

## Full Paper

**Glycoprotein Isolated From *Ulmus davidiana* NAKAI Protects Against Carbon Tetrachloride-Induced Liver Injury in the Mouse**Jeong-Hyeon Ko<sup>1</sup> and Kye-Taek Lim<sup>1,\*</sup><sup>1</sup>#521, Molecular Biochemistry Laboratory, Institute of Biotechnology, Chonnam National University, 300 Yongbong-Dong, Kwangju 500-757, Korea

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**Abstract.** *Ulmus davidiana* NAKAI (UDN) has traditionally been used for healing of inflammatory diseases. This study was carried out to investigate the hepatoprotective effect of the glycoprotein isolated from UDN in carbon tetrachloride (CCl<sub>4</sub>)-induced liver injury. We evaluated the activities of alanine aminotransferase (ALT), lactate dehydrogenase (LDH), thiobarbituric acid-reactive substances (TBARS), and antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)] activities in CCl<sub>4</sub>-treated mice. When mice were treated with CCl<sub>4</sub> in the absence of UDN glycoprotein, the activities of ALT, LDH, and TBARS were increased, while the antioxidant enzymes activities were decreased. However, when the mice were treated with CCl<sub>4</sub> in the presence of UDN glycoprotein, the activities of ALT, LDH, and TBARS were significantly reduced and SOD, CAT, and GPx activities were remarkably increased. In addition, UDN glycoprotein increased the nitric oxide production and decreased the nuclear factor-kappa B and activator protein-1 activation in CCl<sub>4</sub>-treated mice. We also investigated the protective effects of UDN glycoprotein in glucose/glucose oxidase (G/GO)-induced cytotoxicity in primary cultured mouse hepatocytes. UDN glycoprotein markedly inhibited the cell death induced by G/GO. These results suggest that UDN glycoprotein protects against CCl<sub>4</sub>-induced liver injury in the mouse.

**Keywords:** *Ulmus davidiana* NAKAI glycoprotein, antioxidant enzyme, nitric oxide, nuclear factor-kappa B, activator protein-1

**Introduction**

Reactive oxygen species (ROS) including oxygen free radicals are causative factors in the etiology of degenerative diseases, including some hepatopathies (1). The enhanced production of free radicals and oxidative stress can be induced by a variety of factors such as ionizing radiation or exposure to drug and xenobiotics (e.g., carbon tetrachloride). Carbon tetrachloride (CCl<sub>4</sub>) has been used extensively to study liver injury induced by free radicals in an animal model system. Liver damage (inflammation) caused by CCl<sub>4</sub> in mouse model system is closely to analogue of hepatotoxicity in human. In the principle of liver damage, CCl<sub>4</sub> is reduc-

tively bioactivated by cytochrome P450 2E1 into a trichloromethyl radical ( $\cdot\text{CCl}_3$ ), which is subsequently converted into a peroxy radical ( $\cdot\text{OOCCL}_3$ ) in the presence of oxygen. These reactive free radical metabolites may cause cellular damage by initiating lipid peroxidation and covalently binding to macromolecules (2–4).

Antioxidative action plays an important role in protection against CCl<sub>4</sub>-induced liver injury. Protective effects of various natural products against CCl<sub>4</sub> hepatotoxicity have been reported (5–8). *Ulmus davidiana* NAKAI (UDN) is widely distributed in Korea and has been used for treatment of inflammation, edema, mastitis, and gastric cancer in oriental medicine (9). Recently, we have found a glycoprotein with an approximate molecular mass of 116 kDa, isolated from UDN. UDN glycoprotein has pharmacological activities such as anti-apoptotic and anti-oxidative effects, indicating that UDN glycoprotein is a functional substance with multi-

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ple biological activities (10, 11). Based on the results from our experiments, we speculated that UDN glycoprotein might be effective against diseases in which ROS play a role as potent causative factors because it has a strong antioxidant activity.

Therefore, we examined the preventive effect of UDN glycoprotein on CCl<sub>4</sub>-induced mouse liver injury and elucidated the possible mechanisms of these protective effects.

## Materials and Methods

### Chemicals

5-Bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium (BCIP/NBT) mixture solution, collagenase, glutathione peroxidase (GPx),  $\beta$ -nicotinamide adenine dinucleotide ( $\beta$ -NADH), olive oil, superoxide dismutase (SOD), 1,1,1,3-tetraethoxypropane, thiobarbituric acid, and Williams's Medium E were obtained from Sigma (St. Louis, MO, USA). CCl<sub>4</sub> was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). L-Alanine and L-lactate dehydrogenase (LDH) were obtained from Fluka (Buchs, Switzerland). Specific antibodies of nuclear factor-kappa B (NF- $\kappa$ B) (p50), c-fos, c-jun,  $\beta$ -actin, and alkaline phosphatase-conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals and reagents were of the highest quality available.

