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Preventive Effect of Phytoglycoprotein (27 kDa) on Inflammatory Factors at Liver Injury in Cadmium Chloride-Exposed ICR Mice

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ABSTRACT

Cadmium is one of the inflammation-related xenobiotics and has been regarded as a potent carcinogen. *Gardenia jasminoides* Ellis (GJE) has been used to cure inflammation in Korean folk medicine for a long time. The purpose of present study is the inhibitory effect of glycoprotein isolated from GJE (27 kDa) on inflammation mechanism in cadmium chloride-exposed ICR mice. We evaluated the activities of lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and thiobarbituric acid-reactive substances (TBARS), activities of anti-oxidative enzymes [superoxide dismutase (SOD) and gluthathione peroxidase (GPx)], activities of c-Jun N-terminal protein kinase (JNK), heat shock protein 27 (Hsp27), activator protein (AP)-1, nuclear factor (NF)-κB and expression of inflammation-related mediators including tumor necrosis factor (TNF)-α and interleukin (IL)-6 in cadmium chloride-exposed ICR mice using immunoblot analysis, EMSA and RT-PCR. It notes that mice plasma was used to measure ALT, LDH, and TBARS after treatment with cadmium chloride alone or cadmium chloride under the pretreatment with GJE glycoprotein. Liver tissues were used to assess activities of anti-oxidant enzymes, SAPK/JNK, Hsp27, AP-1, NF-κB, TNF-α, and IL-6 in this study. The results obtained from this study revealed that GJE glycoprotein (10 mg/kg) decreased the levels of LDH, ALT and TBARS, whereas increased the activity of hepatic anti-oxidant enzymes (SOD and GPx) in cadmium chloride-exposed ICR mice. Moreover, it decreased the activity of JNK/AP-1, NF-κB, Hsp27, and pro-inflammatory cytokines (TNF-α and IL-6). Taken together, the results in this study suggest that GJE glycoprotein inhibits the expression of inflammation-related cytokines (TNF-α and IL-6) in cadmium chloride-exposed ICR mice. J. Cell. Biochem. 112: 694–703, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: GJE GLYCOPROTEIN (27 kDa); CADMIUM CHLORIDE; HSP27; AP-1; TNF-α; IL-6

Cadmium is an environmental contaminant that has recently gained public attention due to the worldwide increase in discard of this toxic metal [Fouad et al., 2009]. It is used in industry in a variety of modern technologies, such as electroplating, color pigments in paints, stabilizers, and batteries [Järup, 2003]. Cadmium has been regarded as one of the inflammation-related xenobiotics. It also has been classified as carcinogenic in humans, affecting lung, kidney, and liver [Kundu et al., 2009]. Particularly, cadmium hepatotoxicity is closely related to inflammation, since after acute cadmium exposure, the damaged liver tissues are often accompanied with infiltration of inflammatory cells [Yamano et al., 2000].

Inflammation is a common denominator in development and exacerbation of liver disease. Inflammatory phenomena have also been suggested as the most likely events responsible for induction of lipid peroxidase in the liver tissue following acute exposure of mice to cadmium [Tzirogiannis et al., 2003]. Lipid peroxidation and subsequent autocatalytic disruption of membranes have been implicated in the pathogenesis of a number of diseases and clinical conditions including diabetes, atherosclerosis, various inflammatory conditions, and cancer [Boujelben et al., 2006]. Aerobic organisms are protected against free radicals by anti-oxidant defense systems. Antioxidant includes endogenously synthesized compound such as superoxide dismutase (SOD) and gluthathione peroxidase (GPx) [Hudecová and Ginter, 1992]. Oxidative stress can activate the stress-activated protein kinase (SAPK)/c-Jun N-terminal protein kinase (JNK) pathway in response

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to various stimuli [Shen and Liu, 2006]. SAPK/JNK has also been reported to be involved in liver damage, such as cold ischemia/warm reperfusion injury in liver transplantation, hepatocyte death, and non-alcoholic liver disease [Czaja, 2003]. Moreover, there is considerable evidence that JNK activation may act through downstream substrates such as activator protein (AP)-1. As AP-1 is redox sensitive, cadmium may induce the expression of pro-inflammatory cytokines and chemokines by causing a change in cellular redox status. Tumor necrosis factor (TNF)-α and interleukin (IL)-6 are well known as critical important inflammation-related cytokines at the healing stage after partial hepatectomy or cadmium-induced liver injury [Harstad and Klaassen, 2002].

A diverse array of metabolic insults including the exposure of cells to heavy metals, result in the increased expression of gene encoding heat shock protein. Hsps are a family of stress-inducible proteins that show a functional heat shock element in their promoter. Elevated levels of heat shock protein 27 (Hsp27) have been observed to result in cytoprotection and may represent an important cellular defense mechanism against divers forms of cellular and tissue injury produced by hepatotoxicants [Yoo et al., 2000; Urani et al., 2001].

It is a good strategy to use medical plants for the prevention or healing of several diseases caused by toxic heavy metal like cadmium, because most natural compounds (like phytoglycoprotein) have less cytotoxicity than artificial synthetic compounds such as drugs. *Gardenia jasminoides* Ellis (GJE) has been used for a long time to cure febrile diseases, jaundice, acute conjunctivitis, epistaxis, hematemesis, pyogenic infections and ulcers of the skin as traditional medicine in Korea [Chang and But, 1987]. Recently, we isolated a glycoprotein with an approximate molecular mass of 27 kDa from GJE. The glycoprotein has a carbohydrate and protein content of 57.65% and 42.35%, respectively. In our previous study, the GJE glycoprotein was shown to possess a strong anti-oxidant-activity against, anti-inflammatory activity, and hepatoprotective activity [Lee et al., 2006; Oh and Lim, 2006]. Thus, we assume that GJE glycoprotein can probably prevent the liver cell damage cause by cadmium ion. However, it has not been reported that GJE glycoprotein prevents hepatocytes from the inflammation occurred by cadmium chloride.

Therefore, to know whether GJE glycoprotein has inhibitory effect of hepatotoxicity and inflammation in cadmium chloride-exposed ICR mice, we evaluated LDH, ALT and TBARS in the mice blood, and anti-oxidant enzymes (SOD and GPx), JNK/AP-1, Hsp27 and pro-inflammatory cytokines (TNF-α and IL-6) in the mice liver tissues using Western blot, EMSA, and RT-PCR.

