Anti-atherogenic Effects of the Methanol Extract of Sorbus Cortex in Atherogenic-Diet Rats

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The present study was designed to examine whether the methanol extract of Sorbus commixta cortex (MSC) could prevent the development of atherosclerosis through regulating the vascular nitric oxide (NO) and endothelin-1 (ET-1) systems in atherogenic-diet rats. Our findings show that aortic NO production as well as endothelial nitric oxide synthase (eNOS) expression was significantly decreased in atherogenic-diet rats compared with those in the control group. Aortic ET-1 expression was augmented in rats fed an atherogenic-diet while NF-κB p65 was upregulated. Treatment of atherogenic-diet rats with either low (100 mg/kg/d) or high (200 mg/kg/d) doses of MSC led not only to significant increases in the aortic NOS/NO system, but also to decreases in aortic ET-1 expression. The aortic expression level of NF-κB p65 was also attenuated in atherogenic-diet rats by chronic treatment with low or high doses of MSC. Atherogenic-diet induced increases in the expression of adhesion molecules including intercellular adhesion molecules-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin were markedly decreased by treatment with MSC. From the histopathological examination, MSC treatment was shown to lessen the thickening noted in the aortic intima and media of the atherogenic-diet rats. These results suggest that MSC affects the atherogenic process via the suppression of proinflammatory and adhesion molecules in atherogenic-diet rats, which may be, at least in part, causally related with the regulation of vasocoactive systems such as the NO and ET-1 systems.

Key words Sorbus commixta cortex methanol extract (MSC); atherosclerosis; nitric oxide (NO); endothelin-1 (ET-1)

Generally, atherosclerosis is characterized by fatty streak formation which is caused by endothelial dysfunction, macrophage foam cell accumulation, and fibrous plaque formation caused by smooth muscle cell proliferation and connective tissue synthesis.1) It has been well documented that some vasocoactive molecules such as nitric oxide (NO) and endothelin-1 (ET-1) are involved in the vascular inflammatory process that leads to atherosclerosis.2,3) Several lines of evidence implicate the endothelin system in the pathogenesis of atherosclerosis. It has also been demonstrated that ET-1 stimulates the proliferation, migration, and matrix formation of smooth muscle cells in vitro and potentiates neointimal formation after vascular wall injury in vivo. Therefore, suppression of ET-1 expression by the endothelium is believed to play an important role in preventing the development of atherosclerosis.

In addition to its well-known role as a vasodilator, endothelial NO inhibits platelet aggregation, thrombogenesis, leukocyte adhesion, and proliferation of vascular smooth muscle cells.5) Endothelial dysfunctions that are closely related with impaired NO may represent an early stage of vasculopathy that can lead to atherosclerotic cardiovascular disorders.6) Impaired release of NO from vascular beds results in increased leukocyte–endothelium interaction via the up-regulation of endothelial cell adhesion molecules which include E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1).7,8) Under these conditions, numerous leukocytes adhere to the vascular endothelium and transmigrate the endothelium, thus aggravating the endothelial dysfunction and tissue injury.9,10) Systemically administering NO donors to NO-deficient animals preserves endothelial function and attenuates pathological interactions between circulating leukocytes and the vascular endothelium.7,11) Sorbus commixta cortex has long been used in the field of traditional Oriental medicine as a tonic to treat coughing, asthma, and other bronchial disorders.12) From the cortex of S. commixta, triterpenoids such as lupenone and lupeol have been isolated.13) Recently, the methanol extract of Sorbus commixta cortex (MSC) was shown to have a potent radical scavenging activity.14) MSC has also been observed to dilate vascular smooth muscle via the up-regulation of an endothelium-dependent NO-cyclic GMP pathway.15) This pharmacological effect on vascular tissue may be useful for the treatment of cardiovascular diseases such as atherosclerosis. Therefore, the present study was designed to investigate the effects of MSC treatment on the inflammatory process and in the development of atherosclerosis in an atherogenic-diet fed rat model.

MATERIALS AND METHODS

Plant Material and Extraction The stem bark of S. commixta (Malaceae) was purchased from the herbal medicine co-operative association of Junbuk Province, Korea, in October 2003. A voucher specimen (No. BDR 23) was deposited in the Herbarium of the Professional Graduate School of Oriental Medicine, Wonkwang University (Korea). The stem bark of S. commixta (1.0 kg) was air-dried at room temperature and reduced to a fine powder by milling. The powder was subjected to extraction with 800 ml of methanol, three times, 24 h each. The methanol extract was filtered through Whatman No. 3 filter paper and concentrated using a rotary evaporator (61.2 g).

