The Protective Effects of water Extracts of ZoaGumHwan (ZGH) on the Oxidized LDL-induced Monocyte Adhesion to Human Umbilical Vein Endothelial Cells

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Abstract – It is well known that oxidized low-density lipoprotein (oxLDL) is the most characterized humoral factor that plays an important role in the pathogenesis of atherosclerosis. The water extract of the Korean herbal remedy, ZoaGumHwan (ZGH), which is composed of roots of *Coptis chinensis* Franch and fruits of *Evodia officinalis* Dode with the ratio of 6 to 1, reduced the in vitro oxidation of low density lipoproteins (LDL). Also, the ZGH extract and berberine, one of the major components of ZGH, significantly prevented oxLDL-induced adhesion of monocytes to human umbilical vein endothelial cells (HUVEC). Furthermore, the ZGH water extract and berberine decreased oxLDL-induced expression of CC chemokine receptor 2 (CCR2), a dominant monocyte chemotaxis receptor, in U937 human monocytic cells. The protective effects of the ZGH water extract and berberine were similar to those of simvastatin, an effective lipid-lowering drug. The results suggest that Korean herbal remedy, ZGH, seems to have protective effect against oxLDL-induced monocyte chemoattractant protein (MCP)-1/CCR2-dependent monocyte recruitment onto endothelial cells.

Keywords □ oxidized low density lipoprotein, monocyte adhesion, berberine, ZoaGumHwan, monocyte chemoattractant protein-1, CC chemokine receptor 2

INTRODUCTION

Vascular endothelial cells play a pivotal role in the maintenance of vessel wall integrity. Oxidized low-density lipoprotein (oxLDL) has been implicated to induce vascular endothelial dysfunction in the process of atherogenesis which has a characteristic of an atherosclerotic plaque formation. The crucial step to cause atherosclerotic plaque formation is a focal accumulation of monocytes of which process includes monocyte adhesion, migration, and infiltration.

Monocyte chemoattractant protein (MCP)-1, a 14 kDa glycoprotein of the CC chemokine family, is a potent chemoattractant for monocyte recruitment (Navab *et al.*, 1991; Adams and Lloyd., 1997), and it is expressed by various cell types including human umbilical vein endothelial cells (HUVEC) (Sica *et al.*, 1990). MCP-1 has been found as one of the key factors to start the inflammatory process in atherogenesis (Terkeltaub *et*

al., 1998; Navab *et al.*, 1991). Not only *in vitro* cell system, but also in atherosclerotic patients, MCP-1 mRNA expression has been detected in endothelial cells, macrophages, and vascular smooth muscle cells in atherosclerotic arteries (Seino *et al.*, 1995). Therefore, MCP-1 is a critical factor to initiate atherosclerotic lesions.

Unlike most CC chemokines that bind to CC chemokine receptor (CCR)-1 through CCR8, MCP-1 binds only to CCR2 (Charo *et al.*, 1994; Boring *et al.*, 1998; Kurihara and Bravo, 1996). Therefore, the MCP-1/CCR2 binding is the important step of monocyte recruitment and seems to play a primary role in many inflammatory states of vessels. (Takeya *et al.*, 1993; Yang *et al.*, 2004; Kaikita *et al.*, 2004).

ZoaGumHwan (ZGH), a Korean herbal remedy, is composed of roots of *Coptis chinensis* Franch and fruits of *Evodia officinalis* Dode with a ratio of 6 to 1. *Coptis chinensis* is a widely used herb in traditional chinese medicine for the treatment of inflammation related diseases. As one of the major alkaloids in *Coptis chinensis*, berberine has a strong anti-inflammatory activity. Berberine has been shown to have vasorelaxing and anti-proliferating effects (Ko *et al.*, 2000) and

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to reduce serum low density lipoproteins (LDL) cholesterol levels in both hamsters and human patients (Kong *et al.*, 2004). In addition, we reported the inhibitory effects of ZGH water extract and berberine on angiotensin (Ang) II-induced endothelial inflammation (Ko *et al.*, 2007). It has been suggested that berberine is useful for treating cardiovascular disease such as hypertension and atherosclerosis (Lau *et al.*, 2001; Hong *et al.*, 2002).