MATERIALS AND MEHTODS

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's 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 GJE GLYCOPROTEIN

GJE glycoprotein was isolated and purified from GJE with slight modifications, essentially as described previously [Lee et al., 2006]. Samples of GJE were obtained in October 2003 from the Naju traditional market in the Chonnam province, South Korea. They were identified by Dr. H.T. Lim (Chonnam National University) and the fruits (8.20 kg) were chopped into small pieces and soaked in ethanol (7 L) for several months in a dark basement. The ethanol extracts were subsequently filtered through Whatman filter paper (No. 2) and centrifuged at 8,000*g* for 10 min. The collected supernatants were concentrated with a rotary evaporator (B465; Buchi, Switzerland) and the dried matter was 380.05 g. The dried-crude extracts (5 g) were resolved in distilled and subjected to concanavalin sepharose 4B affinity chromatography (24–45 mm, Sigma, C9017). It was 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 6000-8000, CA, USA) against 20 mM Tris–Cl (pH 7.4) at 4°C overnight and lyophilized to yield 2.5 g (1.49%). The final purity of GJE glycoprotein was over 98 ± 2% and consisted of carbohydrate (57.65%) and protein moiety (42.35%). The quantity of GJE glycoprotein yielded was 5.68 mg (0.07%) after lyophilization and stored at 4°C until use.

EXPERIMENTAL ANIMALS (EXPERIMENTAL DESIGN)

Male mice (ICR), aged 5 weeks, were purchased from Daehan Lab. (Animal Research Center Co., Ltd, DaeJeon, Korea) and housed according to Guiding Principles in the Use of Animal in Toxicology, adopted by the Society of Toxicology in 1989 at the Experimental Animal Room of Veterinary College of Chonnam National University (CNU). All mice were fed a commercial diet and water ad libitum, and kept for at least 1 week prior to the experiments. The 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: GJE glycoprotein (10 mg/kg, BW) (n = 6);
- Group 3: Cadmium chloride (2.5 mg/kg) (n = 6);
- Group 4: Cadmium chloride (2.5 mg/kg) + GJE glycoprotein (5 mg/kg, BW) (n = 6);
- Group 5: Cadmium chloride (2.5 mg/kg) + GJE glycoprotein (10 mg/kg, BW) (n = 6).

The extract-exposed groups were pretreated with their respective extracts for 1 week and continued for additional 2 weeks during which treatment groups were intraperitoneally treated with cadmium chloride. Mice were administered with a 5 or 10 mg/kg body weight of GJE glycoprotein once a day for 1 week, and the control was administered with 100 μl of phosphate-buffered saline (PBS). Starting at 1 week, the mice were received intraperitoneal injection of 2.5 mg/kg cadmium chloride dissolved in PBS (pH 7.0) once a day for 2 weeks. At 3 weeks, the blood from mice was collected by a cardiac puncture and centrifuged at 10,000*g* for 5 min at 4°C. The supernatant was separated and stored at −70°C for later measurement of the activity of alanine transaminase (ALT), lactate dehydrogenase (LDH) and formation of thiobarbituric acid reactive

substances (TBARS). It notes that mice plasma was used to measure ALT, LDH, and TBARS after treatment with cadmium chloride or cadmium chloride under the pretreatment with GJE glycoprotein. Liver tissues were used to assess activities of anti-oxidant enzymes, SAPK/JNK, Hsp27, AP-1, TNF-α, and IL-6 in this study. For determining toxicity, relative values of body and liver weights were calculated as shown in the following equations:

$$\text{Relative value of BW} = \text{BWA(g)}/\text{BWB(g)} \times 100$$

$$\text{Relative value of LW} = \text{LW(g)}/\text{BW(g)} \times 100$$

where BWB is the body weight before administration, BWA the body weight after administration, LW the liver weight, and BW the body weight.

LIPID PEROXIDATION ASSAY

Lipid peroxidation was estimated by the amounts of thiobarbituric acid reactive substances (TBARS) in plasma according to the method of Ohkawa et al. [1979]. One volume of sample was mixed thoroughly with two volumes of stock solution 15% (w/v) trichloroacetic acid, 0.375% (w/v) triobarbituric acid, and 0.25 N HCl. The mixture was heated for 30 min in a boiling water bath. After cooling, the flocculate precipitate was removed by centrifugation at 1,000*g* for 10 min and the absorbance (OD) of the sample were measured and calculated. After that it was represented as relative change (percentage) to the control as the standard for TBARS measurement. Data are expressed as percent of control.

LDH AND ALT ASSAY

The levels of LDH and ALT in mice serum were measured according to the method of Bergmeyer and Bernt [1974a,b]. The activities of LDH and ALT were measured as the rate of loss of β-NADH absorption at 340 nm for 2 min.

ASSAY OF ANTIOXIDANT AND GLUTATHIONE-METABOLIZING ENZYME

SOD activity in liver tissue was determined by the method of Kakkar et al. [1984] in which the inhibition of formation of NADPH-phenazine methosulfate nitroblue tetrazolium formazone was measured spectrophotometrically at 560 nm. Glutathione peroxidase activity (GPx) in liver tissue was assayed by the method based on the reaction between glutathione remaining after the action of GPx and 5,5′-dithiobid-2-nitrobenzoic acid to form a complex that absorbs maximally at 412 nm [Rotruck et al., 1973].

IMMUNOBLOT ANALYSIS

Livers were homogenized in ice-cold lysis buffer [150 mM NaCl, 0.5% Triton X-100, 50 mM Tris–HCl (pH 7.4), 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4 and protease inhibitor cocktail tablet]. Lysates were centrifuged at 14,800*g* for 30 min at 4°C. The amount of protein was measured by the method of Lowry et al. [1951], and the cellular proteins were stored at −70°C prior to use. 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 [SAPK/JNK, phospho-SAPK/JNK, Hsp27, phosphor-Hsp27, AP-1 (c-Jun), AP-1 (c-Fos), 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).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