Experimental Animals All animal procedures were carried out in accordance with the National Institute of Health...
Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85—23, revised 1996) and were approved by the Institutional Animal Care and Utilization Committee for Medical Science of Wonkwang University. Male Sprague-Dawley (SD) rats (weighing 170 to 200 g) were purchased from Korean Experimental Animals Co. (Daejeon, Korea) and housed in an animal room with an automatic temperature (22 °C) and lighting (12 h light–dark cycle) control. A 1-week adaptation period for vehicle (tap water) administration and blood pressure measurements was allowed before initiation of the experimental protocol. Over the course of 7 weeks, the experimental protocol was carried out. During the first 3 weeks, the rats were allocated a normal rat chow diet with water alone (control group) or a diet supplemented with an atherogenic-diet in water (ATH group). The rats were then divided into four groups. For the remaining 4 weeks, the rats were treated as follows: 1) control group, 2) ATH group; atherogenic-diet, 3) ATH/MSC1 group; atherogenic-diet with MSC-containing water (100 mg/kg/d), 4) ATH/MSC2 group; atherogenic-diet with MSC-containing water (200 mg/kg/d) (n = 10, each group). The atherogenic diet, containing 1.25% cholesterol, 1.25% casein, and 75% commercial laboratory rat feed, was purchased from ICN Pharmaceutical Inc. (Irvine, CA, U.S.A.). Systolic blood pressure (SBP) was measured weekly in conscious rats by tail-cuff plethysmography. At least six determinations were made in every session and the mean of the lowest three values within 5 mmHg was taken as the SBP level. On the day of sacrifice, rats were decapitated without anesthesia and trunk blood was collected in prechilled tubes containing 1 mg/ml ethylenediaminetraacetic acid (EDTA) to measure the triglyceride (TG) and cholesterol concentrations.

Protein Preparation and Western Blot Analyses

The thoracic aortae were homogenized in solutions containing 250 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 20 mM potassium phosphate buffer, at pH 7.6 with a Polytron homogenizer at 3000 rpm. Large tissue debris and nuclear fragments were removed by two low speed spins in succeesion (10000 g, 5 min; 10000 g 10 min) at 4 °C. Supernatants from these low speed spins were ultracentrifuged at 100000 g for 1 h at 4 °C. The pellets were resuspended for protein blotting and the protein concentrations were determined by the Bradford method (1) with bovine serum albumin as a standard. Protein samples (50 µg) were electrophoretically fractionated with a discontinuous system consisting of 10% or 13% polyacrylamide resolving gels and electrophoretically fractionated with a discontinuous system consisting of 10% or 13% polyacrylamide resolving gels and 7.6 with a Polytron homogenizer at 3000 rpm. Large tissue debris and nuclear fragments were removed by two low speed spins in succeesion (10000 g, 5 min; 10000 g 10 min) at 4 °C. Supernatants from these low speed spins were ultracentrifuged at 100000 g for 1 h at 4 °C. The pellets were resuspended for protein blotting and the protein concentrations were determined by the Bradford method (1) with bovine serum albumin as a standard. Protein samples (50 µg) were electrophoretically fractionated with a discontinuous system consisting of 10% or 13% polyacrylamide resolving gels and 5% stacking gels and then transferred to nitrocellulose membranes (Amersham, Buckinghamshire, England) and a Chemi-doc image analyzer (Bio-Rad, Hercules, CA, U.S.A.). Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using a Trizol reagent (Sigma, MI, U.S.A.) as suggested by the manufacturer. RNA concentrations were determined using a spectrophotometer (Shimadzu, Kyoto, Japan). 5 µg of RNA was used for the reverse transcription-polymerase chain reaction (PCR) using a thermal cycler (MJ Research Inc., Waltham, MA, U.S.A.). The following sequence was performed for each PCR reaction: 94 °C for 1 min (1 cycle); 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min (with a variable number of cycles); and a final extension phase at 72 °C for 7 min. Thirty cycles were run for ET-1 and actin. Primer sequences used for the analyses of ET-1 and GAPDH mRNAs are as follows: ET-1 (sense): 5′-ATG GAT TAT TTT CCC GTG AT-3′; ET-1 (anti-sense): 5′-GGG AGT GTT GAC CCA GAT GA-3′; GAPDH (sense): 5′-TCA TGG ACC TCA ACT ACA-3′; GAPDH (anti-sense): 5′-CAA AGT TGT CAT GGA TGA CC-3′. PCR products were run on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide (EtBr). The lengths of the amplicons were 230 and 460 base pairs for ET-1 and GAPDH, respectively.

