1A). In time-dependent manner, the values were 0.96-, 0.93-, 0.73-, and 0.73-fold for 4, 5, 7, and 9 weeks at 50 μg/ml DEN, respectively (Fig. 1B). As shown Figure 1C,D, the values were not changed by SJSZ glycoprotein (∼less than 50 mg/kg, BW) in dose- and time-dependent.

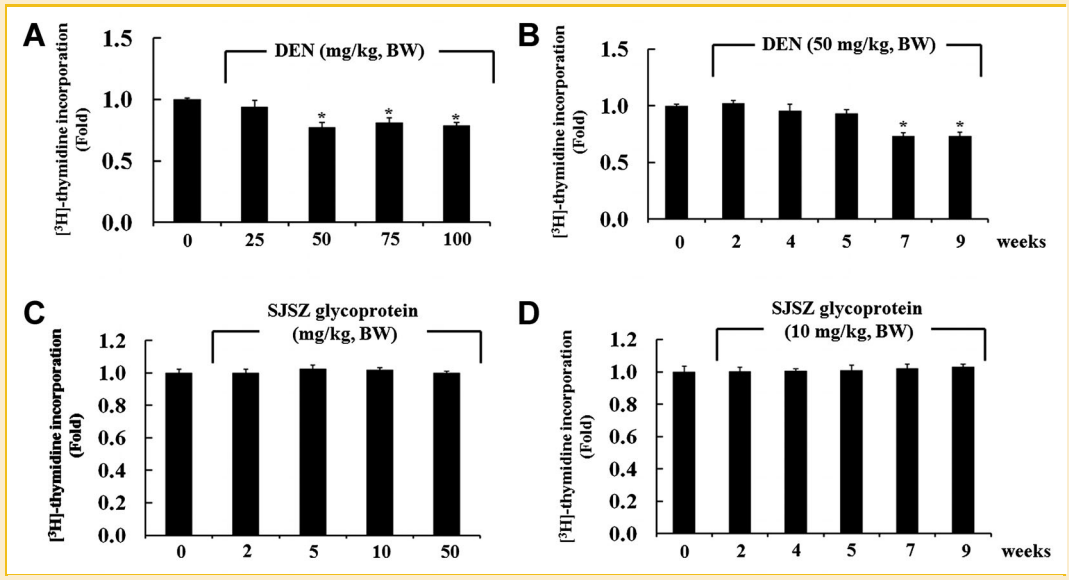

Fig. 1. Determine proper dose and time of DEN and SJSZ glycoprotein in primary cultured splenocytes using [^{3}H] thymidine incorporation. Splenocytes was pulsed with 1 μCi [^{3}H] thymidine for 5 h. All data are mean ± standard error from separate triplicates. (*) Indicates significant difference between treatment (DEN or SJSZ glycoprotein) alone and control, $P < 0.05$.

EFFECT OF THE SJSZ GLYCOPROTEIN ON [^{3}H]-THYMIDINE INCORPORATION IN PRIMARY CULTURED SPLENOCYTES INDUCED BY DEN

As shown in Figure 2, the number of living cells decreased compared to that in controls when splenocytes were treated with DEN (50 mg/kg, BW). However, when the splenocytes were treated with the SJSZ glycoprotein (5 and 10 mg/kg, BW) in the presence of DEN, [^{3}H]-thymidine incorporation increased in a dose-dependent manner compared to DEN treatment alone. For example, the relative values of [^{3}H]-thymidine incorporation increased by 0.16- and 0.27-fold in the 5 and 10 mg/kg SJSZ glycoprotein treatments, respectively, compared to that in the DEN treatment.