To make the double stranded oligonucleotide, both strand of oligonucleotide for AP-1 (5′-TTCCGGCTGACCTCATCAAGCG-3′) and nuclear factor (NF)-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were annealed in TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) by heating at 94°C for 10 min. For probing of AP-1 and NF-κB double stranded oligonucleotide, they were labeled with [γ-^{32}P]dATP (0.25 mCi, Amersham Pharmacia Biotech, Buckinghamshire, UK) by T4 polynucleotide kinase and purified on A QIA quick® Nucleotide Removal Kit according to the manufacturer's protocol (LRS Laboratory, Inc., QIAGEN Distributor, Seoul, Korea). The DNA-protein binding reaction was performed by incubation of the AP-1 and NF-κB probe, and 10 μg of nuclear protein extracts and 0.5 μg/ml poly-dI/dC (Sigma Chemical Co., USA) in a binding buffer [0.2 M DTT, 20 mg/ml BSA, buffer A (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40), buffer D (20% Ficoll 400, 100 mM HEPES, 300 mM KCl)] at room temperature for 1–2 h. The DNA–protein complexes were resolved by applying 6% non-denaturing polyacrylamide gel in 0.5× TBE (45 mM Tris–borate, 1 mM EDTA). Electrophoresis was carried out at 200 V for 3 h in a cold room. Gels were then dried on 3 M blotting paper (Whatman) and exposed to X-ray film at −70°C overnight.

REVERSE TRANSCRIPTION-POLYMERAE CHAIN REACTION (RT-PCR)

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNF-, IL-6, and GAPDH (internal control). Total RNA was isolated from mouse liver using TRIZOL Reagent in according to the manufacture's protocol (Invitrogen Life Technologies, Carlsbad, CA). RT-PCR was performed using the AccessQuick™ RT-PCR System (Promega). Briefly, the RT-PCR mixture (50 μl) containing 1 μg of total RNA, 10 μM gene-specific sense and antisense primers, 1 μl of avian myeloblastosis virus reverse transcriptase, and 25 μl of AccessQuick™ Master Mix was prepared on ice. The following primer sequences of TNF-α, IL-6 and GAPDH were used (sense and antisense, respectively): TNF-α (231 bp), 5′-TCT CAT CAG TTC TAT GGC CC-3′ and 5′-GGG AGT AGA CAA GGT ACA AC-3′; IL-6 (223 bp), 5′-GTT CTC TGG GAA ATC GTG GA-3′ and 5′-TGT ACT CCA GGT AGC TAT GG-3′; and glyceraldehydes-3-phosphate dehydrogenase (GAPDH; 443 bp), 5′-CAA AAG GGT CAT CAT CTC TG-3′ and 5′-CCT GCT TCA CCA CCT TCT TG-3′. The amplifications were conducted with 30 cycles for TNF-α, IL-6 and GAPDH (30 s at 94°C, 30 s at 52°C, and 30 s at 72°C),

TABLE I. Effect of GJE Glycoprotein on LDH and ALT Levels in Cadmium Chloride-Exposed Liver of Control and Experimental Mouse

Treatment	Relative weight (%)			
	Body	Liver	LDH (%)	ALT (U/l)
Control	100 ± 2.5	5.8 ± 0.8	100.0 ± 2.7	17.5 ± 2.7
GJE glycoprotein (10 mg/kg, BW)	102 ± 1.9	5.6 ± 1.2	97.3 ± 4.8	17.0 ± 1.4
Cadmium chloride (2.5 mg/kg, BW)	93 ± 3.5#	7.2 ± 0.1#	149.8 ± 5.49#	56.8 ± 4.8#
Cd + GJE glycoprotein (5 mg/kg, BW)	101 ± 2.7	6.3 ± 0.8	120.7 ± 3.58	41.3 ± 3.4
Cd + GJE glycoprotein (10 mg/kg, BW)	106 ± 1.2*	5.2 ± 0.7*	77.9 ± 2.14*	27.5 ± 2.8*

Cd, cadmium chloride; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; BW, body weight.
Data represented the mean ± SD (n = 6).
#$P < 0.05$ compared with control.
*$P < 0.05$ compared with cadmium chloride treatment alone.

and the RT-PCR amplification products were mixed with 1 μl of loading buffer and separated on a 2% agarose gel. The gels were then stained with 5 μg/ml ethidium bromide and photographed.

STATISTICAL ANALYSIS

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

RESULTS

INHIBITORY EFFECT OF GJE GLYCOPROTEIN ON LDH AND ALT LEVELS IN CADMIUM CHLORIDE-EXPOSED ICR MICE

Cadmium chloride treatment significantly decreased body weight and increased liver weight, compared to the control. However, GJE glycoprotein reversed the body weight loss and liver swelling induced by cadmium chloride to the levels of the control group (Table I). The levels of LDH and ALT were measured in the plasma to evaluate hepatic tissue damage (Table I). The results showed that GJE glycoprotein did not show a significant hepatotoxicity. A significant increase the level of LDH and ALT were observed in the cadmium chloride-exposed group. Administrations of GJE glycoprotein significantly reduced the increased plasma LDH and ALT levels.

INHIBITORY EFFECT OF GJE GLYCOPROTEIN ON LIPID PEROXIDATION IN THE CADMIUM CHLORIDE-EXPOSED ICR MICE

TBARS levels were assessed as an indicator of lipid peroxidation. Cadmium chloride treatment significantly increased the level of TBARS in the plasma. As shown in Figure 1, when cadmium chloride exposed to ICR mice, TBARS levels is significantly increased by 3.30-fold compared to the control. However, GJE glycoprotein (5 and 10 mg/kg) administrated to ICR mice in presence of cadmium chloride, TBARS levels is significantly decreased by 0.24- and 0.38-fold, compared to the cadmium chloride treatment.

EFFECT OF GJE GLYCOPROTEIN ON ACTIVITIES OF ANTIOXIDANT ENZYMES (SOD AND GPX) IN CADMIUM CHLORIDE-EXPOSED ICR MICE

The hepatic anti-oxidant enzyme activities (SOD, GPx) are shown in Figure 2. The SOD and GPx activities in cadmium chloride-exposed mice were decreased, compared to the control. Interestingly, the pretreatment with GJE glycoprotein significantly increased the decreased SOD and GPx activities in the cadmium chloride-treated group.

INHIBITORY EFFECT OF GJE GLYCOPROTEIN ON PHOSPHORYLATION OF SAPK/JNK IN CADMIUM CHLORIDE-EXPOSED ICR MICE

We investigated changes of the phosphorylation of SAPK/JNK by GJE glycoprotein in cadmium chloride-treated mice. As shown in Figure 3, treatment with cadmium chloride significantly increased phosphorylation of SAPK/JNK by 1.53-fold, whereas there was no significant difference in the total expression levels of SAPK/JNK. However, treatment with the GJE glycoprotein resulted in a concentration-dependent inhibition of cadmium chloride-induced SAPK/JNK phosphorylation in mice liver. Administrated GJE glycoprotein significantly inhibited the increase of SAPK/JNK phosphorylation in cadmium chloride-treated mice.