Preparation of Aorta and Recording of Isometric Vascular Tone

Each group of rats was sacrificed by decapitation. The thoracic aortae of these rats were rapidly and carefully dissected and placed into ice-cold Krebs solution (pH 7.4) containing 118 mM NaCl, 4.7 mM KCl, 1.1 mM MgSO4, 1.2 mM KH2PO4, 1.5 mM CaCl2, 25 mM NaHCO3, and 10 mM glucose. The aortae were removed free of connective tissue and fat, and then cut into rings with a width of approximately 3 mm. All dissecting procedures were done with extreme care to protect the endothelium from inadvertent damage. The aortic rings were suspended in a tissue bath containing Krebs solution at 37 °C (pH 7.4). 95% O2–5% CO2 was continuously bubbled through the bath. The baseline load placed on the aortic rings was 1.5 g. Changes in isometric tension were recorded using a force-displacement transducer (Grass FT 03, Quincy, MA, U.S.A.) connected to a Grass polygraph recording system (Model 7E). In the first set of experiments, the aortic rings were contracted with phenylephrine (3 × 10−6 M) to obtain maximal response. The aortic rings were then washed every 20 min with Krebs solution until the tension returned to the basal level. Aortic relaxation was carried out by the cumulative addition of acetylcholine chloride (ACh) or sodium nitroprucide (SNP). After each test, the aortic rings were washed three times with fresh Krebs solution and allowed to equilibrate for 30 min. Relaxant responses are expressed as percentage relaxation from phenylephrine (3 × 10−6 M) pre-constriction levels.

Plasma Triglyceride (TG) and Cholesterol Assay

Plasma triglyceride, total cholesterol, LDL-cholesterol, and HDL-cholesterol levels were assayed enzymatically by an automated commercial method (Behringer Mannheim, Marburg, Germany).

Nitrite Assay

The nitrite (NO2−) concentration in the aortic tissue was measured as an indicator of NO production according to the Griess reaction. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1%
naphthylhexylenediamine dihydrochloride in water). The absorbance of the mixture at 550 nm was determined with a microplate reader (Bio-Rad, Hercules, CA, U.S.A.).

**Histological Examination** The aortae isolated from each group were fixed in 10% (v/v) formalin in 50 mM potassium phosphate buffer (pH 7.0) for 24 h at 4 °C. The tissues were subsequently embedded in paraffin, sectioned (4 μm) and stained with hematoxylin & eosin (H–E). Slides were examined under the light microscope for histopathological changes. Representative sections were photographed using an Olympus automatic photo microscopic system (Tokyo, Japan).

**Statistical Analysis** Results were expressed as means±S.E.M. The statistical significance of the difference between the group means was determined using the one-way ANOVA and Student’s t-test.

**RESULTS**

**Body Weight, Blood Pressure, and Lipid Profile** As shown in Table 1, there were no significant changes in body weight and blood pressure among the experimental groups at the end of experiment. Plasma total cholesterol, HDL-cholesterol, and LDL-cholesterol concentrations were higher in atherogenic-diet rats than in the control group (p<0.01 for total and LDL-cholesterol; p<0.05 for HDL-cholesterol, each). However, treatment of atherogenic-diet rats with either low or high doses of MSC markedly lowered plasma total cholesterol and LDL-cholesterol concentrations (p<0.05, vs. atherogenic-diet rats, each). The plasma HDL-cholesterol concentration remained unchanged. The plasma TG concentration was also higher in atherogenic-diet rats than in the control group (p<0.05). Both low and high dose MSC treatments significantly decreased plasma TG levels in atherogenic-diet rats (p<0.05 vs. atherogenic-diet rats, each).