In spite of all the studies, the effects of berberine and the extract of berberine-containing plants on the oxLDL-induced endothelial expression of adhesion molecules have not been studied. In the present study, we examined whether ZGH water extract and berberine prevents oxLDL-induced expression of inflammatory genes and monocyte adhesion to endothelial cells.

MATERIALS AND METHODS

Preparation of ZGH water extract

ZGH is a mixture of roots of *Coptis chinensis* Franch and fruits of *Evodia officinalis* Dode, which was obtained from the Oriental Herbal Center (OHC) of the Oriental Medical Hospital of Dongguk University College of Oriental Medicine (Kyoungju, Korea). For water extraction, ZGH (210 g) was mixed with 1,500 ml of distilled water and extracted under reflux for 3 h at 100°C. The extract was filtered with a Whatman paper filter. The filtrate was concentrated by a vacuum evaporator, freeze dried, and preserved at -20°C until use. The yield of the dried extract was approximately 18.5 g/l. The extract was suspended in distilled water, and used for experiments.

Cell Culture

HUVECs were purchased from Clonetics (San Diego, CA). HUVECs were grown on 0.2% gelatin coated flask with EBM-2 medium supplemented with 2% fetal bovine serum (FBS), ascorbic acid, hydrocortisone, human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), human epidermal growth factor (hEGF), long R insulin-like growth factor-1 (R³-IGF-1), gentamicin sulfate (GA-1000) and heparin. HUVECs between passage 2 and 6 were used in the experiments.

U937 cells were maintained in a RPMI 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 200 IU/ml penicillin and 200 µg/ml of streptomycin. Culture medium was replaced every other day. After attaining confluence, the cells were subcultured by splitting 1:5 ratio.

Isolation of LDL and generation of oxLDL

Blood from healthy volunteers was obtained by venipunc-

ture and collected in evacuated tubes. Ethylene diamine tetraacetate (EDTA) was used as an anticoagulant and antioxidant when samples were intended for LDL isolation. Blood was centrifuged at 800x g at 4°C for 15 min and then, the separated plasma was employed to prepare LDL (d=1.019 - 1.063 g/ml) by sequential flotation ultracentrifugation at 10°C as previously described (Ramos *et al.*, 1995). The isolated LDL was dialyzed for 48 hr against 10 mM sodium phosphate buffer pH 7.4 containing 150 mM NaCl and 0.1 mM EDTA at 4°C and used within two weeks. The protein content of the LDL was measured by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard.

To obtain oxLDL, LDL oxidation was done by incubating with 10 µM CuSO₄ in 10 mM sodium phosphate buffer at 37°C for 20 h and then, stopped by adding an 10 mM EDTA solution. Oxidation of LDL was monitored by thiobarbituric acid-reactive substances (TBARS) analysis (Buege and Aust, 1978).

In vitro LDL oxidation study

Isolated LDL was preincubated with ZGH water extract for 30 min. LDL oxidation was initiated by adding 10 µM CuSO₄ to the reaction mixture. The TBARS was determined after 20 h. Briefly, 500 µl of reaction mixture was mixed with 1 ml of freshly prepared 20% acetate buffer (pH 3.5) containing 0.046 mol/l thiobarbituric acid and 0.92 mol/l trichloroacetic acid. After 30 min incubation at 100°C, the samples were cooled on ice, centrifuged at 1000 g for 5 min at RT. The absorbance of the supernatant containing malondialdehyde (MDA), TBARS, was read at 532 nm.