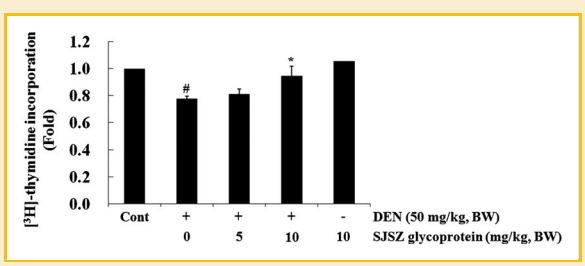

Fig. 2. Effect of the *Styrax japonica* Siebold et al. *Zuccarini* (SJSZ) glycoprotein on [^{3}H] thymidine incorporation in primary cultured splenocytes induced by diethylnitrosamine (DEN). Splenocytes was pulsed with 1 μCi [^{3}H] thymidine for 5 h. All data are mean ± standard error from separate triplicates. (#) Represents a significant difference between DEN treatment alone and control, $P < 0.05$. (*) Indicates significant difference between DEN treatment alone and the SJSZ glycoprotein treatment in the presence of DEN, $P < 0.05$.

EFFECT OF SJSZ GLYCOPROTEIN ON ERK ACTIVITY IN PRIMARY CULTURED SPLENOCYTES INDUCED BY DEN

ERK activity decreased following DEN (50 mg/kg, BW) treatment compared to that in the controls (Fig. 3). For example, the relative ERK band intensity decreased by 0.18-fold with DEN treatment alone. However, when the cells were treated with DEN in the presence of the SJSZ glycoprotein (5 and 10 mg/kg, BW), the relative band intensities were markedly augmented in a dose-dependent manner. That is, when the cells were treated with the SJSZ glycoprotein (10 mg/kg, BW) in the presence of DEN, ERK band intensity increased significantly by 0.80-fold, compared to that in the DEN treatment alone.

EFFECT OF SJSZ GLYCOPROTEIN ON CKI (P53, P21, AND P27) ACTIVITY IN PRIMARY CULTURED SPLENOCYTES INDUCED BY DEN

CKI expression was evaluated by Western blot analysis after treatment with DEN to determine whether the SJSZ glycoprotein inhibits CKI expression in DEN-treated splenocytes. As shown in Figure 4, CKI expression increased after DEN treatment compared to that in the controls. Specifically, the relative p53, p21, and p27 band intensities increased by 2.38-, 1.37-, and 2.74-fold, respectively, following the DEN treatment. However, when the splenocytes were treated with DEN in the presence of the SJSZ glycoprotein (5 and 10 mg/kg, BW), relative band intensities decreased markedly in a concentration dependent manner.

EFFECT OF THE SJSZ GLYCOPROTEIN ON CYCLIN D1/CDK4 AND PCNA EXPRESSION IN PRIMARY CULTURED SPLENOCYTED INDUCED BY DEN

Cyclin D1/CDK4 and PCNA expression was evaluated by Western blot analysis after DEN treatment to determine whether the SJSZ

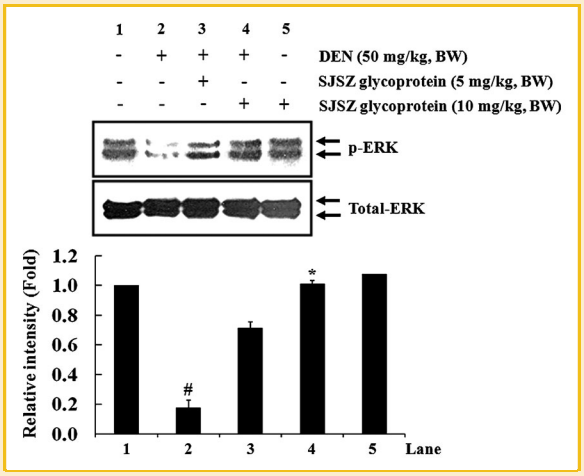

Fig. 3. Effect of the *Styrax japonica* Siebold et al. *Zuccarini* (SJSZ) glycoprotein on extracellular signal regulated kinase (ERK) activity in primary cultured splenocytes induced by diethlynitrosamine (DEN). The relative intensities of the Western blot bands were determined using Scion imaging software. All data are mean ± standard error from separate triplicates. (#) Significant difference between DEN treatment alone and the control, $P < 0.05$. (*) Significant differences between DEN treatment alone and the SJSZ glycoprotein treatment in the presence of DEN, $P < 0.05$. Lane 1, control; lane 2, DEN alone; lane 3, 5 mg/kg SJSZ glycoprotein in the presence of DEN; lane 4, 10 mg/kg SJSZ glycoprotein in the presence of DEN; lane 5, 10 mg/kg SJSZ glycoprotein alone. α-Tubulin was used as the internal standard. BW, body weight.