**NO₂⁻ Level in the Plasma and Aorta** The plasma nitrite concentrations did not differ among the experimental groups. However, in atherogenic-diet rats the aortic nitrite content was decreased compared with that of the control group (p<0.05). Nitrite production in the aortic tissue was markedly restored by treatment with both low and high doses of MSC (p<0.05, vs. atherogenic-diet group, each) (Table 2).

**Aortic Expression of ecNOS** Compared with the control group, the expression level of ecNOS in aortic tissue was significantly greater in the atherogenic-diet group (p<0.01) (Fig. 1). Both low and high dose MSC treatments lowered aortic ecNOS expression in the atherogenic-diet group (p<0.01 vs. atherogenic-diet group, each).

**Aortic ET-1 mRNA Expression** As shown in Fig. 2, aortic ET-1 expression levels in atherogenic-diet rats were greater than in the control group (p<0.01). These levels were attenuated by both the low and high dose MSC treatments (p<0.01 vs. atherogenic-diet group).

**Vascular Tone of Aorta** Addition of ACh produced an endothelium-dependent relaxation in the aortic strips (Fig. 3). The relative aortic relaxation was attenuated in atherogenic-diet rats compared to control group rats. However, the dose-dependent relaxation curves for the MSC-treated atherogenic-diet rat aortae were similar to those for the con-
Vasodilator response to SNP was almost identical among the four groups (data not shown).

**Aortic NF-κB p65 Expression** The aortic expression level of NF-κB p65 was examined by Western blot analyses in control, atherogenic-diet, and both the low and high dose MSC-treated atherogenic-diet groups. Within the atherogenic-diet group, the expression level of NF-κB p65 was significantly increased compared to the control group (p < 0.01). Both the low and high dose MSC treatments restored the upregulated NF-κB p65 protein expression level to normal (p < 0.05 vs. atherogenic-diet group, each) (Fig. 4).

**Aortic Expressions of Adhesion Molecules** Aortic expression levels of VCAM-1, ICAM-1, and E-selectin were determined by Western blots using actin as an internal standard. Aortic expression levels of VCAM-1, ICAM-1, and E-selectin were higher in the atherogenic-diet group than in the control group (p < 0.01 for ICAM-1, VCAM-1, and E-selectin, each) (Fig. 5). Both the low and high dose MSC treatments significantly inhibited these increases in the atherogenic-diet group (p < 0.05 vs. atherogenic-diet group for ICAM-1 and VCAM-1, respectively; p < 0.01 for E-selectin).

**Histological Examination** Figure 6 shows the H–E stained aortic segments from the experimental groups. In the aortic segments from the atherogenic-diet group, there was a
pathological thickening of the intima and media (Fig. 6B). This change was ameliorated by both the low and high dose MSC treatments (Fig. 6C, D).

DISCUSSION

Endothelial dysfunction, defined as an impaired ability of the vascular endothelium to stimulate vasodilation, plays a key role in the development of atherosclerosis. The major cause of endothelial dysfunction is a decrease in the bioavailability of nitric oxide (NO), a potent biological vasodilator produced in the vascular endothelium from L-arginine by the endothelial NO synthase (NOS). In vascular diseases, the bioavailability of NO can be impaired by various mechanisms, including a decreased NO production by ecNOS, and/or an enhanced NO breakdown due to increased oxidative stress. After observing that MSC activates an endothelium-dependent NO/cGMP signaling pathway, we began to investigate the effects of MSC treatment on vascular inflammation and the pathophysiological changes induced by an atherogenic-diet. In rats fed an atherogenic-diet, a plasma TG levels as well as cholesterol levels were increased; (b) endothelium-dependent vascular relaxation in response to ACh was impaired; (c) aortic ecNOS expression was attenuated; (d) aortic ET-1 expression was augmented; (e) aortic NF-κB expression and several adhesion molecules were up-regulated. These results are in line with the results obtained from previous reports. Treatment of atherogenic-diet rats with either low or high doses of MSC markedly lowered plasma total and LDL-cholesterol concentrations. HDL-cholesterol concentrations remained unchanged. Plasma TG concentrations were suppressed by MSC treatment. Treatment of atherogenic-diet rats with MSC restored aortic nitrate levels and aortic ecNOS expression levels. In addition, endothelium-dependent aortic relaxation in response to ACh was almost completely restored by chronic treatment with MSC.