### Preparation of UDN glycoprotein

Samples of UDN were obtained from the Naju traditional market in the Chonnam province of South Korea, and glycoprotein was isolated from it as described previously (10). Briefly, UDN was chopped into small portions and soaked in 99% ethanol for several months in a dark basement. The ethanol extract was filtered through Whatman filter paper (No. 2) and concentrated with a rotary evaporator (B465; Buch, Flawil, Switzerland). The concentrated solution was dried with a freeze-dryer (SFDS06; Sam Won, Seoul, Korea). Five grams of dried-crude ethanol extract dissolved in distilled water was applied to the silica gel column (4  $\times$  28 cm, 28–200 mesh, 22 Å). An ordered elution was performed using distilled water, 70% ethanol, absolute ethanol, and 5% acetic acid. Only the solution eluted with 70% ethanol was collected and lyophilized to 2.5 g (50% of the initial amounts), because it was shown to have a biological function in trials before the experiment. The dried powder was dissolved again with distilled water. The solution was precipitated with 80% ammonium sulfate and then dialyzed with a dialysis membrane (Spectra/por,

MWCO 6000-8000; Spectrum, Pasadena, CA, USA) against 20 mM Tris-HCl (pH 7.4) at 4°C overnight. After dialysis, the contained solution was dried with a freeze-dryer and stored at -70°C. After electrophoresis, the gels were stained with a Schiff reagent (12) for the glycoprotein from UDN. The UDN glycoprotein was eluted with an electro-eluter (Mini Whole Gel Eluter; Bio-Rad, San Francisco, CA, USA). The final amount of UDN glycoprotein was 5 mg (0.1%) from the initial UDN sample. In this study, we used UDN glycoprotein with a molecular weight of 116 kDa.

### Animals and treatments

Male ICR mice, aged 5 weeks, were purchased from Daehan Lab. (Animal Research Center Co., Ltd., Dae Jeon, Korea) and housed according to the animal care guidelines approved by the Animal-care Committee of the American Society of Mammalogists (13) at the Experimental Animal Room of Veterinary College of Chonnam National University. All mice were fed a commercial diet and water ad libitum, and kept for at least 1 week prior to the experiments.

Mice were divided into the following five study groups:

- 1) Control (n = 6),
- 2) Control + UDN glycoprotein (80 mg/kg) (n = 6),
- 3) CCl<sub>4</sub> (n = 6),
- 4) CCl<sub>4</sub> + UDN glycoprotein (40 mg/kg) (n = 6),
- 5) CCl<sub>4</sub> + UDN glycoprotein (80 mg/kg) (n = 6).

UDN glycoprotein was dissolved in PBS and then administered orally with 40 and 80 mg/kg body weight UDN glycoprotein into mice once a day for 3 days. One hour after the final treatment, mice were treated with CCl<sub>4</sub> at a dose of 0.5 ml/kg body weight intraperitoneally dissolved in olive oil (1:1, v/v). At 24 h after CCl<sub>4</sub> administration, the mice of each group were anesthetized with ether and the blood drawn by cardiac puncture. The samples were centrifuged, the plasma was isolated and stored at -70°C for use future experiments. Mice were weighed before and after the defined treatments for each group. Livers were also extracted after dissection and then were weighed.

### Plasma alanine transaminase (ALT) and LDH activities

The activity of plasma ALT and LDH were measured as a marker of liver injury by the method of Bergmeyer and Bernt (14, 15).

### Lipid peroxidation

Lipid peroxidation was estimated by measuring the formation of thiobarbituric acid-reactive substances (TBARS). The method of Uchiyama and Mihara (16) was used for the hepatic TBARS. Briefly, 1 g of liver

was homogenized in 9 ml of KCl 1.15% (w/v) and the homogenate was filtered through 4 layers of gauze. A volume of 0.5 ml of liver homogenate was mixed with 3 ml of  $\text{H}_3\text{PO}_4$  1% (v/v) and 1 ml of TBA 0.6% (w/v) and heated at  $100^\circ\text{C}$  for 45 min. The samples were allowed to reach room temperature and 3 ml of *n*-butanol was added. After a vigorous shaking, the butanolic phase was obtained by centrifugation at  $4,000 \times g$  for 10 min to determinate the absorbance at 535 nm. For determination of plasma TBARS level according to the method of Buege and Aust (17), one volume of sample was mixed thoroughly with two volumes of stock solution consisting of 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, and 0.25 N HCl. The mixture was heated for 30 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at  $1,000 \times g$  for 10 min and the absorbance of the sample was measured at 535 nm. 1,1,1,3-Tetraethoxypropane was used as the standard.

#### *Antioxidant enzymes activities*

The antioxidant enzyme activities in the liver were measured as described previously (18) according to the methods of Beauchamp and Fridovich (19), Thomson et al. (20), and Paglia and Valentine (21).

