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Inhibitory Effects of the Ethanol Extract of *Ulmus davidiana* on Apoptosis Induced by Glucose-glucose Oxidase and Cytokine Production in Cultured Mouse Primary Immune Cells

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The bark of *Ulmus darvidiana var. japonica* Nakai (UDN) has been used for a long time to cure inflammation in oriental medicine. In the present study, two types of extracts, *Ulmus* water-eluted fraction (UWF) and *Ulmus* ethanol-eluted fraction (UEF), were prepared from the UDN stem bark, and employed to test the extracts to see if they had anti-oxidative properties against hydroxyl radicals that could alter immune reactivity in mouse immune cells. Deoxyribose assay, DNA nicking assay, and glucose/glucose oxidase assay showed that both fractions had scavenging activity against oxygen free radicals at 50 mg/ml. In addition, hydroxyl radical-mediated apoptosis in mouse thymocytes was not protected by UEF treatment, but the apoptosis was protected by UWF at the same concentration. DNA synthesis and cytokine production that were induced in splenocytes by mitogens (Concanavalin A and lipopolysaccharide) were reduced by the addition of both fractions. These results indicate that both extracts that were prepared from the UDN stem bark have anti-oxidative activities, anti-apoptotic effects, and inhibitory effects on DNA synthesis and cytokine production in mouse immune cell cultures.

Keywords: Antioxidant, Apoptosis, Cytokine, Mouse immune cells, *Ulmus davidiana*

Introduction

Small amounts of reactive oxygen species (ROS), including hydroxyl radicals (·OH), superoxide anions (O_2^{-}), and hydrogen peroxide (H_2O_2), are constantly generated during aerobic metabolism in organisms. However, ROS can be detoxified by certain enzymatic and nonenzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbic acid, α-tocopherol, and glutathione (GSH) (Ames and Shinenaga, 1993; Lee *et al.*, 1999b; Fiander and Schneider, 2000). In a normal biological system, the activity of an antioxidant defense system, and the amount of ROS generated by the normal metabolic process, is well balanced. If this balance is broken by a certain factor(s), ROS may attack several biomolecules that might be important for metabolism in life (Lee *et al.*, 1999c). These effects account for several biological abnormalities, such as an abnormal signal transduction, mutagenesis, and apoptotic cell death (Sen and Packer, 1996; Mates and Sanchez-Jimenez, 2000). Also, oxidative damage to the cells leads to the initiation and progression of many degenerative diseases, such as atherosclerosis, dysfunction of immune systems , and cancer (Wiseman and Halliwell, 1996; von Harsdorf *et al.*, 1999).

Apoptotic cell death that is induced by oxidative stresses could be blocked by endogenous and exogenous antioxidants (Mates and Sanchez-Jimenez, 2000). Based on this concept, many scientists have tried to treat the oxidation-associated diseases with natural antioxidants that are isolated from natural products, such as plants, fruits, and even vegetables. For example, a crude ethanol extract from *Rhus verniciflua* Stokes was found to have antioxidative effects against hydroxyl radicals, anti-proliferative activity in HeLa cells, and stimulating activity on detoxifying enzymes in hepatocytes (Kim *et al.*, 1997; Jung, 1998; Lee *et al.*, 1999a; Lee, 2000; Lim *et al.*, 2000; Lee *et al.*, 2001). In addition, it has been reported that *in vivo* supplementation of ascorbic acid and α-tocopherol, which have antioxidative activity, reduced the immune dysfunction that is caused by virus infection (Lee *et al.*, 1998).

Ulmus darvidiana var. japonica Nakai (UDN) is a deciduous tree that is widely distributed in Korea. In oriental medicine, the bark of the stem and root of the plant have been used traditionally for treating edema, mastitis, gastric cancer, and

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inflammation (Lee, 1996). It has also been reported that *Ulmus davidiana* Planch (UDP), a kind of Ulmaceae, has strong antioxidative activity on lipid peroxidation and an inhibitory effect on an endogenous NO-induced apoptotic cell death (Kim *et al.*, 1996; Jun *et al.*, 1998). Even though the UDN stem has been used for treating inflammatory diseases, the mechanism of its biological function has not been understood well. In this study, we initially prepared two fractions from the extracts of UDN stem bark by eluting the ethanol extract of UDN stem bark with water and ethanol. It was named UWF (*Ulmus darvidiana* Water-eluted Fraction) and UEF (*Ulmus darvidiana* Ethanol-eluted Fraction), respectively. Also, we investigated whether the extracts of UDN stem bark could inhibit hydroxyl radical-mediated apoptosis and cytokine production in mouse immune cell culture.

Materials and Methods

Chemicals and mice Unless otherwise specified, the chemicals and plastics used in this study were purchased from Sigma Chemical Co. (St. Louis, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, USA), respectively. The chemicals for cytokine ELISA that were used in this study were obtained from PharMingen (San Diego, USA). Murine INF-γ (1×10^5 U/mg) was purchased from Genzyme (Munich, Germany). Cell culture reagents, including RPMI 1640 and fetal bovine serum, were purchased from Gibco BRL Life Technologies (Grand Island, USA). Four to six-week old inbred female BALB/c mice were purchased from Damul Science (Yoosung, Korea) and were fed *ad libitum* under a day/night rhythm.