U937 adhesion assay

Monocyte-endothelial adhesion was evaluated using the human leukemia pro-monocytic U937 cells which were labeled with 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM, 10 µg/ml) for 1 h at 37°C. HUVECs cultured in 24-well plate were pretreated with ZGH water extract or berberine for 30 min and then incubated with Ang II for an additional 3 h. Then, HUVECs were co-incubated with BCECF/AM-prelabeled U937 cells (1 × 10⁶ cells/well) for 30 min at 37°C. Non-adhering U937 cells were removed, and the cells were washed twice with PBS. A set of cells was taken and imaged by inverted microscopy connected to digital camera (TMS; Nikon, Japan), and in other sets, cells were lysed in 0.1% Triton X-100 in 0.1 mol/l Tris. Fluorescence was measured by using a Fluostar optima microplate reader (BMG LABTECH GmbH, Germany) using excitation at 485 nm and

emission at 520 nm.

RT-PCR (mRNA analysis)

The total cellular RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and RT-PCR was performed as previously described (Park *et al.*, 2007). Briefly, cDNA was synthesized using a Ready-To-Go T-Primed First Strand Kit (Amersham Biosciences, USA). PCR was performed in the presence of 0.5 U Taq DNA polymerase (Takara, Japan) using primer sets that were specific to the following: MCP-1 (5'-CCC TTC TGT GCC TGC TGC TCA T-3', 5'-TTT CCC CAA GTC TCT GTA TCT-3'; expected 300bp fragment); CCR-2 (5'-GGA TTG AAC AAG GAC GCA TT-3', 5'-TCT CAC TGC CCT ATG CCT CT-5'; expected 766bp fragment); lectinlike oxLDL receptor(LOX)-1 (5'-ACA GAT CTC AGC CCG GCA ACA AGC A-3', 5'-GGG AGA CAG CGC CTC GGA CTC TAA AT-3'; expected 463bp fragment); and GAPDH (5'-GGT GAA GGT CGG AGT CAA CG-3', 3'-CCA GTA GGT ACT GTT GAA AC-5'; expected 496bp fragment). The PCR products were separated on a 2% agarose gel containing ethidium bromide (0.5 µg/ml), visualized, and photographed using a gel documentation system (UVP, Cambridge, UK).

DPPH radical scavenging assay

To measure the antioxidant activity of the samples, free radical scavenging activity was detected using 1,1-diphenyl-2-picryl-hydroazyl (DPPH) as previously reported (Blois, 1958). Briefly, 200 µM solution of DPPH in methanol was prepared. DPPH solution (190 µl) was added to 10 µl of sample solution, and mixed with vigorous shaking for 10 s. After thirty minutes, the absorbance was measured at 517 nm. DPPH radical scavenging ability was calculated following equation in which H and H_0 were optical density of with and without sample, respectively.

$$\text{Radical scavenging activity (\%)} = \{(1 - H) / H_0\} \times 100$$

Data analysis

The data are expressed as means $\pm$ S.E.M. and were analyzed using one-way analysis of variance (ANOVA) and the Student–Newman–Keul's test for individual comparisons. P values of <0.05 were considered statistically significant.

RESULTS

ZGH water extract and Berberine inhibits oxLDL-induced monocyte adhesion to endothelial cells

To determine whether ZGH water extract and berberine have an inhibitory action in oxLDL-induced vascular inflammation, we first examined the effect of ZGH water extract on Ang II-induced monocyte adhesion to endothelial cells, an initial step of monocyte recruitment, by using U937 human premonocytic cell line (Carvalho *et al.*, 1996; Lazzarino *et al.*, 2001). As shown in Fig. 1, oxLDL (50 µg/ml) stimulation for 3 h significantly enhanced U937 adhesion to HUVECs. ZGH water extract and berberine inhibited oxLDL-induced monocyte adhesion in a concentration-dependent manner (Fig. 1A and Fig. 1B). Since the inhibitory effect of simvastatin, a 3-

(A)

(B)

Fig. 1. Inhibitory effects of ZGH water extract and berberine on oxLDL-induced monocyte adhesion to endothelial cells. The adhesion of fluorescence-labeled U937 cells to HUVECs was detected by microscopic images (A), and quantitated by using a fluorescence-detecting microplate reader (B). HUVECs were treated with different concentrations of ZGH water extract (1 and 10 µg/ml), berberine (1 and 10 µM), and simvastatin (5 µM) for 30 min before oxLDL (50 µg/ml) treatment for 3 h. Data are expressed as the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.01$ compared to untreated control group. # $P < 0.01$ compared to the oxLDL-treated group.

hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, on MCP-1/CCR2-mediated monocyte recruitment has been known (Han *et al.*, 2005), we used simvastatin as a positive control. The effect of ZGH water extract at 10 $\mu\text{g/ml}$ concentration was similar to that of simvastatin (5 μM).