#### *Western blot analysis*

Liver tissues were homogenized in lysis buffer (0.6% NP-40, 150 mM NaCl, 10 mM HEPES (pH 7.9), 1 mM EDTA, and 0.5 mM PMSF) at  $4^\circ\text{C}$ . Nuclear extracts were prepared by modification of the method of Deryckere and Gannon (22). Protein concentrations were measured by the method of Lowry et al. (23). Fifty micrograms of protein was fractionated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were incubated with primary antibodies overnight at  $4^\circ\text{C}$  using 1:3,000 dilution of goat polyclonal anti-rabbit NF- $\kappa\text{B}$  (p50), c-fos, c-jun, and  $\beta$ -actin antibodies. After washing, the membranes were incubated with 1:10,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG as the second antibody for 1 h. Antibody binding was visualized by incubation with BCIP/NBT mixture solution. Bands of NF- $\kappa\text{B}$  and activator protein-1 (AP-1) in the results of Western blot analysis were quantified using Scion Imaging Software (Scion Image Beta 4.02; Scion, Frederick, MD, USA) and represented in the relative intensities to the control.

#### *Determination of nitric oxide (NO)*

NO production was indirectly assessed by measuring

the nitrite levels in plasma determined by a calorimetric method based on the Griess reaction (24). Plasma samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at  $10,000 \times g$  for 5 min at room temperature, 100  $\mu\text{l}$  supernatant was applied to a microtiter plate well, followed by 100  $\mu\text{l}$  of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid). After 10 min of color development at room temperature, the absorbance was measured at 540 nm with a Micro-Reader (Hyperion, Inc., Miami, FL, USA). Nitrite was quantified by using sodium nitrate as a standard curve.

#### *Primary culture of mouse liver tissue*

Hepatocytes were made from liver tissue of ICR mouse (5- to 7-week-old male) using the two-step collagenase perfusion technique of Seglen (25) with some modifications. Cell viability measured by trypan blue exclusion was more than 85%–90%. The single hepatocytes were plated at a density of  $1 \times 10^6$  cells/ml into 6-well plates or 96-well plates for cytotoxicity assays, which were coated with mouse tail collagen according to the method of Michalopoulos and Pitot (26) and were incubated in Williams's Medium E containing 10% FBS, 5  $\mu\text{g}/\text{ml}$  insulin, 2  $\mu\text{g}/\text{ml}$  dexamethasone, 5 KIU/ml aprotinin, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere. After an attachment period of 4 h, the medium was replaced by fresh serum-free medium, and cells were cultured overnight and then used for experiments.

#### *MTT assay*

Cell viability was assessed by MTT assay as described previously (27). Briefly, the cultured hepatocytes were washed with fresh serum-free medium, and then treated with glucose/glucose oxidase (G/GO) in the same medium containing 0.5%  $\beta$ -D-glucose in the presence and absence of UDN glycoprotein (50 and 100  $\mu\text{g}/\text{ml}$ ). Following exposure, 2  $\mu\text{l}$  of the MTT solution (5 mg/ml) was added into each well, and the plates were incubated at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  atmosphere for an additional 4 h. After removing the medium, acidic isopropanol (70  $\mu\text{l}$ ) was added into each well. The plates were read on a microplate reader (Dynatech Microelisa Reader, S/N UVT06235; Molecular Devices, Sunnyvale, CA, USA) at 570 nm.

#### *Statistical analyses*

All experiments were done in triplicate, and the results were represented as the mean  $\pm$  S.D. The Duncan

test and a one-way analysis of variance (ANOVA) were used to compare the means of separate replicates (SPSS program, ver. 10.0).

## Results

### *Effect of UDN glycoprotein on hepatotoxicity in CCl<sub>4</sub>-treated mouse*

CCl<sub>4</sub> significantly decreased body weight and increased liver weight, compared to the control. However, UDN glycoprotein reversed the body weight loss and liver swelling induced by CCl<sub>4</sub> to the levels of the control group (Table 1). The levels of ALT and LDH were measured in the plasma to evaluate hepatic tissue damage (Table 1). The results showed that UDN glycoprotein did not show a significant hepatotoxicity. A significant increase the levels of ALT and LDH were observed in the CCl<sub>4</sub>-treated group. Administrations of UDN glycoprotein significantly reduced the increased plasma ALT and LDH levels.

### *Effect of UDN glycoprotein on lipid peroxidation in CCl<sub>4</sub>-treated mouse*

TBARS levels were assessed as an indicator of lipid peroxidation. CCl<sub>4</sub> treatment significantly increased the level of TBARS in the plasma and liver tissue.

However, pretreatment of UDN glycoprotein significantly decreased the increased level of TBARS in both the plasma and liver tissue (Tables 1 and 2).

### *Effect of UDN glycoprotein on antioxidant enzymes activities in CCl<sub>4</sub>-treated mouse liver*

The hepatic antioxidant enzyme activities (SOD, catalase (CAT), and GPx) are shown in Table 2. The SOD, CAT, and GPx activities in CCl<sub>4</sub>-treated mice were decreased, compared to the control. Interestingly, the pretreatment with UDN glycoprotein significantly increased the decreased SOD, CAT, and GPx activities in the CCl<sub>4</sub>-treated group.