INHIBITORY EFFECT OF GJE GLYCOPROTEIN ON PHOSPHORYLATION OF HSP27 IN CADMIUM CHLORIDE-EXPOSED ICR MICE

We investigated phosphorylation of Hsp27 by GJE glycoprotein in cadmium chloride-exposed mice. As shown in Figure 4, when mice were treated with cadmium chloride (2.5 mg/kg), activity of phosphorylation of Hsp27 was significantly increased by 6.28-fold compared to the control. However, when mice were treated with GJE

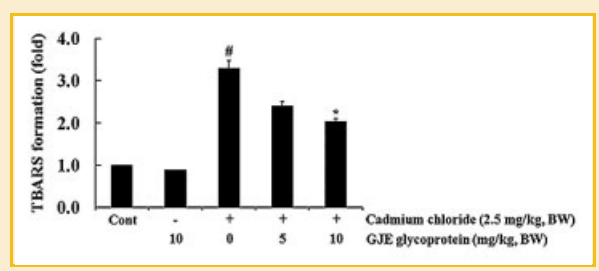

Fig. 1. Inhibitory effect of GJE glycoprotein on lipid peroxidation activity in cadmium chloride-exposed ICR mice. Lipid peroxidation was estimated by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et al. [1979]. All data are the means ± SE from triplicate experiments (n = 6), separately. # Significant difference between treatments and control, $P < 0.05$. * Significant differences between cadmium chloride treatment alone and the GJE glycoprotein treatment in the presence of cadmium chloride, $P < 0.05$. BW: body weight.

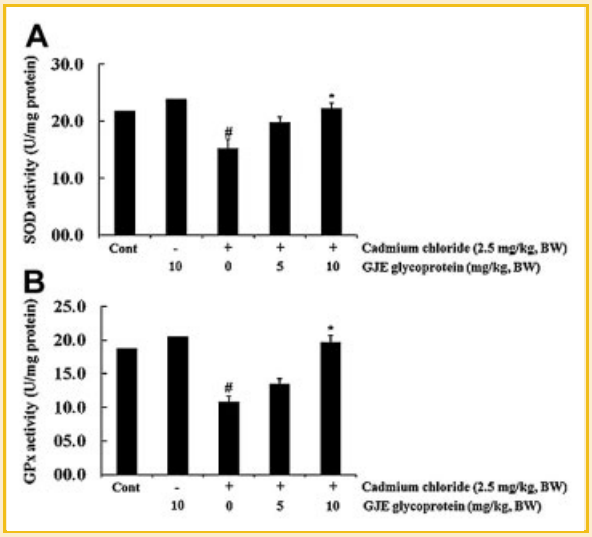

Fig. 2. Effect of GJE glycoprotein on activities of anti-oxidant enzymes (SOD and GPx) in cadmium chloride-exposed ICR mice. The activities of hepatic SOD and GPx in mice liver were measured at 560 and 412 nm, respectively. All data are the means ± SE from triplicate experiments (n = 6), separately. # Significant difference between treatments and control, $P < 0.05$. * Significant differences between cadmium chloride treatment alone and the GJE glycoprotein treatment in the presence of cadmium chloride, $P < 0.05$. BW: body weight.

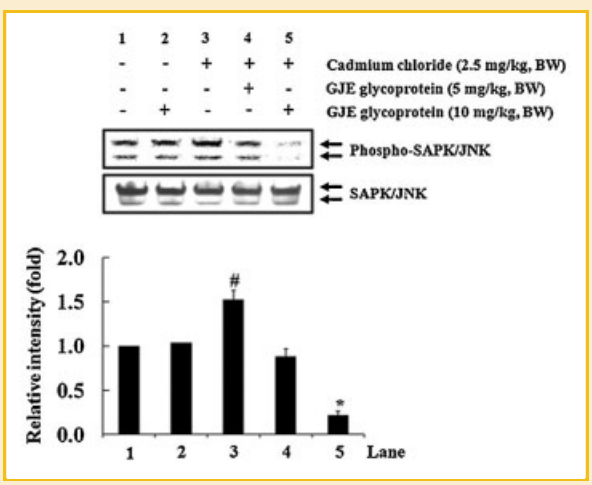

Fig. 3. Inhibitory effect of GJE glycoprotein on expression of SAPK/JNK in cadmium chloride-exposed ICR mice. The relative intensities of bands obtained from Western blot were calculated using Scion Imaging Software (Scion Image Beta 4.02, Frederick, MD). All data are the means ± SE from triplicates, separately. # Significant difference between cadmium chloride treatment alone and control, $P < 0.05$. * Significant differences between cadmium chloride treatment alone and the GJE glycoprotein treatment in the presence of the cadmium chloride, $P < 0.05$. Lane 1, control; lane 2, 10 mg/kg GJE glycoprotein alone; lane 3, cadmium chloride alone; lane 4, 5 mg/kg GJE glycoprotein in the presence of the cadmium chloride; lane 5, 10 mg/kg GJE glycoprotein in the presence of the cadmium chloride. Blot was re-probed for total SAPK/JNK protein to demonstrate equal loading. BW: body weight.

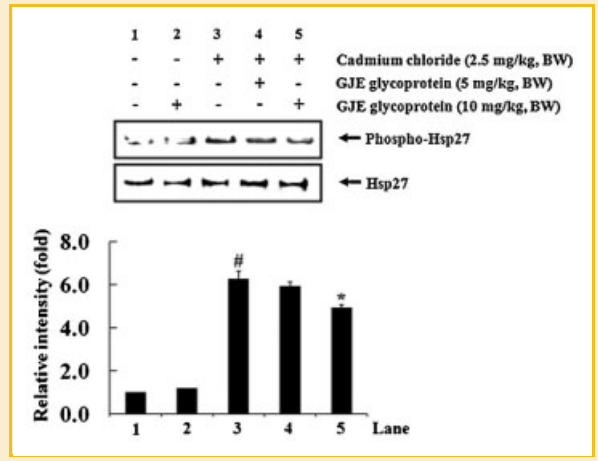

Fig. 4. Inhibitory effect of GJE glycoprotein on phosphorylation of Hsp27 in cadmium chloride-exposed ICR mice. The relative intensities of bands obtained from Western blot were calculated using Scion Imaging Software (Scion Image Beta 4.02, Frederick, MD). All data are the means ± SE from triplicates, separately. # Significant difference between cadmium chloride treatment alone and control, $P < 0.05$. * Significant differences between cadmium chloride treatment alone and the GJE glycoprotein treatment in the presence of the cadmium chloride, $P < 0.05$. Lane 1, control; lane 2, 10 mg/kg GJE glycoprotein alone; lane 3, cadmium chloride alone; lane 4, 5 mg/kg GJE glycoprotein in the presence of the cadmium chloride; lane 5, 10 mg/kg GJE glycoprotein in the presence of the cadmium chloride. Blot was re-probed for total Hsp27 to demonstrate equal loading. BW: body weight.