Preparation of UDN extracts The stem bark of UDN was obtained in October, 2000 from the Naju traditional market in Chonnam province, South Korea, and identified by Dr. H. T. Lim (Chonnam National University). Five kilograms of fresh stem bark of UDN were air-dried in a dark room and cut into approximately 5 cm size of small, long pieces. Extracts were obtained with a treatment of absolute ethanol onto the pieces of stem bark. The ethanol extract was filtered with Whatman filter paper (No. 2) and concentrated with a rotary evaporator (R110, Buchi, Switzerland). The crude ethanol extract (5 g) was fractionated on a silica gel column (2.5 × 60 cm, 22Å, 28-200 mesh) with distilled water and then with absolute ethanol. UDN fractions were named UWF and UEF for the water-eluted fraction (2.5 g, 0.05%), and ethanol-eluted fraction (1.1 g, 0.022%), respectively. Both of the eluted solutions were then freeze-dried and dissolved with distilled water. The solutions were precipitated with 80% ammonium sulfate. then dialyzed with a dialysis membrane (Spectra/por, MWCO 6000-8000) against 20 mM Tris-HCl (pH 7.4) overnight. After dialysis, each sample was freeze-dried and stored at –20°C until use.

SDS-PAGE analysis SDS-PAGE was performed with the 50 mg/ml of UWF that contained 0.1% SDS, using a Mini-Protean II cell (Bio-Rad) electrophoresis unit with a 15% polyacrylamide gel. The gels were stained (according to the method of Neville and Glossmann, 1974) for glycoproteins, and with Coomassie brilliant blue for non-glycoprotein.

Cell cultures For the primary culture of immune cells (thymocytes, bone marrow, and splenocytes), each tissue was cut out from BALB/c mice and dissected into small pieces with a razor blade. The pieces were then gently passed through a stainless steel net (82 and 256 mesh) in a phosphate buffered saline solution (PBS; pH 7.4) that contained 5% FBS (HyClone, Logan, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The bone marrow cells were collected by flushing the femurs and tibias with PBS using a syringe that contained 5% FBS and 1 mM of hydrocortisone. The cells were then gently resuspended by pipetting in the RPMI 1640 medium that was supplemented with 50 U/ml of penicillin, 50 μg/ml of streptomycin, and 10% FBS, then centrifuged at $1,000 \times g$ for 10 min. The cells (10^6 cells/ml) were divided into 35 mm culture dishes (2 ml/dish) or 96-well flat bottom plates (100 μl/well). The culture medium was changed with the RPMI 1640 that was supplemented with 0.5% FBS 24 h before treatment of the UDN extracts.

Antioxidative activity assay The antioxidative activity of the UDN extracts was measured using a deoxyribose assay, a DNA nicking assay, and a glucose/glucose oxidase (G/GO) assay. The deoxyribose assay was conducted according to the methods of Halliwell *et al.* (1987). Briefly, the UDN fractions (UWF and UEF) were mixed with a Haber-Weiss reaction buffer (10 0μM $FeCl_3$, 104 μM EDTA, 1.5 mM H_2O_2, 2.5 mM deoxyribose, and 100 μM L-ascorbic acid, pH 7.4), which made a final volume of 1.0 ml, and then incubated for 1 h at 37°C. One milliliter of 2-TBA (0.5% 2-TBA in 0.025 M NaOH) and 1 ml of 2.8% trichloroacetic acid were mixed and heated for 30 min at 80°C in a water bath. The mixture was then cooled on ice. The absorbance was measured at 532 nm using a Spectrophotometer (Beckman, DU 530, Germany). The inhibitory effects on the generation of hydroxyl radicals were calculated by the following equation: Inhibitory effect (%) = $[(OD_{control} - OD_{sample})/OD_{control}] \times 100$.

For the DNA nicking assay, the pBR322 plasmids were prepared from DH5α using the Wizard® Plus SV Minipreps (Promega, Madison, WI, USA). Plasmid DNA (0.5 μg) was added to the mixing solution (30 mM H_2O_2, 50 μM ascorbic acid, and 80 μM $FeCl_3$) that contained varied concentrations of UDN fractions. The final volume of the mixture was brought up to 20 μl with deionized distilled water. The mixture was then incubated for 30 min at 37°C. Finally, the DNA was separated on 1% agarose gel.

The G/GO assay was performed as described previously (Michikawa *et al.*, 1994). Briefly, the cultured immune cells in 96-well plates were exposed for 4 h to hydroxyl radicals that were generated by G/GO (27.75 mM D-glucose and 20 mU/ml glucose oxidase in RPMI 1640) in the presence of UDN fractions. Thereafter, 10 μl of the MTT solution (5 mg/ml in PBS as stock solution) was added into each well. Then the cells were further incubated for 4 h at 37°C. To measure the absorbance, 70 μl of acidic isopropanol was added to each well, and the plates were read at 560 nm using a SpectraCount™ (Packard, Instrument Co., Downers Grove, USA) ELISA reader.

Apoptosis assay For the DNA fragmentation and TUNEL assays, the cultured immune cells were exposed to 5 mU/ml GO in RPMI 1640. DNA fragmentation was analyzed by electrophoretic analysis. Briefly, the immune cells (2×10^6 cells) from the culture

plates were harvested by centrifugation at 1,000 × *g* for 5 min and then lysed with the addition of a lysis buffer [50 mM Tris-HCl, 20 mM EDTA, 1% (v/v) NP-40, and 1% (v/v) SDS, pH 8.0] at 65°C for 1 h. DNA was extracted twice using phenol/chloroform methods. The extracts were digested with 1 μg/ml of RNase A and 10 μg/ml of proteinase K. Finally, the DNA was separated on a 2% agarose gel and stained with ethidium bromide.

The terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was used to detect DNA fragmentation *in situ,* and performed as described previously (Gorczyca *et al.*, 1993). Briefly, thymocytes were fixed with 1% formaldehyde on ice for 1 h and treated with 80% ice-cold ethanol. After fixation, the cells were reacted in a TdT buffer (30mM trizma base, 140 mM sodium cacodylate, 1 mM cobalt chloride, 3 U/ml terminal transferase, and 0 .4nM Rhodamine-labeled dUTP, pH 7.2) for 30 min at 37°C and washed three times with PBS. Then, the cells were observed with a fluorescence microscope (Zeiss, Germany).

Mitogenesis assay To investigate the effects of UDN on DNA synthesis, the splenocytes were incubated for 24 h with varied concentrations of UDN fractions before the treatment with mitogens. The culture medium was then gently replaced and the cells were stimulated for either 20 h with 5 μg/ml of Con A , or 44 h with 10 μg/ml of LPS for the mitogenesis. Subsequently, 50 μl of 1 μCi/ml [*methyl*-^{3}H] Thymidine deoxyribose was added into each well 6 h before the last incubation period. Finally, the cells were collected with a cell harvester (Inotech Inc., Switzerland) and the tritium uptake was measured with a liquid scintillation counter (Packard Instrument Co.) and presented as count per minute (cpm).

Measurement of cytokines To determine the level of cytokine production, ELISA was performed as described previously (VanCott *et al.*, 1996). Briefly, the supernatant of splenocytes was collected 72 h after treatment with varied concentrations of UDN fractions. The appropriate amount of supernatant was divided into 96-well ELISA plates, which had been pre-coated with rat anti-mouse INF-γ and IL-4 in a 0.1 M bicarbonate buffer overnight at 4°C. On the following day, biotinylated-secondary antibodies were added onto each well. Finally, the substrate was added and the absorbance was measured at 405 nm with an ELISA reader.

Statistical analysis All data are expressed as mean ± SEM of the three independent experiments. A one-way ANOVA was used for multiple comparisons (SPSS program, ver 10.0).

Results

SDS-PAGE analysis As shown in Fig. 1, the gels were stained (according to the method of Neville and Glossmann, 1974) for glycoproteins and with Coomassie brilliant blue for non-glycoproteins. An analysis of both fractions (UWF and UEF) by SDS-PAGE exhibited similar band patterns (Fig.1). For example, SDS-PAGE of UWF indicated two bands. One was at approximately 116 kDa and the other at 14 kDa (lane 1). However, UEF showed only one band around 14 kDa (lane 2). In the UWF sample, the band at about 116 kDa was strongly stained by the method for glycoprotein staining (lane 1), while the band about 14 kDa was strongly stained by Coomasie brilliant blue (lane 2).

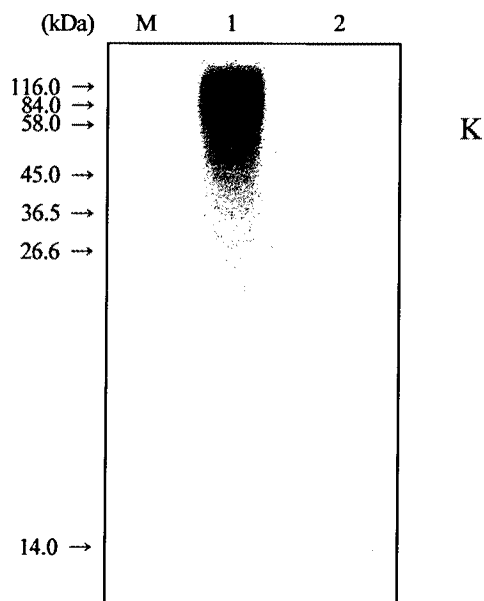

Fig. 1. SDS-PAGE analyses of UWF and UEF. Electrophoresis and staining of the gel were performed as described in the Materials and Methods Section. M, 1, and 2 represent the prestained molecular weight marker, two bands of glycoprotein (116 kDa and 14 kDa) in UWF, and one band of non-glycoprotein (14 kDa) in UEF, respectively.

Anti-oxidative activity of UDN extracts The antioxidative activity of each of the preparations of the UDN fractions, UWF and UEF, was assessed by measuring the level of TBA-MDA adduct formation, described in Materials and Methods (Table 1). Hydroxyl radical-induced deoxyribose degradation was effectively inhibited by the addition of both UDN fractions. The inhibitory effect of UWF on hydroxyl radicals was found to be 1.06% at 0.01 mg/ml, 4.47% at 0.1 mg/ml, 13.19% at 1 mg/ml, 22.34% at 2 mg/ml, and 28.93% at 3 mg/ml, compared to that of the control. In general, the inhibitory effect of UEF on hydroxyl radicals was lower than that of UWF at the same concentration.

In the DNA nicking assay, antioxidative activity was assessed by measuring the degree of protection on DNA scission that was induced by the attack of OH radicals, which was shown by the agarose electrophoretic pattern (Fig. 2). As shown in Fig. 2A, when pBR322 plasmid DNA was exposed to the mixing solution, the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$) took place and caused a change of the DNA band from Form I (native plasmid DNA) to Form II (single-stranded, nicked circular plasmid DNA), or to Form III (linear plasmid DNA). It was apparent that hydroxyl radical-mediated DNA nicking occurred within 30 min of the reaction (Fig. 2A, lanes 2-5). Interestingly, the hydroxyl radical-mediated DNA nicking was gradually protected when