glycoprotein increases cyclin D1/CDK4 and PCNA expression in DEN-treated splenocytes. When the splenocytes were treated with DEN, the cyclin D1/CDK4 expression decreased compared to that in the control. That is, the cyclin D1/CDK4 relative band intensities following treatment with DEN alone were augmented by 0.54- and 0.26-fold compared to that in the controls. However, when splenocytes were treated with the SJSZ glycoprotein (10 mg/kg, BW) in the presence of DEN, the cyclin D1/CDK4 relative band intensities increased significantly by 0.39- and 0.63-fold, respectively, compared to those in DEN alone treatment.

As shown in Figure 5, expression of PCNA decreased after DEN treatment compared with that in the controls. For example, the relative PCNA band intensity decreased by 0.75-fold following DEN treatment. However, when the splenocytes were treated with DEN in the presence of the SJSZ glycoprotein (5 and 10 mg/kg, BW), the relative band intensities increased markedly in a concentration-dependent manner. That is, the PCNA band intensity following treatment with the SJSZ glycoprotein (5 and 10 mg/kg, BW) in the presence of DEN decreased significantly by 0.17- and 0.40-fold, compared to that in the DEN treatment alone.

EFFECT OF SJSZ GLYCOPROTEIN ON CELL CYCLE IN PRIMARY CULTURED SPLENOCYTES INDUCED BY DEN

To investigate whether SJSZ glycoprotein has normalizing of effect on the molecular mechanisms underlying cell cycle arrest in DEN-induced splenocytes, we evaluated cell cycle analysis using FACS analysis. When cells treated with DEN, 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