glycoprotein (5.0 and 10 mg/kg) in the presence of cadmium chloride (2.5 mg/kg), they were significantly increased by 0.03- and 0.28-fold, respectively, compared to the treatment with cadmium chloride alone. Total expression levels of Hsp27 as internal standard were not changed, even though the concentrations or treatments were differently.

INHIBITORY EFFECT OF GJE GLYCOPROTEIN ON EXPRESSION OF AP-1 AND NF-κB IN CADMIUM CHLORIDE-EXPOSED ICR MICE

As shown in Figure 5, treatment with cadmium chloride increased the expression level of AP-1 in the Western blot. The relative intensities of AP-1 (c-Jun) and AP-1 (c-Fos) increased by 1.57- and 1.69-fold in the presence of cadmium chloride, respectively, compared to the control group. However, administration of the GJE glycoprotein inhibited the cadmium chloride-induced increase in expression of AP-1 (c-Jun) and AP-1 (c-Fos) in a concentration-dependent manner. For example, GJE glycoprotein (10 mg/kg) reduced by 0.58- and 0.52-fold in the presence of cadmium chloride, respectively, compared to the cadmium chloride group. Expression of NF-κB in the results of Western blot, the relative intensities of NF-κB (p50) and NF-κB (p65) increased by 2.86- and 1.70-fold in the presence of cadmium chloride, respectively, compared to the control group. However, administration of the GJE glycoprotein inhibited the cadmium chloride-induced increase in expression of NF-κB (p50) and NF-κB (p65) in a concentration-dependent manner. For example, GJE glycoprotein (10 mg/kg) reduced by 0.82- and 0.71-fold in the presence of cadmium chloride,