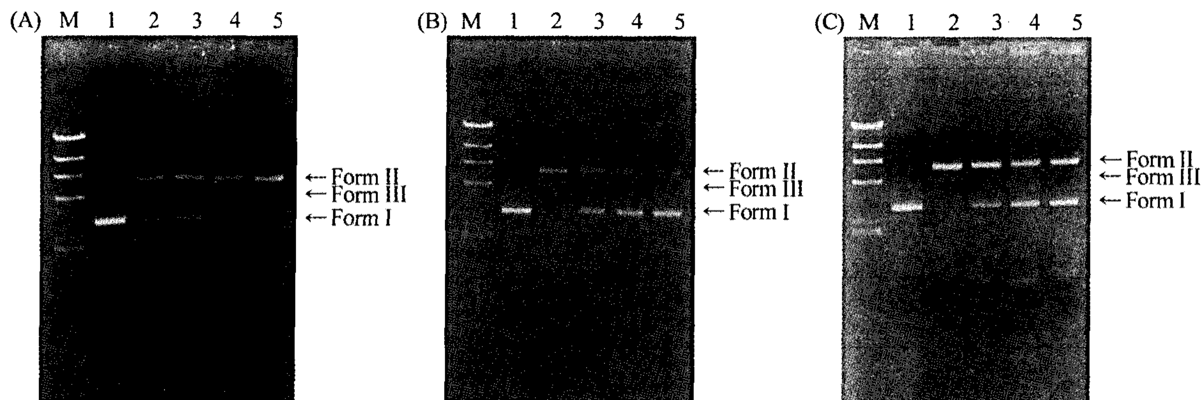

Fig. 2. Inhibitory effect of UDN fractions on DNA nicking induced by hydroxyl radicals. (A) Each reaction solution (20 μl) contained 30 mM H_2O_2, 50 μM ascorbic acid, and 80 μM $FeCl_3$. The DNA nicking was initiated by mixing 0.5 μg of pBR322 plasmid DNA with the reaction solution for 5, 10, 20, and 30 min (lanes 2, 3, 4, and 5) at 37°C. The reaction was carried out for 30 min and stopped by adding 4 μl of the loading buffer. The addition of (B) UWF and (C) UEF [10 μg (lane 3), 20 μg (lane 4), and 40 μg (lane 5)] prohibited the DNA nicking reaction. M, 1, and 2 represent the DNA marker, pBR 322 plasmid DNA without the reaction solution as a control, and pBR 322 DNA plasmid DNA + reaction solution, respectively.

the UWF was added into the reaction solution (Fig. 2B). For example, an addition of 40 μg of UWF clearly increased the presence of Form I and mediated an almost complete loss of the hydroxyl radical-mediated Form II formation (Fig. 2B, lane 5). Similar protective activity on hydroxyl radical-mediated DNA nicking was also observed in UEF, although its scavenging activity on hydroxyl radical was weaker than that of UWF. It should be noted, however, that the hydroxyl radical-mediated Form II DNA formation was not completely inhibited by the UEF treatment (Fig. 2C, lane 5).

Antioxidative activity of UDN fractions was confirmed by a G/GO assay as described in Materials and Methods (Fig. 3). In the presence of GO in culture, H_2O_2 was continuously produced through the oxidization of glucose by glucose oxidase, and lead to ·OH accumulation via the Fenton and/or Haber-Weiss reaction within the cells (Rollet-Labelle *et al.*, 1998; Panayiotidis *et al.*, 1999). As shown in Fig. 3, after the addition of 20 μg/ml UWF, viability of the immune cells increased ($P<0.05$), compared to that obtained without UWF treatment. The trend of an increase in viability after the addition of UDN fractions was shown in the experimental systems, using both thymocytes and bone marrow cells. For example, the viabilities obtained after the addition of 50 μg/ml UWF were 65.22% and 63.34% in thymocytes and bone marrow cells, respectively. The viabilities obtained without UWF were 51.50% in thymocytes and 51.38% in bone marrow cells (Figs. 3A and 3B). In general, the protective effects of UWF against hydroxyl radicals were higher than those of UEF. When 50 μg/ml of both UDN fractions without GO were added, the viabilities were 97.26% in UWF and 94.98% in UEF, and showed more than 90% in both the thymocytes and bone marrow cells. At 50 μg/ml, and in concert, both fractions showed no cytotoxic effects on the thymocytes and bone marrow cells (data not shown).

Inhibition of radical-mediated apoptosis of thymocytes by UDN fraction The hydroxyl radical-induced apoptotic nature of thymocytes was confirmed by DNA fragmentation analysis (Fig. 4). A characteristic ladder pattern of DNA fragmentation was observed when the thymocytes were treated with the varied concentration of G/GO for 90 min (Fig. 4A). DNA fragmentation was hardly noticeable in the control (Fig. 4A. lane 1), while 200-bp multiples of DNA fragments were observed after the treatment of thymocytes with 5 mU/ml G/GO for 90 min (Fig. 4A, lane 2). Interestingly, the appearance of the DNA fragments gradually reduced with the treatment of an increasing concentration of UWF (Fig. 4B). However, UEF did not inhibit the DNA fragmentation that was induced by the G/GO treatment (Fig. 4C). Moreover, when the thymocytes were treated with high doses (>50 μg/ml) of UEF in the presence of G/GO, the DNA fragmentation was more accelerated than that with the G/GO treatment alone (Fig. 4C, lanes 4 and 5).