Native and oxidized LDLs are involved in the atherogenic process and affect endothelium-dependent vascular tone through their interaction with NO. A systemic deficit of NO may increase the levels of LDL-cholesterol in hypercholesterolemia by modulating its synthesis and metabolism in the liver. Cholesterol lowering drugs, for example statins, have been shown to improve endothelial functions in patients with hypercholesterolemia and atherosclerosis. Previous studies have suggested that the effects of MSC in atherosclerotic rats are similar to the effects of L-arginine on atherosclerosis; the MSC-induced up-regulation of the vascular NOS/NO system may be the pivotal event improving the vascular endothelial function in rats fed an atherosclerotic-diet.

In general, the endothelial dysfunction associated with atherosclerosis has been attributed to either alterations in the NOS/cGMP pathway or an excess of ET-1. ET-1 is a potent vasoconstrictr peptide involved in homeostatic regulation of vascular smooth muscle tone. An increase in circulating ET-1 is associated with many cardiovascular disorders including congestive heart failure, hypertension, and atherosclerosis. ET-1 is primarily released from endothelial cells and exerts its biological effect through the activation of specific ET receptors. ET-1 production is increased in systemic cardiovascular diseases like atherosclerosis and hypercholesterolemia. This can disrupt the delicate balance between endothelium-derived vasoactive factors. Circulating endothelin-1 immunoreactivity has been shown to be elevated in patients with diffuse atherosclerotic disease, as well as in animal models of early coronary atherosclerosis and coronary endothelial dysfunction. Tissue endothelin-1 levels have shown to be increased in atherosclerotic coronary arteries and aortae. Coronary and peripheral endothelial function has been restored in experimental hypercholesterolemia during ET blockage, supporting the role of ET-1 as a contributor to endothelial dysfunction in hypercholesterolemia. Here, we observe that co-administering MSC attenuates the enhanced expression of ET-1 in aortae from the atherogenic-diet rats. These findings suggest that the restoration of vascular function with MSC is due not only to an upregulation of the vascular NOS/NO system, but also to a down-regulation of aortic ET-1.

Inflammation is a basic pathological mechanism that underlies a variety of diseases. Atherosclerosis is clearly a result of an inflammatory response. Interrupting the function of an inflammatory mediator can decrease the atherosclerotic lesion size. The vascular inflammatory reaction involves complex interactions between inflammatory cells and vascular cells. NF-κB is an important mediator in the vascular inflammatory processes caused by vasoactive molecules. NF-κB is a ubiquitously expressed multiunit transcription factor that is activated by diverse signals; possibly via phosphorylation of the I-κB subunit, its dissociation from the inactive cytoplasmic complex, and translocation of the active dimer, p50 and p65, to the nucleus. NF-κB is activated by vasoac-
tive molecules such as ET-1 in monocytes/macrophages and vascular smooth muscle cells (VSMCs). Activation results in the up-regulation of inflammatory and cell adhesion molecules.\(^3\)\(^8\) Vascular NO could inhibit the expression of NF-κB and several cell adhesion molecules in the vascular endothelium.\(^7\)\(^10\) The suppressive effect of NO on the expression levels of adhesion molecules may represent one of its important anti-inflammatory and anti-atherosclerotic effects. In rats fed an atherogenic-diet, the vascular expression levels of NF-κB and several cell adhesion molecules, ICAM-1, VCAM-1, and E-selectin, were significantly higher than in the control group. MSC treatment suppressed these atherogenic-diet-induced increases in the vascular expression of NF-κB and the cell adhesion molecules. In this experiment model, MSC treatment seemed to decrease the expression of the vascular adhesion molecules through the suppression of NF-κB. This seems to be, at least in part, causally related with the modulation of vasoactive systems such as the NOS/NO/cGMP and ET-1 systems.

In accordance with the molecular changes of the aortic segments from the atherogenic-diet rats, histological examinations revealed a thickening of aortic intima and media that is compatible with the processes of atherosclerosis. Even this morphological change could be prevented by treatment with MSC. This finding suggests a novel anti-inflammatory effect of MSC beyond the salutary effects on endothelial dysfunction.

In conclusion, our study demonstrates that MSC treatment not only improves the vascular dysfunction but suppresses the atherosclerotic processes in atherogenic-diet rats, which may be causally related with the restoration of vasoactive systems such as the eNOS/NO/cGMP and/or ET-1 systems.

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