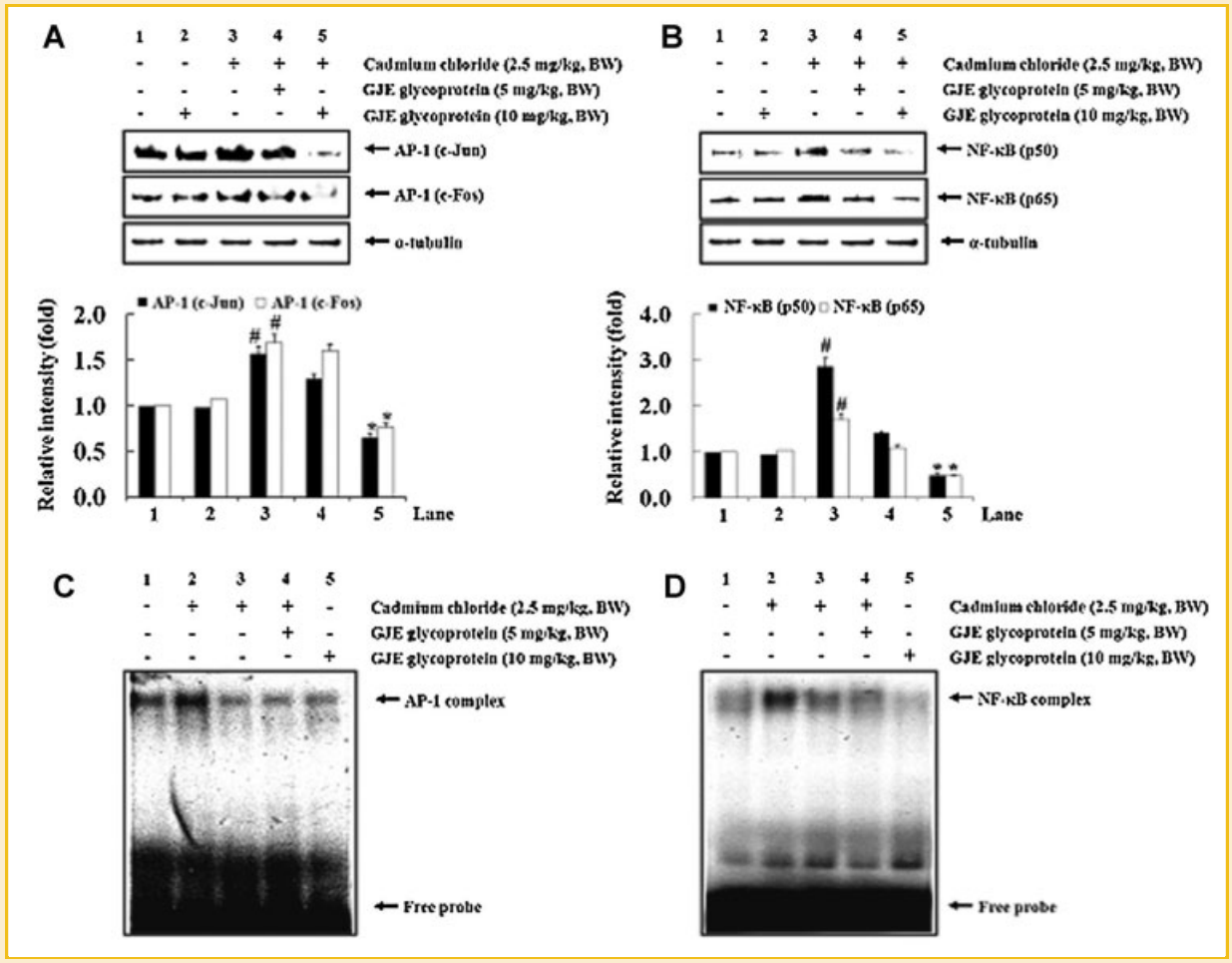

Fig. 5. Inhibitory effect of GJE glycoprotein on expression of AP-1 (c-Jun and c-Fos) and NF-κB (p50 and p65) in cadmium chloride-exposed ICR mice using Western blot and EMSA. The relative intensities of bands obtained from Western blot were calculated using Scion Imaging Software (Scion Image Beta 4.02, Frederick, MD). All data are the means ± SE from triplicates, separately. The subparts A and C indicate the results of AP-1 obtained from Western blot and EMSA. The subparts B and D indicate the results of NF-κB obtained from Western blot and EMSA. # Significant difference between cadmium chloride treatment alone and control, $P < 0.05$. * Significant differences between cadmium chloride treatment alone and the GJE glycoprotein treatment in the presence of the cadmium chloride, $P < 0.05$. Lane 1, control; lane 2, 10 mg/kg GJE glycoprotein alone; lane 3, cadmium chloride alone; lane 4, 5 mg/kg GJE glycoprotein in the presence of the cadmium chloride; lane 5, 10 mg/kg GJE glycoprotein in the presence of the cadmium chloride. α-Tubulin was used as internal standard. BW: body weight.

respectively, compared to the cadmium chloride group. In the results of EMSA for transcriptional factors (AP-1 and NF-κB), it appeared similar tendency like Western blot.

INHIBITORY EFFECT OF GJE GLYCOPROTEIN OF PRO-INFLAMMATORY CYTOKINES (TNF-α AND IL-6) IN CADMIUM CHLORIDE-EXPOSED ICR MICE

As shown in Figure 6, treatment with cadmium chloride increased the expression levels of TNF-α and IL-6 mRNA as compared to the control. However, administration of the GJE glycoprotein inhibited the expression of TNF-α and IL-6 as compared to the cadmium chloride treatment alone. For example, the band intensity of TNF-α and IL-6 significantly decreased 1.27- and 0.58-fold the administration of the GJE glycoprotein (10 mg/kg) in the presence of cadmium chloride.

DISCUSSION

The liver plays a critical role in metabolizing foreign substances, including nutrients, therapeutic drugs, and environmental toxicants, some of which can have an adverse effect on it [Palsamy et al., 2010]. Cadmium is harmful to human beings because of its long lifetime and can cause a number of lesions in many organs like liver. It has been reported that cadmium which has a potential of carcinogen can induce inflammatory responses in several cell types [Waalkes, 2003; Souza et al., 2004] and can induce inflammation in rodents at treatment with chronic or acute cadmium intoxication [Kataranovski et al., 1998; Dan et al., 2000]. Particularly, acute cadmium exposure in experimental animals results primarily in accumulation of the metal in the liver and acute hepatotoxicity, which is well-studied experimental toxicological model [Dudley et al., 1982]. In this study, we therefore tried to understand the

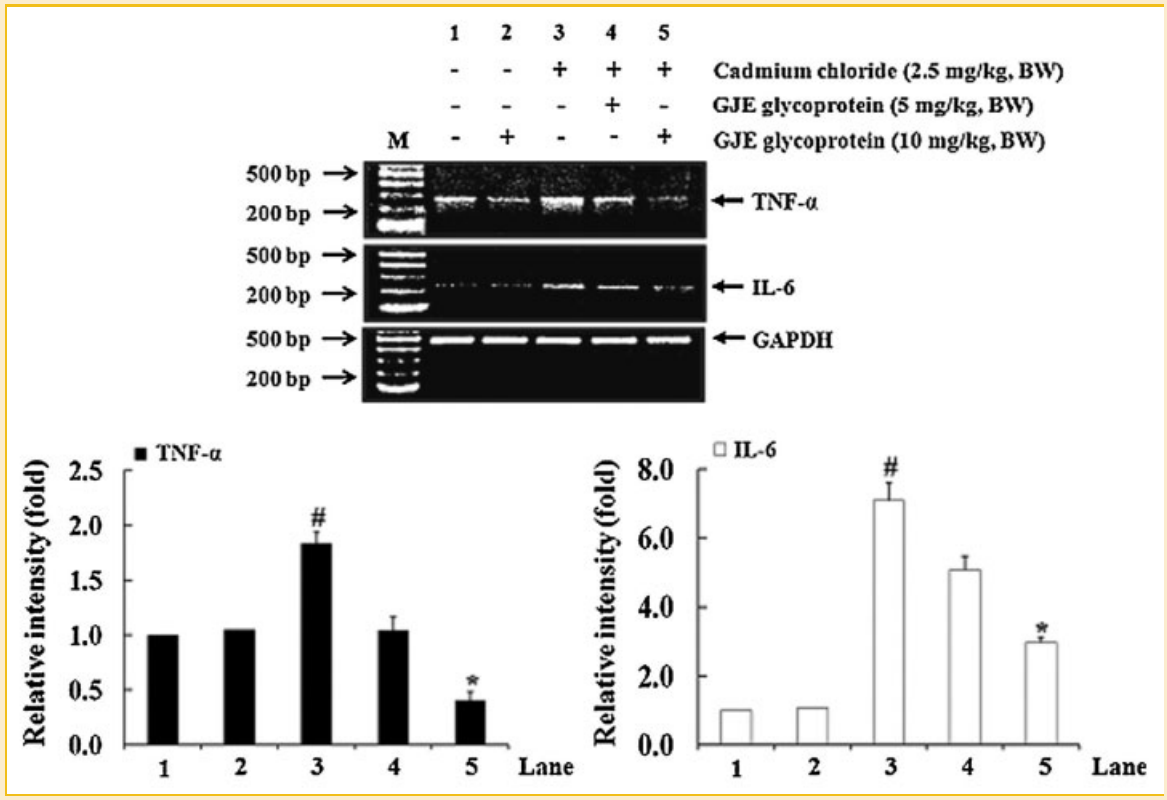

Fig. 6. Inhibitory effect of GJE glycoprotein on expression of pro-inflammatory cytokines (TNF-α and IL-6) in cadmium chloride-exposed ICR mice. The expressions of TNF-α and IL-6 mRNAs were determined by RT-PCR using total RNA isolated from cadmium chloride-exposed mouse liver tissue. The relative intensities of bands were calculated using Scion Imaging Software (Scion Image Beta 4.02, MD). # Significant difference between treatments and control, $P < 0.05$. * Significant difference between cadmium chloride treatment alone and treatment with the GJE glycoprotein in the presence of cadmium chloride, $P < 0.05$. M, molecular weight maker; lane 1, control; lane 2, 10 mg/kg GJE glycoprotein alone; lane 3, cadmium chloride alone; lane 4, 5 mg/kg GJE glycoprotein in the presence of the cadmium chloride; lane 5, 10 mg/kg GJE glycoprotein in the presence of the cadmium chloride. GAPDH was used as an internal control. BW: body weight.

possible mechanism of inflammation and to elucidate inhibitory effect of GJE glycoprotein in cadmium chloride-exposed ICR mice.