In the TUNEL assay, the majority of the thymocytes showed a positive staining pattern in the presence of G/GO (Fig. 5). For example, when the thymocytes were incubated for 90 min in the presence of 5 mU/ml of G/GO, most of the cells were positively stained with Rhodamine labeled dUTP (Fig. 5A). Such positive staining of the thymocytes was reduced by the addition of 50 μg/ml of UWF (Fig. 5B). However, the addition of UEF showed no detectable inhibition against the radical-mediated apoptosis in the thymocytes (data not shown). This result suggests that UWF was stronger in a protective effect than UEF in a 50 μg/ml concentration, when comparing the anti-apoptotic effects on hydroxyl radicals between UWF and UEF in the thymocyte culture.

Effect of UDN fractions on mitogenesis and cytokine productions As shown in Fig. 6, proliferation of mitogen-

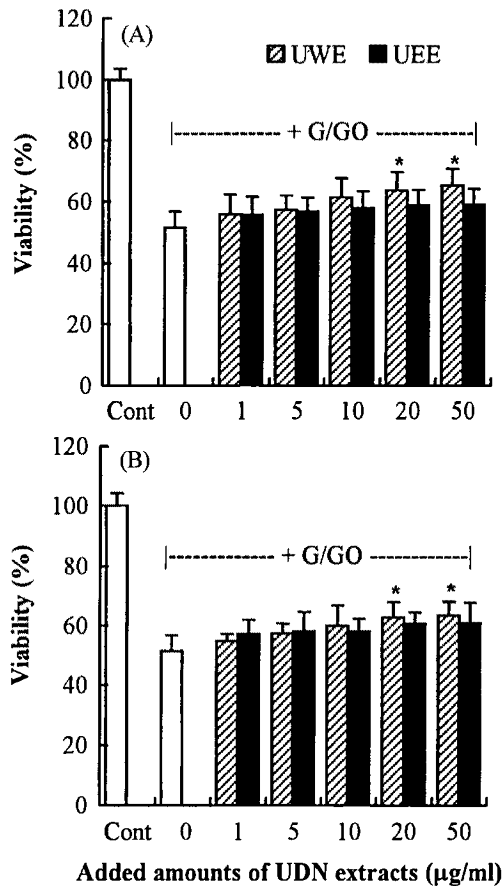

Fig. 3. Protective effect of UDN fractions on hydroxyl radical-induced cytotoxicity in thymocytes and bone marrow cells. The immune cells were exposed to hydroxyl radicals generated by G/GO (5 mU/ml), then incubated for 4 h in the presence of UWF and UEF (1 to 50 μg/ml). Viability of the thymocytes (A) and bone marrow cells (B) was measured through the MTT assay. Each bar represents the mean ± SED of the three separate experiments. An * represents a significant difference between the values of treatments and the control (*P*<0.05).

treated splenocytes was inhibited when the cells were treated with UWF and UEF. It was significantly reduced by the addition of UEF at the defined concentrations before mitogen treatment. For example, when the splenocytes were pretreated with UWF (10 to 100 μg/ml), Con A (5 μg/ml)-induced proliferation of splenocytes was reduced by 5.16% at 10 μg/ml, 10.09% at 50 μg/ml, and 25.62% at 100 μg/ml (Fig. 6A). However, the LPS (10 μg/ml)-induced proliferation of splenocytes declined by 2.54% at 10 μg/ml, 11.20% at 50 μg/ml, and 60.50% at 100 μg/ml with respect to the levels obtained after the Con A or LPS treatment alone (Fig. 6B). In contrast, the pre-addition of UEF to splenocytes reduced the ratios of Con A-induced proliferation of splenocytes by 9.53% at 10 μg/ml, 14.27% at 50 μg/ml, and 43.34% at 100 μg/ml. However, the reduction was 3.20% at 10 μg/ml, 44.26% at 50 μg/ml, and 97.13% at 100 μg/ml in the LPS-induced proliferation of splenocytes with respect to the level obtained after each mitogen treatment alone (Figs. 6A and 6B).

In order to understand the characteristics of T cell responses in splenocytes, we determined the level of Th1-specific cytokine, INF-γ, and Th2-specific cytokine, IL-4 (Fig. 7). The level of INF-γ production by the splenocytes that was obtained with Con A (5 μg/ml) stimulation was 3.63 ng/ml. However, the level was 3.61 and 3.44 ng/ml after treatment with 5 μg/ml of UWF and UEF, respectively. Interestingly, when the cells were pretreated with 50 μg/ml of UWF and UEF, the level was determined as 2.37 and 2.08 ng/ml, respectively (Fig. 7A). A similar inhibitory effect was observed in IL-4 production only when UEF was added. For example, with the addition of 50 μg/ml of UEF, the level of the produced IL-4 was 96 pg/ml. This represents a 31.4% reduction from that obtained by LPS treatment alone (140 pg/ml) (Fig.7B). Unexpectedly, cytokine production was increased in IL-4 by pre-treatment of the cells with UWF (50 μg/ml) and UEF (5 μg/ml).

Discussion

In the analysis of SDS-PAGE, both UWF and UEF showed a similar pattern. However, there were two bands at approximately 116 kDa and 14 kDa in UWF, and only one band at 14 kDa in UEF. The band at 116 kDa was strongly stained by the method of Neville and Glossmann (1974), which is also known to stain glycoprotein. The band at 14 kDa was stained well with Coomasie brilliant blue. These results strongly suggest that the bands at 116 kDa of UWF represent a glycoprotein that contains high amounts of mannose. Also, the 14 kDa band from UEF is not a glycoprotein. In addition, glycoproteins have a high hygroscopic character in the air, because of air's high viscosity. On the other hand, non-glycoproteins were in the form of a fine powder.