### *Effect of UDN glycoprotein on NO production in CCl<sub>4</sub>-treated mouse*

As shown in Fig. 1, the production of NO in mouse plasma was significantly reduced in CCl<sub>4</sub>-treated mice, compared to the control group. However, pretreatment of UDN glycoprotein increased the NO production in CCl<sub>4</sub>-treated mice. For example, NO production in the control and UDN glycoprotein treatment group was 18.0 and 18.9  $\mu$ M, while it was 10.9  $\mu$ M at the treatment of CCl<sub>4</sub>. However, the NO production in the CCl<sub>4</sub>-treated mice was significantly augmented by 3.9 and 7.5  $\mu$ M at 40 and 80 mg/kg UDN glycoprotein pretreatment,

**Table 1.** Effects of UDN glycoprotein on plasma ALT, LDH, and TBARS in CCl<sub>4</sub>-treated mice

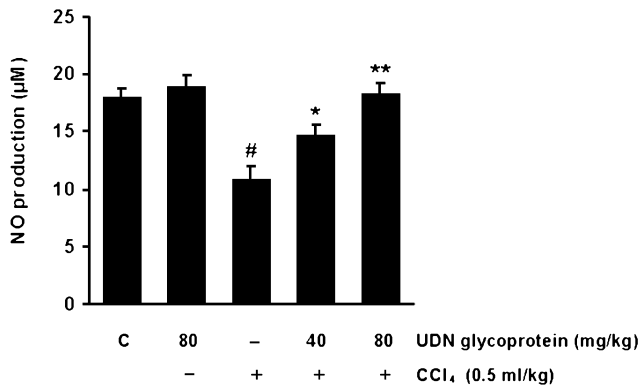
|                     | Control          | UDN (80 mg/kg)   | CCl <sub>4</sub>               | CCl <sub>4</sub> + UDN (mg/kg) |                    |
|---------------------|------------------|------------------|--------------------------------|--------------------------------|--------------------|
|                     |                  |                  |                                | 40                             | 80                 |
| ALT (U/L)           | 11.3 $\pm$ 1.4   | 13.8 $\pm$ 1.2   | 39.4 $\pm$ 3.1 <sup>##</sup>   | 27.6 $\pm$ 1.1*                | 15.8 $\pm$ 0.8**   |
| LDH (U/L)           | 234.6 $\pm$ 44.6 | 248.6 $\pm$ 37.3 | 539.7 $\pm$ 91.3 <sup>##</sup> | 398.4 $\pm$ 24.0*              | 298.6 $\pm$ 15.3** |
| TBARS ( $\mu$ M/L)  | 0.25 $\pm$ 0.02  | 0.25 $\pm$ 0.04  | 0.50 $\pm$ 0.08 <sup>#</sup>   | 0.44 $\pm$ 0.02                | 0.30 $\pm$ 0.02*   |
| Relative weight (%) |                  |                  |                                |                                |                    |
| Body                | 99.03 $\pm$ 0.06 | 98.82 $\pm$ 1.15 | 90.60 $\pm$ 1.28 <sup>#</sup>  | 94.73 $\pm$ 0.42               | 97.11 $\pm$ 0.84*  |
| Liver               | 3.91 $\pm$ 0.05  | 4.02 $\pm$ 0.11  | 5.46 $\pm$ 0.21 <sup>#</sup>   | 5.27 $\pm$ 0.06                | 4.80 $\pm$ 0.07*   |

ALT, alanine aminotransferase; LDH, lactate dehydrogenase; TBARS, thiobarbituric acid-reactive substances. The values are mean  $\pm$  S.D. from six mice done in triplicates. <sup>#</sup>*P*<0.05, <sup>##</sup>*P*<0.01, as compared to the control group. \**P*<0.05, \*\**P*<0.01, as compared to the CCl<sub>4</sub>-treated group.

**Table 2.** Effects of UDN glycoprotein on antioxidant enzymes (SOD, CAT, GPx) activities and TBARS in the liver of CCl<sub>4</sub>-treated mice

|                          | Control         | UDN (80 mg/kg)              | CCl <sub>4</sub>             | CCl <sub>4</sub> + UDN (mg/kg) |                  |
|--------------------------|-----------------|-----------------------------|------------------------------|--------------------------------|------------------|
|                          |                 |                             |                              | 40                             | 80               |
| SOD (U/mg protein)       | 21.8 $\pm$ 1.2  | 25.9 $\pm$ 1.0 <sup>#</sup> | 13.6 $\pm$ 0.8 <sup>#</sup>  | 17.8 $\pm$ 1.1*                | 24.3 $\pm$ 0.7** |
| CAT (U/mg protein)       | 17.3 $\pm$ 0.8  | 20.5 $\pm$ 1.1 <sup>#</sup> | 10.7 $\pm$ 0.7 <sup>#</sup>  | 14.5 $\pm$ 0.9*                | 19.7 $\pm$ 1.2** |
| GPx (U/mg protein)       | 10.8 $\pm$ 0.7  | 12.5 $\pm$ 0.8 <sup>#</sup> | 8.2 $\pm$ 0.5 <sup>#</sup>   | 9.3 $\pm$ 0.7                  | 11.4 $\pm$ 0.9*  |
| TBARS ( $\mu$ M/g liver) | 0.54 $\pm$ 0.01 | 0.53 $\pm$ 0.02             | 1.15 $\pm$ 0.07 <sup>#</sup> | 0.67 $\pm$ 0.02*               | 0.54 $\pm$ 0.04* |

SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; TBARS, thiobarbituric acid-reactive substances. The values are mean  $\pm$  S.D. from six mice done in triplicates. <sup>#</sup>*P*<0.05, as compared to the control group. \**P*<0.05, \*\**P*<0.01, as compared to the CCl<sub>4</sub>-treated group.



**Fig. 1.** Effect of UDN glycoprotein on NO production in CCl<sub>4</sub>-treated mice. The mice were pretreated with UDN glycoprotein (40 and 80 mg/kg body weight) orally once a day for 3 days. One hour after the final treatment, mice were treated with CCl<sub>4</sub> (0.5 ml/kg body weight, i.p.). The mice were killed 24 h after CCl<sub>4</sub> treatment. The production of NO was assessed indirectly by measuring the nitrite levels in plasma determined by the Griess reaction. The values are mean  $\pm$  S.D. from six mice done in triplicate. # represents the difference between treatment and control,  $P < 0.01$ ; \* represents the difference between treatment and CCl<sub>4</sub>-treated group,  $P < 0.05$ ; \*\* represents the difference between treatment and CCl<sub>4</sub>-treated group,  $P < 0.01$ .

respectively.

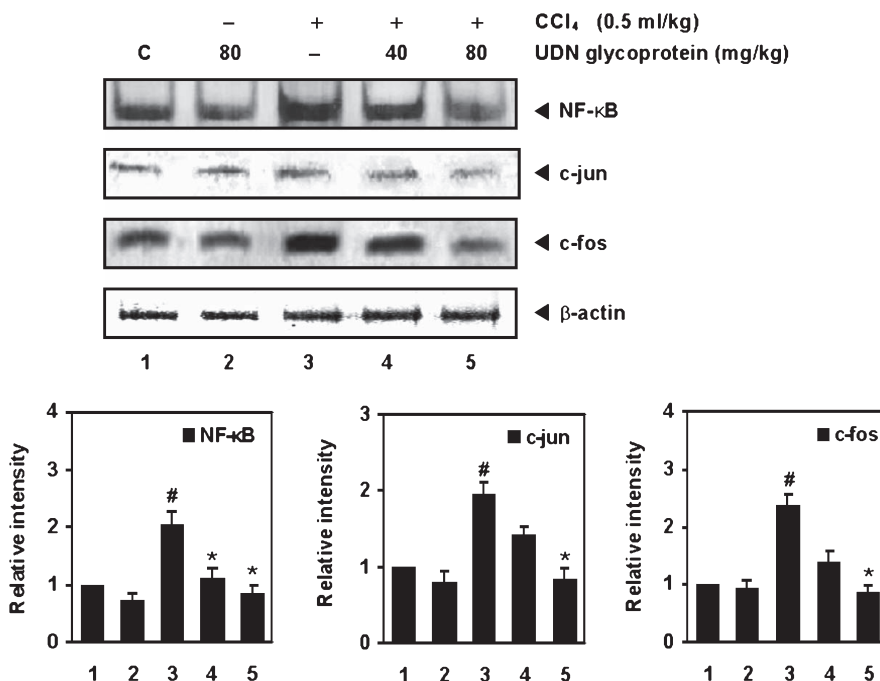
#### Effect of UDN glycoprotein on activities of NF- $\kappa$ B and AP-1 in CCl<sub>4</sub>-treated mouse liver

We investigated the changes of the activation of NF- $\kappa$ B or AP-1 by UDN glycoprotein in CCl<sub>4</sub>-treated mice (Fig. 2). The relative intensities of bands obtained