In order to know GJE glycoprotein has inhibitory effect to hepatotoxicity, we firstly evaluated liver swelling and anti-oxidant activity by cadmium chloride. LDH is an intracellular enzyme, the increase of which in serum is an indicatory of cell damage [Kim et al., 2001]. It has been reported that liver injury followed by cadmium exposure is well established by the elevated levels serum hepatic marker enzymes indicating the cellular leakage and loss of functional integrity of hepatic membrane architecture [Yogalakshmi et al., 2010]. Also, high levels of ALT are the crucial parameters to detect liver damage [Yogalakshmi et al., 2010]. As shown in Table I, the liver weights at treatment with cadmium chloride are significantly increased and swollen, the reason why the liver weight is increased by treatment with cadmium chloride, is probably caused by swelling liver mitochondria. When the broken traffic balance of cation ion occurs because of having similar radius of both metal ion (Cd^{2+} and Ca^{2+}), the mono- and di-cations penetrate from extracellular membrane into cytoplasm. Thus, it appears osmotic pressure at which the mitochondria of liver cells are swollen. Subsequently, it results in the gaining liver weight [Korotkov et al., 1998; Swandulla and Armstrong, 1989]. Additionally, cadmium chloride-induced hepatic damage was indicated by a marked increase of LDH and ALT levels in serum (Table I). However, GJE glycoprotein significantly decreased LDH and ALT levels in cadmium chloride exposed to ICR mice. It suggesting GJE glycoprotein may be potent to protect the hepxototoxicity.

It has been reported that activation of inflammatory cells induces generation of reactive oxygen species and lipid peroxidation which are implicated as a key event in cadmium hepatotoxicity [Liu et al., 2004]. Cadmium is known to produce oxidative damage in the liver by enhancing peroxidation of membrane lipids, a deleterious process solely carried out by free radicals [Hassoun and Stohs, 1996]. The elevated levels of lipid peroxides in the liver reveal the degree of lipid peroxidation in hepatic tissues and are considered as the indicator of hepatocyte damage [Albano, 2008]. Our results indicated that the GJE glycoprotein reduced the levels of the TBARS formation in cadmium chloride-induced liver (Fig. 1).

In order to more clarify the mechanisms of hepatoprotective activity of GJE glycoprotein, the effect on hepatic anti-oxidant defense system was explored. SOD and GPx are two anti-oxidant enzyme which play a critical role in the cellular defense against the deleterious action of ROS and cellular products of free radical chain reactions. While SOD catalyzes the conversion of superoxide free radical to less toxic hydrogen peroxide, GPx catalyzes the breakdown of hydrogen peroxide into water and oxygen and can

also directly detoxify lipid peroxides generated by ROS [Castro and Freeman, 2001]. However, distinct responses of anti-oxidant defense have been reported in animals exposed to cadmium [Liu et al., 2009]. SOD and GPx were well known as anti-oxidative enzyme in the existing liver tissue. In general, the activities of those enzymes are increased at treatment with strong anti-oxidants, while they are deactivated at exposure of toxicants to liver tissue. The results obtained from this study pointed that the activities of SOD and GPx are remarkably reduced at treatment with cadmium chloride alone (i.p.), while their activities are definitely augmented at treatment with cadmium chloride (i.p.) under condition of the pretreatment with GJE glycoprotein. The possible mechanism of the decreasing activity of SOD may be that cadmium contributes either to bind active site of three-dimensional structure of SOD or to disturb Cu/Zn or Mn, because they exist in the SOD enzyme itself. There are two kinds of SOD, containing Cu/Zn in cytoplasm or Mn in mitochondria. The decreasing activity of SOD could be deduced from cadmium-induced lipid peroxidation [Tang et al., 1998; Joseph, 2009]. In the other hand, the possible mechanism of the decreasing activity of GPx might be that cadmium contributes either to bind active site of three-dimensional structure of GPx or to disturb Se, because selenium exists in the GPx enzyme itself [Gambhir and Nath, 1992; Joseph, 2009]. In the increasing activities of SOD and GPx, the reason why the results obtained from treatment with GJE glycoprotein shows the increasing activities of SOD and GPx, might be caused by scavenging the intracellular ROS produced by cadmium-induced lipid peroxidation, because GJE glycoprotein has a strong anti-oxidative character. Otherwise, GJE glycoprotein blocks function of cadmium chloride which has divalent positive ion, because GJE glycoprotein has many hydroxyl ion which has negative charge on the carbohydrate in the whole molecule of glycoprotein. Thus, the activities of anti-oxidative enzymes are appeared in the increasing tendency. The results obtained from this study indicated that activities of these enzymes (SOD and GPx) were reduced by treatment with cadmium chloride alone, whereas their activities were augmented at the orally administration of GJE glycoprotein in the presence of cadmium chloride (Fig. 2). Therefore, these finding suggest that GJE glycoprotein might be to use for prevention of cadmium chloride-induced liver injury because of having strong anti-oxidant character as one of component of health supplemental food.

The mitogen-activated protein kinases (MAPK) pathways are important for signal transduction in response to oxidative stress. Especially, JNK (one of MAPKs) is well known for specifically pro-inflammatory cytokine and AP-1 as inflammation-related transcription factor. A number of cell responses to stress factors are mediated by SAPK pathways [Tibbles and Woodgett, 1999]. The SAPK/JNK and p38 MAPK targets are all nuclear and induce dramatic and controversial effects on transcriptional and post-transcriptional processes, growth arrest, inflammation and transformation [Tibbles and Woodgett, 1999]. Also, it has been reported that cadmium is known to activate the MAPK pathway via generation of ROS [Qu et al., 2006]. We therefore investigated that whether GJE glycoprotein inhibits the expression of JNK1/2 in cadmium chloride-exposed ICR mice. The results obtained from Western blot assay indicated that SAPK/JNK is significantly activated by cadmium chloride treatment, while it is deactivated by treatment with GJE glycoprotein in the presence of cadmium chloride (Fig. 3). In other words, the reason why SAPK/JNK is deactivated by GJE glycoprotein, is that intracellular ROS as upstream signal factor was already blocked by GJE glycoprotein which has a strong anti-oxidative character. Therefore, activity of SAPK/JNK as downstream signal was apparently diminished at treatment with GJE glycoprotein in the result of Western blot assay.