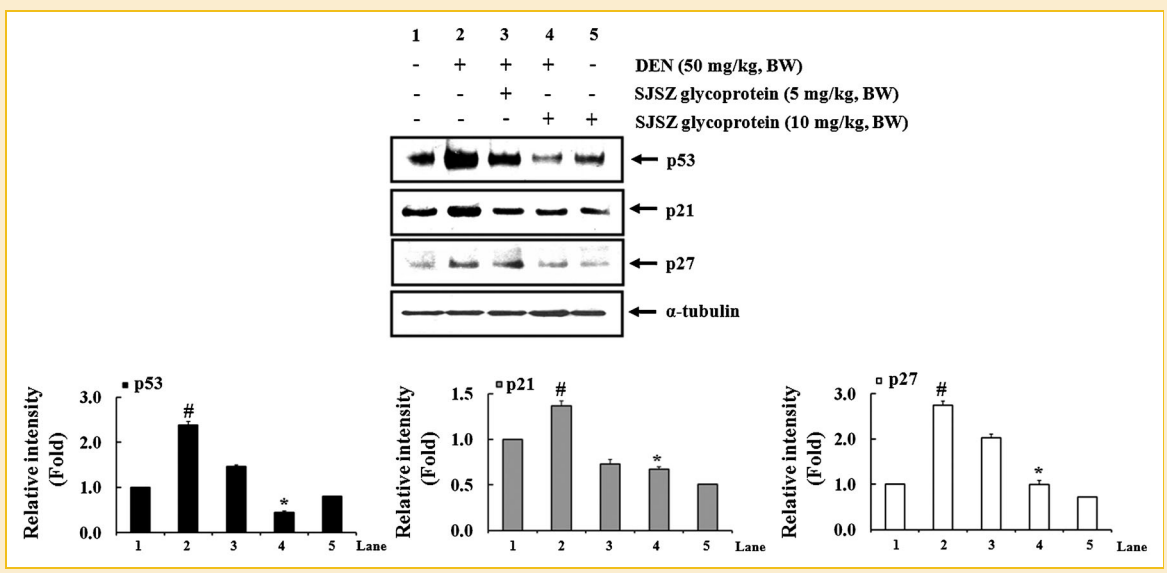

Fig. 4. Effect of the *Styrax japonica* Siebold et al. *Zuccarini* (SJSZ) glycoprotein on activity of cell cycle kinase inhibitors (CKIs) (p53, p21, and p27) in primary cultured splenocytes induced by diethlynitrosamine (DEN). The relative intensities of the Western blot bands were determined using Scion imaging software. All data are mean ± standard error from separate triplicates. (#) Significant difference between the DEN treatment alone and control, $P < 0.05$. (*) Significant difference between DEN treatment alone and the SJSZ glycoprotein treatment in the presence of DEN, $P < 0.05$. Lane 1, control; lane 2, DEN alone; lane 3, 5 mg/kg SJSZ glycoprotein in the presence of DEN; lane 4, 10 mg/kg SJSZ glycoprotein in the presence of DEN; lane 5, 10 mg/kg SJSZ glycoprotein alone. α-tubulin was used as the internal standard. BW, body weight.