Both UDN fractions showed strong scavenging activity against hydroxyl free radicals. This was verified by independent assays for measuring antioxidant activity in this study. The results indicate that UWF has not only an inhibiting effect on radical-mediated apoptosis, but also a stimulating activity on mitogen-induced cytokine production in immune cells. These findings suggest that UDN fractions have a component(s) that potentially works as a natural biodefensive substance. Recently it was reported that *Ulmus davidiana,* a family of UDN, has an anti-oxidative activity on lipid peroxidation (Kim *et al.*, 1996), and inhibitory activities on endogenous NO-induced apoptosis in murine macrophages (Jun *et al.*, 1998). However, the specific scavenging activity of UDN against hydroxyl radicals in immune cells has not been investigated. In this study, the results obtained from the deoxyribose assay and the DNA nicking assay showed that both UWF and UEF have scavenging activity against ·OH. An increase in ·OH-induced deoxyribose degradation was prevented by the UWF and UEF treatment in a dose-dependent manner. A form of plasmid DNA was changed from intact DNA (Form I) to single-stranded nicked DNA

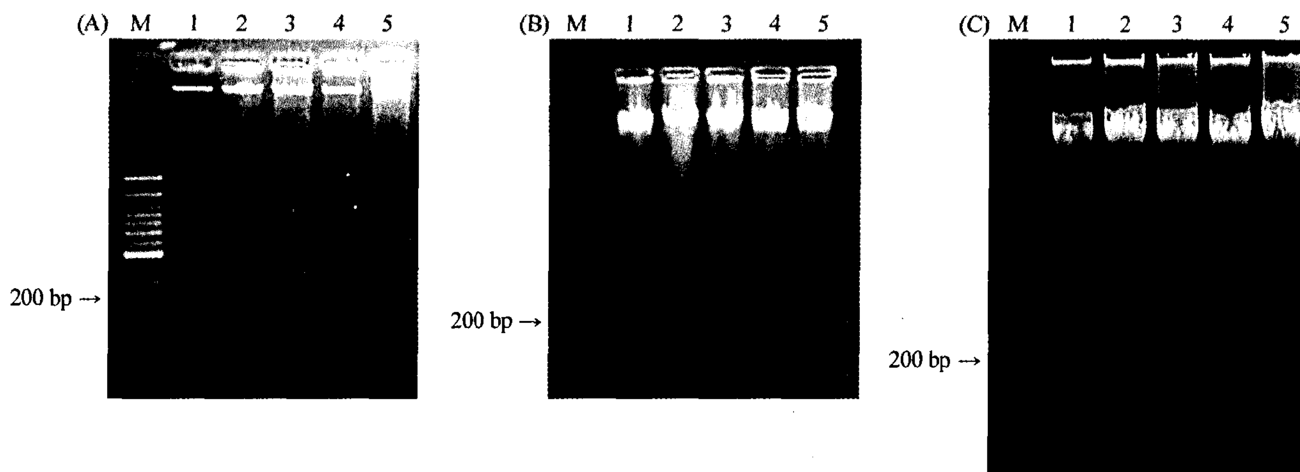

Fig. 4. Inhibitory effects of UDN fractions to DNA fragmentation caused by G/GO in the thymocyte culture. (A) Thymocytes were incubated in RPMI 1640 and exposed with 1 (lane 2), 5 (lane 3), 10 (lane 4), and 20 mU/ml of GO (lane 5) for 90 min. Also, thymocytes were incubated with 5 mU/ml GO and 27.75 mM glucose in the absence (lane 2) and presence of 10 (lane 3), 50 (lane 4), and 100 μg/ml (lane 5) of UWF (B) or UEF (C). M: DNA marker (Hind III/λDNA), lane 1: control. DNA was detected by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

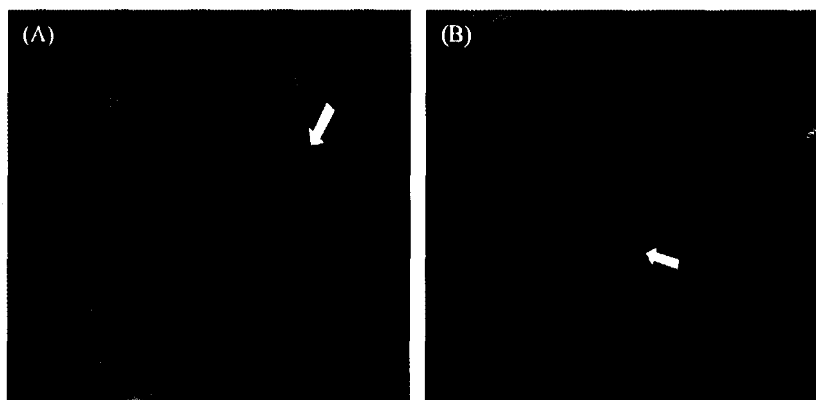

Fig. 5. TUNEL assay of thymocytes. Thymocytes were cultured in RPMI 1640, including 0.5% FBS, and exposed to G/GO in the absence (A) or presence (B) of 50 μg/ml UWF for 90 min. The cells were fixed with formaldehyde and stained with Rhodamine dUTP, described in Materials and Methods. They were observed with a fluorescence microscope. Arrows mean the apoptotic cells with TUNEL positive staining (A) and non-apoptotic viable cells with TUNEL negative staining (B), respectively. Original magnification is ×400.