from Western blotting were calculated with the use of the Scion imaging software. The results showed that CCl<sub>4</sub> treatment increases the activation of NF- $\kappa$ B. For example, in the CCl<sub>4</sub> treatment group, the relative intensity of NF- $\kappa$ B band was increased by 2.1, compared to the control. However, the treatment of UDN glycoprotein decreased the NF- $\kappa$ B activation in CCl<sub>4</sub>-induced mice. Namely, the relative intensities of bands about NF- $\kappa$ B activation increased by 1.0 and 1.3 at 40 and 80 mg/kg of UDN glycoprotein, respectively, compared to CCl<sub>4</sub> treatment alone. In activation of AP-1 (c-jun and c-fos) (Fig. 2), the activations of c-jun and c-fos proteins were increased by the CCl<sub>4</sub> treatment alone, compared to the control. However, when the mice were pretreated with UDN glycoprotein (40 and 80 mg/kg), c-jun and c-fos activities were reduced in the CCl<sub>4</sub>-treated mice. In fact, at CCl<sub>4</sub> treatment alone, the relative intensities of bands about c-jun and c-fos proteins activation increased by 2.0 and 2.4 compared to the control. However, the pretreatment of UDN glycoprotein reduced the relative intensities of c-jun bands by 0.6 and 1.2 at 40 and 80 mg/kg in the CCl<sub>4</sub>-treated mice, respectively, compared to CCl<sub>4</sub> treatment alone. UDN glycoprotein also decreased the relative intensities of c-fos bands by 1.0 and 1.5 at 40 and 80 mg/kg, respectively, compared to CCl<sub>4</sub> treatment alone.

#### Protective effect of UDN glycoprotein on G/GO-induced toxicity in primary cultured mouse liver

We examined the effects of UDN glycoprotein against G/GO-induced cytotoxicity in the primary cultured



**Fig. 2.** Effect of UDN glycoprotein on CCl<sub>4</sub>-induced activation of NF- $\kappa$ B and AP-1 in mice. The mice were pretreated with UDN glycoprotein (40 and 80 mg/kg body weight) orally once a day for 3 days. One hour after the final treatment, mice were treated with CCl<sub>4</sub> (0.5 ml/kg body weight, i.p.). Mice were sacrificed 24 h after administration. NF- $\kappa$ B and AP-1 activation were determined by Western blot analysis with anti-p50, c-fos, and c-jun antibodies. The relative intensities were evaluated with the use of the Scion imaging software.  $\beta$ -Actin was used as internal control for equal loading of proteins. The values are mean  $\pm$  S.D. from six mice done in triplicates. # represents the difference between treatment and control,  $P < 0.01$ ; \* represents the difference between treatment and CCl<sub>4</sub>-treated group,  $P < 0.05$ .

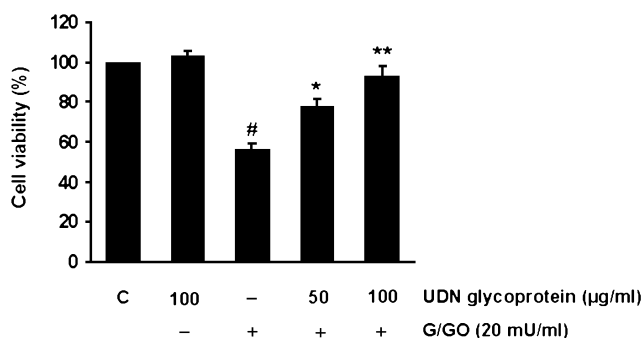
mouse liver tissues. In the cytotoxicity assay, the experimental condition was set at 20 mU/ml G/GO and 4 h of incubation time to approximate the inhibitory concentration 50% (IC<sub>50</sub>). The effect of UDN glycoprotein on cytotoxicity was evaluated in the presence or absence of G/GO. Interestingly, the results showed that UDN glycoprotein does not have any cytotoxic effect. When the cells were exposed to 20 mU/ml G/GO, the viability of the cells was decreased to 43.6%, compared to the control. In contrast, when the cells were treated with 50 and 100 µg/ml UDN glycoprotein in the presence of G/GO (20 mU/ml), the viability of the cells increased significantly, compared to G/GO treatment alone (Fig. 3). For example, the cell viabilities at 50 and 100 µg/ml of UDN glycoprotein were 67.6% and 92.7%, respectively.

## Discussion

Liver injuries induced by CCl<sub>4</sub> are the distinct symptom of xenobiotic-induced hepatotoxicity and commonly used models for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs (3, 28). The principle causes of CCl<sub>4</sub> induced hepatic damage is lipid peroxidation and decreased activities of antioxidant enzymes and generation of free radicals (1, 29). For the therapeutic strategies of liver injury and disease, we postulate that it is important to find antioxidant compounds that are able to block liver injuries through free radical generated by CCl<sub>4</sub>. Therefore, we strongly speculated that UDN glycoprotein protects against diseases that are caused by ROS because it has radical scavenging ability (10, 11). Actually it is restrictively