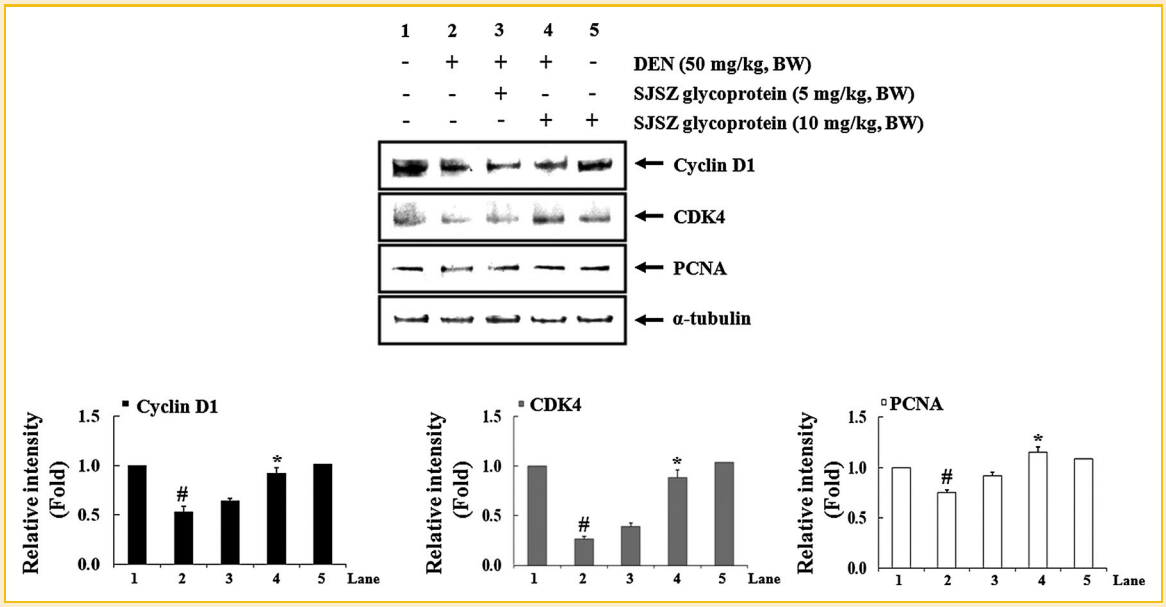

Fig. 5. Effect of the *Styrax japonica* Siebold et al. *Zuccarini* (SJSZ) glycoprotein on activity of cyclin D1/CDK4 and proliferating cell nuclear antigen (PCNA) in primary cultured splenocytes induced by diethlynitrosamine (DEN). The relative intensities of Western blot bands were determined using Scion imaging software. All data are mean ± standard error from separate triplicates. (#) Significant difference between DEN treatment alone and control, $P < 0.05$. (*) Significant difference between DEN treatment alone and the SJSZ glycoprotein treatment in the presence of DEN, $P < 0.05$. Lane 1, control; lane 2, DEN alone; lane 3, 5 mg/kg SJSZ glycoprotein in the presence of DEN; lane 4, 10 mg/kg SJSZ glycoprotein in the presence of DEN; lane 5, 10 mg/kg SJSZ glycoprotein alone. α-tubulin was used as the internal standard. BW, body weight.

increased 51.1% in treatment with DEN (50 mg/kg, BW) compared to the control, whereas in S phase, it decreased 28.4% in treatment with DEN (50 mg/kg,BW) compared to the control. However, G_0/G_1 phase was normalized in presence of SJSZ glycoprotein compared to the DEN treatment alone.

EFFECT OF THE SJSZ GLYCOPROTEIN ON IFN-γ EXPRESSION IN PRIMARY CULTURED SPLENOCYTES INDUCED BY DEN

qRT-PCR was used to study the effects of the SJSZ glycoprotein on IFN-γ expression. As shown in Figure 7, IFN-γ expression in cells that were treated with DEN was lower than that in the control. However, when cells were treated with the SJSZ glycoprotein (10 mg/kg, BW) in the presence of DEN, IFN-γ expression was significantly higher than that of DEN treatment alone.

DISCUSSION

The immune system plays an important role in anti-tumor defense. Progressive tumor growth is frequently accompanied by concomitant immunosuppression, regardless of tumor location and etiology. Thus, this is one of the ways to prevent progressive tumor growth. According to some studies, immunosuppression and spleen lymphocyte proliferation are closely related [Elvinger et al., 1992]. Although administration of chemotherapeutic agents is the primary approach to cancer treatment, severe adverse effects, such as immune system damage, limit their use and development. Moreover, immunomodulatory agents, including natural and synthetic products, may be a possible means to inhibit tumor growth without harming the host. We previously reported that antitumoric effect of SJSZ glycoprotein inhibits metastasis and arrests cell cycle [Lee and Lim, 2011ab]. Therefore, we evaluated whether the SJSZ glycoprotein could inhibit defective splenocyte proliferation induced by DEN.

Firstly, we evaluated splenocyte proliferation and cytokine production to partially uncover the anti-immunosuppressive properties of the SJSZ glycoprotein in HCC models and its effect on the immune system. We incorporated [^{3}H]-thymidine into DEN-induced Balb/c mice after SJSZ glycoprotein exposure. Compared with the untreated cells, the SJSZ glycoprotein-treated cells did not show any changes in viability. The ability of lymphocytes to proliferate in the DEN-induced mice was lower than that of normal mice. The data demonstrate that the SJSZ glycoprotein increased cell proliferation in DEN-induced splenocytes (Fig. 2).

The ERK1/2 pathway plays an important role regulating IFN-γ expression and proliferating T cells [Acuña-Castillo et al., 2005]. Additionally, ERK promotes G_1 progression and S phase entry in the cell cycle in vitro. As shown in Figure 3, the SJSZ glycoprotein induced ERK expression.

Cell cycle-related proteins such as p53, p21, p27, cyclones, and CDKs arrest cells at the G_0/G_1 and S phases. p53 plays a crucial role as a checkpoint protein in the human and murine cell cycle [May and May, 1999]. The Western blot results revealed that the SJSZ glycoprotein inhibited p53 expression in splenocytes exposed to DEN (Fig. 4). Arrest of the p53-dependent cell cycle is achieved by p21 expression following exposure of cells to arsenic as well [Nuntharatanapong et al., 2005]. CDKs, which build complexes with cyclin as a complex molecule with cyclin/CDK, are well-known stimulators of the cell cycle from the G_0/G_1 to the S phase, whereas