(Form II) via the Fenton reaction, and to a double-stranded nicked and liner DNA (Form III) when the concentration and time of the hydroxyl radical treatment was increased. Although the DNA nicking was inhibited by the addition of UWF, it was not completely prevented by the addition of UEF. On the other hand, the scavenging activity of UDN fractions against ·OH was significantly increased by the addition of UWF in immune cell culture. With the addition of 50 μg/ml UWF, the viabilities increased 1.27-fold in thymocytes and 1.23-fold in bone marrow cells, respectively, with respect to the value that was obtained by the GO treatment alone. This increasing trend in viability continued until the addition of 50 μg/ml of UWF and UEF (Fig.). However, the addition of 50 μg/ml UEF did not significantly increase the viability of the immune cells. In addition, when the immune cells were incubated for 4 h in 50 μg/ml of UWF, or UEF without G/GO, the cells were not damaged (data not shown). These results suggest that the UDN fractions have a protective effect on ·OH-mediated cytotoxicity, and that the activity of UWF is stronger than that of UEF.

An apoptosis is know to be one possible reason for thymocyte death (Starke and Farber, 1985; Oyama *et al.*, 1999). In general, apoptosis differs from cell death in terms of cell shrinkage, membrane blebbing, oligonucleosomal DNA laddering, and chromatin condensation (Slater and Orrenius, 1995). Apoptosis of thymocytes can be inhibited by the addition of UWF to the culture media. Low concentrations of hydroxyl radicals are required for cell death through apoptosis, when compared to the necrosis levels that are induced by a high concentration of hydroxyl radicals. DNA

Table 1. Hydroxyl radical scavenging activities of UDN fractions

Addition of UDN extracts (mg/ml)	Optical density (A_{532} nm)	Inhibitory effect on hydroxyl radicals (%)
Control	0.470 ± 0.055	0
UWF		
0.01	0.465 ± 0.036	1.06
0.1	0.449 ± 0.037	4.47
1	0.408 ± 0.042	13.19
2	0.365 ± 0.043	22.34
3	0.334 ± 0.035	28.93
UEF		
0.01	0.456 ± 0.040	2.98
0.1	0.434 ± 0.020	7.66
1	0.410 ± 0.015	12.76
2	0.384 ± 0.052	18.29
3	0.373 ± 0.038	20.64

The experimental conditions are described in the Materials and Methods Section. The control contains only the reaction buffer solution. Values of the absorbance are expressed as the meanS.E.D from triplicates. UDN: *Ulmus darvidiana var. japonica* Nakai; UWF: *Ulmus darvidiana* water- eluted fraction; UEF: *Ulmus darvidiana* ethanol-eluted fraction.

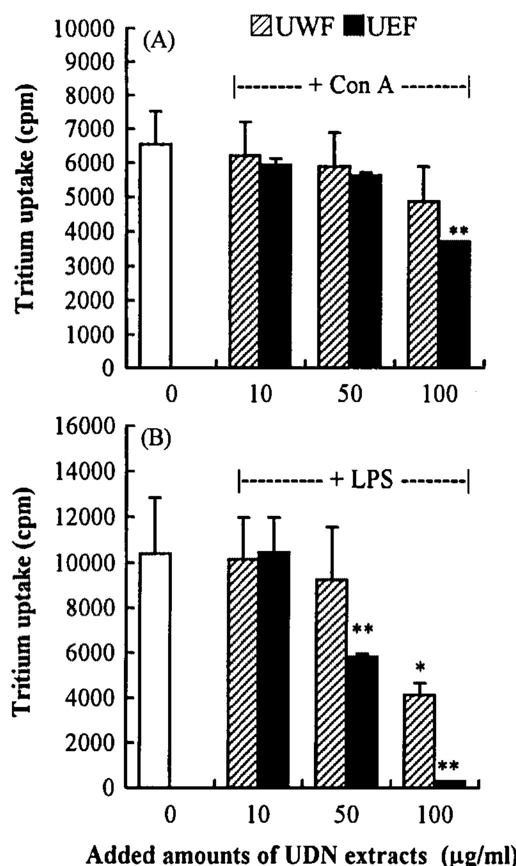

Fig. 6. Effects of UDN fractions on mitogenesis in splenocytes. Splenocytes were treated with UWF and UEF (10 to 100 μg/ml) 24 h prior to mitogen treatment, and stimulated with 5 μg/ml Con A (A) and 10 μg/ml LPS (B). The cells were incorporated with [*methy*-^{3}H] Thymidine (1 μCi/ml) and harvested as described in Materials and Methods. Tritium uptake was determined by a liquid scintillation counter. Each bar represents the mean ± SED of the triplicate experiments. **P*<0.05 and ***P*<0.01 are significantly different from that of the control.

fragmentation was stimulated with 5 mU/ml GO for 90 min. Although such DNA fragmentation was not completely inhibited by the addition of UWF and UEF, the intensity of the DNA band at 200-bp was weakened. In the TUNEL assay, when the thymocytes were treated with 5 mU/ml of G/GO for 90 min, a large number of the cells were positively stained. Such positive staining of the cells clearly decreased when more than 50 μg/ml of UWF was added. This means that UWF can play beneficial roles for protection against apoptosis that is caused by hydroxyl radicals that are generated from the G/GO system. Apoptosis can be mediated by an excess generation of ROS, caused from exo- and endogenous sources (Sinha *et al.*, 1988; Slater and Orrenius, 1995; Mates and Sanchez-Jimenez, 2000). Such oxygen free radicals lead to the activation of the nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which are well-known transcriptional factors (Schwartz *et al.*, 1999; Vollgraf *et al.*, 1999). There is a close correlation between the level of oxygen free radicals and apoptosis, and antioxidants and anti-apoptosis. In general, antioxidants have the capacity to modulate the expression of genes that are involved in apoptosis (Sen and Packer, 1996; Fernandez *et al.*, 1999). Natural antioxidants are known as general methods of treatment for a wide variety of diseases that are caused by oxygen free radicals. UWF, which has a scavenging activity against hydroxyl radicals, showed an ability to inhibit the apoptotic DNA fragmentation that is induced by hydroxyl radicals in thymocytes, although the precise mechanism remains undetermined.