expressed as a proteoglycan and consists of proximately 225 amino acids and 530 saccharides. It has been reported that the absorption of glycoprotein is achieved by the following mechanism. Amino acid, peptide, and glucose are absorbed from intestinal lumen into the blood stream using a specific carrier because they have either a charge or polarity. Therefore, they can not permeate the membrane of intestinal wall against a concentration gradient and their vectorial transport is the combined results of several separate membrane events. The possible explanation about the mechanism of glycoprotein absorption is that it is firstly divided by proteoglycosylase into two parts, protein and carbohydrate, and then they are absorbed by carrier-mediated transport from the lumen into blood stream in the small intestine. Namely, although amino acid transport and peptide transport in the small intestine have different characteristics, they both use a carrier-mediated transporter cross the intestinal wall, such as ASCT-1, EEAT-3, and pep T1. On the other hand, glucose also uses carriers such as SGLT (sodium glucose transporter) and GLUT (glucose transporter) (30, 31). After permeation across the intestinal wall, amino acids and glucoses together bring into the entire molecule of glycoprotein. Then, the glycoprotein molecules are moved to the outside of liver cell membrane through the blood stream. The glycoprotein interacts with an extracellular receptor on outside of liver cell membrane. Their interaction consequently results in transmission of a signal to the cytoplasm. Such a signal is further transmitted to activate a down stream signal transduction cascade resulting in activation of specific transcriptional factors to express anti-inflammation.

This study was carried out by oral administration of UDN glycoprotein. The results of the present study demonstrate that the oral administration of UDN glycoprotein effectively protected mice against CCl<sub>4</sub>-induced acute liver damage. There are two possible explanations about the hepatoprotective effect of orally administered UDN glycoprotein. One of them is that the carbohydrate portion of UDN glycoprotein cannot be digested in the mammalian small intestine and forms a viscous solution, which is also thought to delay the absorption of various chemicals including CCl<sub>4</sub>, suggesting that the viscous UDN glycoprotein can interfere with CCl<sub>4</sub> absorption in the small intestine, especially the ileum (32–34). Also, UDN glycoprotein might improve liver antioxidant status through its biological activity in the CCl<sub>4</sub>-induced system. The other explanation is that UDN glycoprotein may have an ability to modulate the signal transduction to liver cells through binding to the several receptors of intestinal epithelial cells during CCl<sub>4</sub>-induced liver injury. From these speculations, we suggest that pre-



**Fig. 3.** Protective effects of UDN glycoprotein on G/GO-induced hepatotoxicity in cultured mouse hepatocytes. Hepatocytes were treated with 50 and 100 µg/ml UDN glycoprotein in the presence of G/GO (20 mU/ml) for 4 h, the viability of the cells was determined by MTT assay. Results are the mean  $\pm$  S.D. from three independent experiments. \* represents the difference between treatment and control,  $P < 0.05$ , \*\* represents the difference between treatment and control,  $P < 0.01$ ; # represents the difference between treatment and G/GO alone,  $P < 0.01$ .

treatment of UDN glycoprotein has indirect protective effects against CCl<sub>4</sub>-induced liver injury.

The general indicators of CCl<sub>4</sub>-induced hepatotoxicity such as histopathological lesions were not observed, but biological and hepatic damage by CCl<sub>4</sub> administration was clearly evident from the severe body weight loss and liver swelling (Table 1). CCl<sub>4</sub> is known to cause hepatic damage with a marked elevation in serum levels of LDH and aminotransferases enzymes (AST and ALT) because these enzymes are cytoplasmic in location and are released into the blood after cellular damage (3, 28). In agreement with those investigations, our results showed that a significant increase in the activities of ALT and LDH in CCl<sub>4</sub>-treated mice (Table 1). The increase in the activities of these enzymes in plasma suggests enhanced hepatocellular damage by CCl<sub>4</sub>. The activities of these enzymes were found to decrease after pretreatment with UDN glycoprotein (Table 1). UDN glycoprotein also showed the ability to prevent a CCl<sub>4</sub>-induced increment of plasma and hepatic TBARS level, suggesting that UDN glycoprotein inhibits lipid peroxidation and its propagation (Tables 1 and 2). Moreover, antioxidant enzymes (SOD, CAT, and GPx) activities were increased by UDN glycoprotein treatment (Table 2). One of possible mechanisms for lowering of SOD, CAT, and GPx activities is that the treatment of mouse liver with CCl<sub>4</sub> concurrently induces both processes in acute injury and regeneration; injury events are dominantly expressed in the early stage but this regeneration process is latent (35). Therefore, the SOD, CAT, and GPx were degraded or saturated to block the CCl<sub>4</sub>-induced massive free radical production at in the early stage. By contrast, a possible reason why SOD, CAT, and GPx activities after UDN glycoprotein treatment were augmented in both the absence of CCl<sub>4</sub> and in the presence of CCl<sub>4</sub> is that UDN glycoprotein possesses not only the capacity to scavenge the small number of ROS that are inevitably generated due to the incomplete reduction of O<sub>2</sub> in electron transfer reactions in normal aerobic metabolisms, but also the capacity to block the CCl<sub>4</sub>-induced massive ROS production. The excessive activities of SOD, CAT, and GPx and antioxidative activity of UDN glycoprotein can together contribute to synergic/additive scavenging activity against radicals, suggesting that the antioxidative potential of UDN glycoprotein seems to stimulate the activities of antioxidant enzymes, compared to the control. Therefore we speculate that UDN glycoprotein particularly plays a role during the early stage in CCl<sub>4</sub>-induced liver injury. These findings indicated that administration of UDN glycoprotein decreased lipid peroxidation, improved antioxidant status, and thereby prevented the damage to the liver and leakage of enzymes ALT and LDH. These

results clearly show that the antioxidant action of UDN glycoprotein significantly reduces the damage of CCl<sub>4</sub>-induced liver injury and activates the biological defense system of the liver.