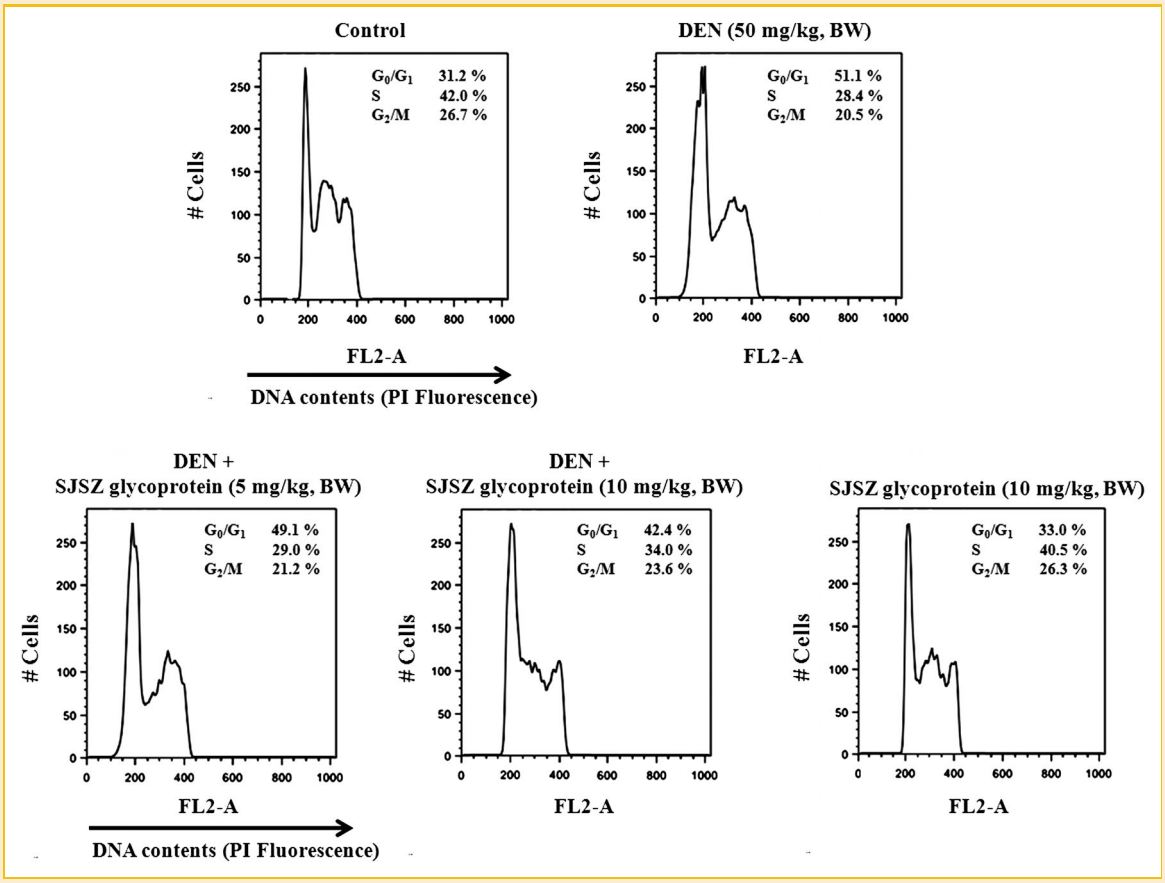

Fig. 6. Effect of the *Styrax japonica* Siebold et al. *Zuccarini* (SJSZ) glycoprotein on cell cycle arrest in primary cultured splenocytes induced by diethlynitrosamine (DEN). Cell cycle analysis was carried out using flow cytometry (FACS) staining with propidium iodide (PI). Distribution histogram of nuclear DNA detected by fluocytometry was indicated according to cellular cycle phase.

p21 and p27 are important inhibitors. When the level of p27 expression in the G_0/G_1 phase is maximal, there is little progression of the cell cycle from the G_1 phase to the S phase [He et al., 2005; Miyake et al., 2010]. Our results suggest that the SJSZ glycoprotein inhibited p21 and p27 expression, whereas DEN stimulated their expression (Fig. 4).