Effects of UDN fractions on DNA synthesis and cytokine production in splenocytes were assessed by a [*methyl*-^{3}H] thymidine incorporation assay and an ELISA test. In general, the UDN fractions inhibited the growth of splenocytes and had no mutagenic ability. Con A- and LPS-mediated mitogenesis of thymocytes was significantly prevented by the addition of 50 μg/ml of UEF (*P*<0.01) and UWF (*P*<0.05). This indicates that UDN fractions, especially UEF, may cause a growth arrest at more than one stage of the cell cycle, depending on the time and dose of the treatment. Thus, UEF could play its inhibitory role on DNA synthesis through a different pathway.

Cytokines that are produced in T cells by mitogens have distinctive biological functions (Paul and Seder, 1994; Cerwenka *et al.*, 1998). CD4^{+} Th cells are divided into two distinct categories, based on their patterns of cytokine production, in response to antigenic stimulation. Not only are the amount of cytokines that are produced from helper cells (Th1 and Th2) important, but the fact that they maintain a balanced level in the immune system is also important for

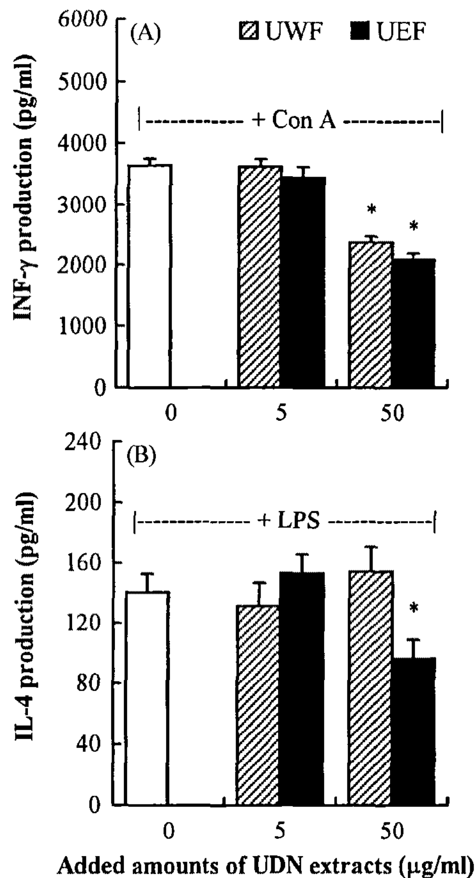

Fig. 7. Production of INF-γ and IL-4 in cultured splenocytes. Splenocytes were incubated in RPMI 1640 that contained 0.5% FBS, and exposed to 5 μg/ml Concanavalin A, or 10 μg/ml lipopolysaccharide in the presence of different concentrations of UWF and UEF. The supernatants were collected 72 h after treatment of UDN fractions, and measured by a cytokine ELISA kit. INF-γ in Fig. 7A and IL-4 in Fig. 7B were induced with mitogen (Con A and LPS). Results are expressed as mean ± SEM from the three separate experiments. *$P<0.05$ means the difference from the positive control.

their critical protective role on stimulators (Mikszta and Kim, 1996). Th1 cells, which produce IL-2 and INF-γ, are involved mostly in cell-mediated reactions, while the Th2-specific cytokines, such as IL-4, IL-5, IL-10, and IL-13, are commonly found in association with strong antibodies and an allergic response (Mosmann *et al.*, 1986; Paul and Seder, 1994). Therefore, these cytokines are the major factors that determine the differentiation of Th cells. When looking at the effects of UDN fractions on the T cell proliferative responses, we determined the INF-γ and IL-4 levels in splenocytes. The levels of INF-γ in splenocytes clearly decreased after treatment with 50 μg/ml UWF and UEF. In contrast, the IL-4 production changed very little after the addition of 50 μg/ml UWF, while it significantly decreased after the addition of 50 μg/ml UEF ($P<0.05$). Our results show that the suppression and production of INF-γ and IL-4 by UEF is more effective than UWF. These results strongly indicate the possibility that decreased levels of T cell specific cytokine production, which in turn affected various immune responses (such as inflammation and allergies), caused the decreased tritium uptake by the nuclei of splenocytes. The results show that both fractions from UDN inhibited the hydroxyl radical-induced apoptosis, as well as DNA synthesis and production of INF-γ and IL-4, when the immune cells were stimulated with Con A and LPS.

In conclusion, the fractions showed strong scavenging activities against oxygen free radicals, detected by different assay systems for antioxidant activity. UWF was especially shown to have inhibitory effects on the radical-mediated apoptosis in immune cells, while UEF showed no anti-apoptotic effects. In the mitogen-mediated cytokine production and cell proliferation, both fractions showed inhibitory effects. In spite of the similar polarities of ethanol and water as an eluted solution for both fractions, both fractions in this study seemed to have different activities. It is assumed that they are composed of the same compounds, proteins having a molecular size of over 10 kDa. Due to the strong anti-oxidative effects of UDN stem bark, and their ability to modulate the mitogen-stimulated cytokine production, we strongly believe that they can play roles in curing inflammation by enhancing inducible immunity. However, more research needs to be conducted in order to elucidate the chemical properties of the UDN fractions, the precise mechanisms on anti-apoptosis, and the regulation of immunity by UDN extracts.

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