ZGH water extract and berberine inhibits oxLDL-induced expressions of MCP-1 in HUVECs and CCR2 in U937 cells

Since expression level of CCR2 and MCP-1 is important in controlling the process of monocyte recruitment (Han *et al.*, 1998; Han *et al.*, 1999; Han *et al.*, 2000), we examined whether ZGH water extract and berberine inhibits oxLDL-induced expression of both MCP-1 and CCR-2. The treatment with oxLDL (50 $\mu\text{g/ml}$) for 3 h significantly increased MCP-1 mRNA level in HUVECs (Fig. 2A). Similarly, CCR2 mRNA expression in U937 cells, a receptor for MCP-1, was increased by the treatment with oxLDL (50 $\mu\text{g/ml}$) for 3 h (Fig. 2B). However, co-treatment with ZGH water extract and berberine significantly suppressed the oxLDL-increased MCP-1 and CCR2 mRNA, which corresponds to its suppressive effect on the adhesion of U937 to HUVECs. The inhibitory effects of ZGH water extract and berberine were similar to that of simvastatin (5 μM) on the oxLDL-induced MCP-1 and CCR2 expression.

The effects of ZGH water extract and berberine on oxLDL-induced lipid peroxidation *in vitro* and DPPH radical generation

Since it is known that oxidative modification of LDL plays a crucial role in the development of atherosclerosis and in cardiovascular disease (Penn and Chisolm, 1996; Ehara *et al.*, 2001), we also examined possible antioxidant activity of ZGH water extract at the concentrations tested for monocyte adhesion. LDL oxidation which was measured by the extent of TBARS was dependent upon LDL concentration, as shown in Fig. 3A, which was prevented by ZGH water extract in a concentration-dependent manner, but a limited degree (Fig. 3B). Such limited effect on LDL oxidation was correlated with its antioxidant ability. As shown in Fig. 3C, neither of the berberine, simvastatin, or low concentration of ZGH water extract showed DPPH radical scavenging activity. Only 10 $\mu\text{g/ml}$ concentration of ZGH water extract showed DPPH radical scavenging activity about 29% of Vit.C and Vit. E.

(A)

(B)

Fig. 2. Inhibitory effects of ZGH water extract and berberine on oxLDL-induced expressions of MCP-1 in HUVECs (A) and CCR2 in U937 cells (B). HUVECs and U937 cells were pretreated with ZGH water extract (10 $\mu\text{g/ml}$), berberine (10 μM), or simvastatin (5 μM) for 1 h, and then, treated with 50 $\mu\text{g/ml}$ oxLDL for 3 h. MCP-1 (A) and CCR2 (B) mRNA expression levels were detected in HUVECs and U937 cells (B), respectively, by using RT-PCR. M represents a 100 bp DNA ladder size marker. In the bar graph, the expressions of MCP-1 and CCR2 were normalized to GAPDH using a densitometer. Data are expressed as the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.01$ compared to untreated control group. # $P < 0.01$ compared to the oxLDL-treated group.

The effects of ZGH water extract and berberine on Ang II-induced LOX-1 mRNA expression

Ang II, a potent vasoconstrictor, has been implicated in the progression of cardiovascular diseases and arteriosclerosis (Dzau, 2001). Since it is also known that Ang II increases the expression of LOX-1, we examined any effect of ZGH water

Fig. 3. Effect of ZGH water extract on the *in vitro* lipid peroxidation induced by oxLDL (A, B) and DPPH radical scavenging activity (C). Lipid peroxidation by oxLDL with CuSO₄ was determined by measuring MDA (A). Lipid peroxidation by oxLDL in the absence or presence of ZGH water extract (0.1, 1, and 10 μg/ml) was measured, and the data points were expressed as nmole MDA (B). The antioxidant activity of ZGH water extract (1 and 10 μg/ml), berberine (1 and 10 μM), and simvastatin (5 μM) was measured by DPPH radical scavenging activity (C). **P*<0.05, compared to control; #*P*<0.05, compared to oxLDL-treated group.