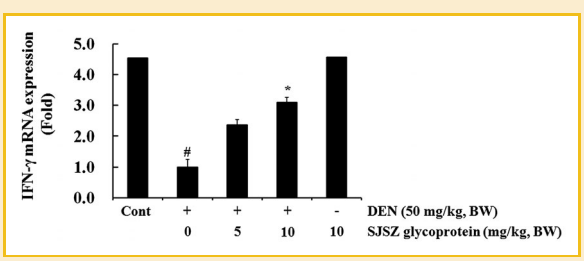

Fig. 7. Effect of the *Styrax japonica* Siebold et al. *Zuccarini* (SJSZ) glycoprotein on expression of interferon (IFN)-γ in primary cultured splenocytes induced by diethlynitrosamine (DEN). IFN-γ mRNA expression was determined by qRT-PCR using total RNA isolated from DEN-induced splenocytes. (#) Significant difference between treatments and control, $P < 0.05$. (*) Significant difference between DEN treatment alone and treatment with the SJSZ glycoprotein in the presence of DEN, $P < 0.05$. GAPDH was used as an internal control. BW, body weight.

CDKs consist of catalytic and activation subunits and participate actively in cell cycle progression. In particular, the formation of two major protein complexes (CDK and cyclin) is absolutely required for the G_1/S transition to permit progression. Cyclin D1 among cyclins plays a pivotal role in cell cycle progression from the G_0/G_1 to the S phase [Zhao et al., 2001; Stacey, 2003]. Interestingly, CDK4 is a specific mediator for transition from the G_0/G_1 into the S phase. Our results appear to indicate that the SJSZ glycoprotein reduced cyclin D1 and CDK4 suppression, whereas DEN stimulated cyclin D1 and CDK4 suppression (Fig. 5). PCNA has been identified as a cyclin or an auxiliary protein for DNA polymerase-δ [Bravo et al., 1987]. Our results demonstrate that the SJSZ glycoprotein increased DEN-induced PCNA activity, which plays a pivotal role in DNA repair, cell proliferation, and cell cycle control [Gramantieri et al., 2003]. Cell proliferation caused by various external mitogenic factors is ultimately linked to the cell cycle. Thus, the cell cycle process depends on precise and rigorous regulation mediated by many cell cycle regulatory factors.

Moreover, in the present study evaluated that cell cycle arrest using the FACS analysis, when splenocytes were exposed to DEN, the number of cells in the G_0/G_1 phase was higher than the number in other cell phases. However, SJSZ glycoprotein attenuated cell cycle arrest (Fig. 6). Thus, it means that cell cycle well not worked in transition of G_1 and G_2

phase. Therefore, SJSZ glycoprotein can prevents to arrest cell cycle in splenocytes.

IFN-γ is an important immune factor secreted by helper T lymphocytes, which promotes immune cell proliferation and differentiation. The interaction between T lymphocytes and antigens initiates a cascade of gene expression such as interleukin-2 and IFN-γ. In the present study, the SJSZ glycoprotein strongly increased cytokine IFN-γ expression in splenocytes induced by DEN, suggesting that SJSZ could improve cellular immune function in DEN-induced mice.

In conclusion, the results of this study suggest that the SJSZ glycoprotein (10 mg/kg, BW) increased [^{3}H]-thymidine incorporation in DEN-treated Balb/c mice. The SJSZ glycoprotein (50 μg/ml) also significantly suppressed p53, p21, and p27 expression, whereas cyclin D1/CDK4 activity was enhanced. Taken together, the results suggest that the SJSZ glycoprotein (10 mg/kg, BW), as a natural compound, significantly normalized the G_0/G_1 arrest induced by DEN. However, the precise mechanism by which the SJSZ glycoprotein arrests the cell cycle stage remains to elucidated.

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