Figure 1 shows that the level of NO in plasma decreased significantly with administration of CCl<sub>4</sub>. UDN glycoprotein blocked the reduction of plasma NO level in CCl<sub>4</sub>-treated mice. There are two possible explanations for the observed decrease in NO levels after CCl<sub>4</sub> treatment in our case. First, one or more of the substrates or any of the cofactors of nitric oxide synthase (NOS) was depleted or damaged, decreasing the NO production. Second, NO was used up continuously after the injury. It is possible that another mechanism of protective action of UDN glycoprotein against CCl<sub>4</sub>-induced hepatotoxicity is due to the increased NO production. Several studies have found that NO protected against CCl<sub>4</sub>-induced liver injury using a NOS knockout mice or a NOS inhibitor (36, 37). The mechanism underlying the protective effects of NO in CCl<sub>4</sub>-induced hepatotoxicity has not been elucidated and may be related to its antioxidant properties (38, 39). NO has also been shown to interfere directly with the progression of lipid peroxidation (39), which may contribute to its protective actions in this model (40).

CCl<sub>4</sub> is also one of the well-known inducers of NF- $\kappa$ B and AP-1 in apoptotic response of hepatocytes, inflammatory response of Kupffer cells, and fibrogenetic response of stellate cells on liver injury (41–44). In the present study, CCl<sub>4</sub> treatment increased the activations of NF- $\kappa$ B and AP-1 (c-jun and c-fos), while the CCl<sub>4</sub>-induced activations of these transcriptional signals markedly inhibited by treatment with UDN glycoprotein in the CCl<sub>4</sub>-induced system (Fig. 2). It has reported that NF- $\kappa$ B activity is increased by metabolites of CCl<sub>4</sub>, an agent that enhances hepatic cell injury and necrosis. The main action of NF- $\kappa$ B in liver injury is to mediate the release of cytotoxic cytokines and inflammatory cytotoxins such as TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$  (45). Therefore, reduction of NF- $\kappa$ B activity may decrease cell necrosis, perhaps through affecting cytokine expression (46). AP-1 participates in cell proliferation and differentiation by positively or negatively regulating the transcription of genes that contain AP-1 binding sites including c-jun and c-fos expression (47, 48). AP-1 activity has been demonstrated to be sensitive to ROS (49) as well as to lipid peroxidation end-products (42). Recently, it has been reported that c-jun and c-fos expression are elevated in livers of rodents following treatment with necrogenic doses of CCl<sub>4</sub> (50, 51). The results in our experiments indicated that CCl<sub>4</sub>-induced acute liver injury, which is mediated by the activation of NF- $\kappa$ B and AP-1 as a result of increased oxidative stress caused

by  $\text{CCl}_4$ . Thus, the antioxidative properties of UDN glycoprotein may be of importance for their protective effect.

According to our results, UDN glycoprotein has properties to inhibit lipid peroxidation, to stimulate increasing activities of antioxidant enzymes, to increase in NO production, and to inhibit activities of NF- $\kappa$ B and AP-1. It was reported that transcriptional factors (NF- $\kappa$ B and AP-1) are activated at inflammation but their activities are reduced by treatment of certain active compound such as curcumin, preventing inflammation (52). Also we examined the protective effects of UDN glycoprotein against G/GO-induced oxidative damage in primary cultured mouse hepatocytes. Primary hepatocyte cultures provide a suitable in vitro system for screening plant extracts for compounds with antioxidative properties and for elucidating their mechanism of action (53). In the oxidative stress induced by G/GO system,  $\text{H}_2\text{O}_2$  is continuously generated by glucose oxidase (GO) acting on  $\beta$ -D-glucose, which is expressed as G/GO.  $\text{H}_2\text{O}_2$  easily penetrates cell membranes and is formed via the iron-mediated Fenton reaction hydroxyl radicals (54). In this study, the effects of G/GO-mediated oxidative stress were investigated on cell viability. We demonstrated here that cell death following the oxidative stress was significantly decreased, when the cells were treated with UDN glycoprotein (Fig. 3). This means that UDN glycoprotein has a scavenging ability for radicals generated by G/GO. That is why the viability increased when UDN glycoprotein was added into culture media in hepatocytes with the G/GO system.

In conclusion, the results in this study indicate that UDN glycoprotein can protect mouse liver from injury induced by  $\text{CCl}_4$  in vivo and by G/GO in vitro through free radical scavenging ability. Nevertheless, further research must be carried out to elucidate the mechanisms of the hepatoprotective effect by UDN glycoprotein at the molecular level.

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