It has been reported that small heat shock proteins (Hsp) play critical roles in variety of cellular type and tissue such as to suppress protein aggregation, to move cytoskeletal proteins, and to differentiate cells [Perng et al., 1999]. Hsp27 is a ubiquitously expressed member of the heat shock protein family that has been implicated in various biological functions including the response to heat shock, oxidative stress, and cytokine treatment. Also, Hsp27 is a powerful ATP-independent chaperone and generally lowers in unstressed cells. During stress response, Hsp27 levels increased dramatically, and Hsp27 becomes phosphorylated on its serine residues, assuming a cytoprotective role. As shown in Figure 4, treatment cadmium chloride increased Hsp27 phosphorylated. However, GJE glycoprotein treatment in the presence cadmium chloride decreased Hsp27 phosphorylated. Recently, it has been reported that the exposure to heavy metals such as cadmium may induce the expression of Hsp27 [Croute et al., 2005]. Also, Hsp genes have been found to have a putative AP-1 binding site by SAPK/JNK [Assimakopoulou and Varakis, 2001].

AP-1 and NF-κB have been implicated as playing important roles in the regulation of a vast variety of genes involved in the modulation of processes importation in inflammatory event [Koj, 1996]. Also, it has been reported that induction of AP-1 and NF-κB by pro-inflammatory cytokines is mostly mediated by the JNK cascade [Shaulian and Karin, 2002]. As shown in Figure 5, GJE glycoprotein inhibited activity AP-1 and NF-κB in mouse liver in a dose-dependent manner. Thus, the results of this study assume that GJE glycoprotein has an inhibitory effect in pro-inflammatory cytokine caused by cadmium chloride.

It has been reported that AP-1 and NF-κB activation are closely related to the regulation of pro-inflammatory cytokine expression (TNF-α and IL-6) [Yang et al., 2009]. It has reported that Hsp27 stimulate NF-κB (p65 and p50) and then the activated NF-κB contributes to expression of TNF-α and IL-6 [Miwa et al., 2000; Singh-Jasuja et al., 2000; Parcellier et al., 2003; Park et al., 2003; Leal et al., 2007; Guelden et al., 2008]. Several cytokines such as TNF-α and IL-6 are important in the initiation stage of inflammatory responses. Increased levels of TNF-α and IL-6 activity are indicators of inflammation at the systemic level and might result from cadmium-induced cell injury or cytokine from local inflammation in the peritoneal cavity [Kataranovski et al., 2009]. A role for the pro-inflammatory cytokine TNF-α and IL-6 has been shown in cadmium-mediated hepatotoxicity [Kayama et al., 1995]. The results in the present study indicated that activities of TNF-α and IL-6 are increased by cadmium chloride but significantly decreased by treatment with the GJE glycoprotein in the presence of cadmium chloride. It notes that GJE glycoprotein is orally administrated from 1 week before injection (i.p.) of cadmium chloride to the end of experimental period (Fig. 6). These results suggest that the

hepatoprotective effect of the GJE glycoprotein result from its ability to reduce liver expression of pro-inflammatory proteins.

The results obtained from JNK phosphorylation experiment (Fig. 3) indicated that the intensity of GJE glycoprotein (5 mg/kg) in the presence of cadmium chloride (2.5 mg/kg) was a little higher than that of the control. However, the intensity of GJE glycoprotein (10 mg/kg) in the presence of cadmium chloride (2.5 mg/kg) was significantly lower than that of control. The possible explanations for the mechanisms what the lowering inhibitory effect of GJE glycoprotein (10 mg/kg) indicate compared to the control, might be follows. Treatment of GJE glycoprotein (5 mg/kg) interacts with cadmium chloride (2.5 mg/kg) just like in the equivalent molar reaction (GJE glycoprotein/cadmium = 2:1), because GJE glycoprotein has many hydroxyl functional group (OH–) but cadmium chloride has divalent cation character. However, when the concentration of GJE glycoprotein (10 mg/kg) which means excess, is more than equivalent molar reaction, the exceeded GJE glycoprotein might block the phosphorylation of JNK, because phosphorylation of certain kinases (JNK) in the intracellular signal pathway can be competed with glycosylation in order to activate kinases (JNK). Another assumption is that the cells and tissues treated by cadmium chloride were more susceptible to react than in the normal cells and tissues. Subsequently they were responded by the exceeded GJE glycoprotein much more than that of in the normal cells and tissues which were treated by the control or by just GJE glycoprotein alone. Thus, JNK phosphorylation at GJE glycoprotein (10 mg/kg) in the presence of cadmium chloride (2.5 mg/kg) in this study was shown lower than that of control due to block phosphorylation through glycosylation. From point of these views, the results in this study might be brought about that the intensities of AP-1 (Fig. 5) and TNF-α (Fig. 6) were systemically lowered compared to the control, as if expressions of AP-1 (Fig. 5) and TNF-α are depended on JNK activation at treatment of GJE glycoprotein (10 mg/kg) in the presence of cadmium chloride (2.5 mg/kg). By the way, when GJE glycoprotein (10 mg/kg, exceeded more than equivalent molarity) treatment alone (without cadmium chloride) is, it could not react with none. That is why GJE glycoprotein cannot influence any intensity, even though it was changed tiny (trace, minute) intensity.

In conclusion, the results in the current study indicated that GJE glycoprotein decrease ALT, LDH, and TBARS levels, whereas increase the anti-oxidant enzymes (SOD and GPx). In the possible signal pathway, GJE glycoprotein prevents liver injury from expression of the inflammation-related factors (TNF-α and IL-6) activated by cadmium chloride through inactivation of JNK, Hsp27, and transcriptional factors (AP-1 and NF-κB). Thus, the results obtained from this study indicate that GJE glycoprotein might be used to prevent liver inflammation. Still, problem remains to elucidate what kind of signal factors are modulated by GJE glycoprotein from membrane receptor to gene expression of inflammation-related mediators, systemically.

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