Fig. 4. Effect of ZGH water extract on the Ang II-induced mRNA expression of LOX-1. HUVECs were pretreated with ZGH water extract (10 μg/ml), and then, treated with Ang II (100 nM) for each designated time. LOX-1 mRNA expression was detected by using RT-PCR. The size of RT-PCR product for LOX-1 and GAPDH are 463 bp and 496 bp, respectively. M: 100 bp DNA ladder size marker.

extract and berberine on the LOX-1 expression. As shown in Fig. 4, Ang II induced LOX-1 expression, which was significantly inhibited by ZGH water extract and berberine.

DISCUSSION

The initial phase of the atherosclerosis process is characterized by an increased adhesion of monocytes to vascular endothelium. Such monocyte adhesion requires the expression of specific chemokines such as MCP-1 on endothelial cells. In the present study, we showed ZGH water extract and berberin blocked monocyte adhesion onto HUVECs by suppressing the MCP-1 expression.

It has been shown that CCR2 expression level is as important as MCP-1 expression in the arterial wall for controlling the process of monocyte recruitment (Han *et al.*, 1998; Han *et al.*, 1999; Han *et al.*, 2000). Our result showed that ZGH water extract and berberine inhibited the expression of both MCP-1 and CCR2, suggesting that ZGH water extract and berberine may be effective agents for suppressing oxLDL-induced monocyte recruitment, an initial step in atherogenesis.

Oxidative modification of LDL plays a crucial role in the development of atherosclerosis and in cardiovascular disease (Penn and Chisolm, 1996; Ehara *et al.*, 2001). Also, there is extensive evidence that intracellular redox status is a key modulator of the inflammatory response (Winyard and Blake, 1997), including the modulation of MCP-1 expression (Anjali *et al.*, 1999). However, the inhibition degree of LDL oxidation by ZGH water extract was only about 8 to 12%. Similarly, the ability of ZGH water extract and berberine as an antioxidant was negligible compared to well-known antioxidants such as

Vit. C and Vit. E.

Ang II is known to be an important factor in atherogenesis. It is also widely appreciated that Ang II and oxLDL are important factors in inducing endothelial dysfunction and injury. Recent studies suggest an interaction between hyperlipidemia, activation of rennin-angiotensin system, and atherosclerotic disease (Nickenig and Bohm, 1997; Keidar *et al.*, 1995; Li *et al.*, 1999). It has been demonstrated that Ang II induces LOX-1 (Morawietz *et al.*, 1999). LOX-1 is a human endothelial receptor that mediates uptake of oxLDL and structurally distinct from scavenger receptors of macrophages (Sawamura *et al.*, 1997). In the present study, we clearly showed ZGH water extract and berberine suppressed the Ang II-induced expression of LOX-1. We have also previously reported that ZGH water extract and berberine inhibits Ang II-induced MCP-1 expression and monocyte adhesion through inhibition of reactive oxygen species (Ko *et al.*, 2007). These results indicate that usefulness of ZGH water extract and berberine for the therapy of anti-atherosclerotic disease.

Berberine in *Coptis chinensis* Franch and evocarpine, a quinolone alkaloid in *Evodia officinalis* Dode, have also been demonstrated as vasorelaxants (Ko *et al.*, 2000; Yamahara *et al.*, 1988), our results further indicate the potential usefulness of ZGH water extract for the therapy of hypertension-accelerated vascular inflammatory disease. However, this study considers the effect of ZGH in the in vitro vascular inflammation, and thus, it should be followed by in vivo experiments to consolidate the application of ZGH for vascular inflammatory disease.

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