Effect of Methanol Extract of Sorbus Cortex in a Rat Model of L-NAME-Induced Atherosclerosis

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Chronic inhibition of nitric oxide (NO) synthesis by administration of high dose of N\(^\text{G}\)-nitro-L-arginine methyl ester (L-NAME) induces vascular inflammation and subsequent atherosclerosis. We aimed to investigate whether the methanol extract of Sorbus commixta cortex (MSC) is able to prevent inflammatory process in a rat model of L-NAME-induced atherosclerosis. Chronic treatment with low or high doses of MSC prevented the L-NAME-induced increase in monocyte chemoattractant protein-1 (MCP-1) and nuclear factor-κB (NF-κB) p65 expressions as well as adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and E-selectin in aorta. In addition, increased endothelin-1 (ET-1) and angiotensin converting enzyme (ACE) expressions and decreased endothelial cell NO synthase (ecNOS) expression in aorta from L-NAME treated group was reversed by treatment with MSC. From the histological examination, aortic segment from the L-NAME-treated rats revealed a thickening of intima and media, which was ameliorated by treatment with MSC. In conclusion, our results indicate that MSC can prevent atherosclerosis by inhibiting vascular over-expressions of vasoactive materials, pro-inflammatory transcription factor, and adhesion molecules and by augmenting ecNOS in chronic L-NAME-treated rat model.

Key words Sorbus commixta cortex; atherosclerosis; N\(^\text{G}\)-nitro-L-arginine methyl ester (L-NAME)

In addition to its well-known vasodilating action, endothelial nitric oxide (NO) inhibits platelet aggregation, thrombogenesis, leukocyte adhesion, and proliferation of vascular smooth muscle cells.1—3) Endothelial dysfunction that is closely related with impaired NO actions may represent a early stage of vasculopathy leading to atherosclerotic cardiovascular disorders.4,5) Accordingly, chronic inhibition of NO synthesis by administration of high dosage of N\(^\text{G}\)-nitro-L-arginine methyl ester (L-NAME) to rats induces an early hypertensive state associated with vascular inflammation in a week, and then severe atherosclerosis in coronary artery in 4—8 weeks.6)

Impaired release of NO from vascular beds results in increased leukocyte–endothelium interaction via 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 vascular endothelium and transmigrate the endothelium, thus aggravating endothelial dysfunction and tissue injury.9,10) On the contrary, systemic administration of NO donors to NO-deficient animals preserves endothelial function and attenuates pathological interactions between circulating leukocytes and vascular endothelium.11,12)

Sorbus commixta cortex (MSC) (Malaceae) has long been used in the field of traditional Oriental medicine as a tonic and for the control of cough, asthma, and other bronchial disorders.13) Recently, MSC was shown to have a potent radical scavenging activity.14) We also made an observation that MSC relax vascular smooth muscle via up-regulation of endothelium-dependent NO-cyclic guanosine-3’,5’-cyclic monophosphate (GMP) pathway.15) This pharmacological effect of MSC on vascular tissue may be useful for the treatment of various cardiovascular diseases such as hypertension and atherosclerosis. Therefore, we aimed to investigate in vivo anti-inflammatory and anti-atherosclerotic activities of MSC in a rat model of NO-deficient atherosclerosis.

MATERIALS AND METHODS

Plant Material and Extraction The stem bark of S. commixta cortex was purchased from the herbal medicine co-operative association of Junbuk Province (Iksan, Korea), in October 2003. Voucher specimen (NO. BDR 23) has been deposited in the Herbarium of the Professional Graduated School of Oriental Medicine, Wonkwang University (Iksan, Korea). S. commixta cortex (1.0 kg) was air-dried at room temperature and reduced to fine powder by milling. The powder was subjected to extraction procedures with 800 ml of 100% methanol, three times, 24 h each. The methanol extract was filtered with Whatman No. 3 filter paper, concentrated using rotary evaporator (61.2 g), and used in the present study.

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) purchased from Korean Experimental Animals Co. (Daejeon, Korea) were housed in an animal room with an automatic temperature (22 °C) and lighting (12 h light–dark cycle) control. An adaptation period of 1 week for vehicle (tap water) administration and blood pressure measurements was allowed before initiation of the experimental protocol. A total of 6 weeks of experiments were planned. The rats were allocated to normal rat chow diet with water alone or with additional L-NAME in water (1 mg/ml) during the first 3 weeks of experiments. Then, these two groups were further divided into five groups
according to MSC-treatment or MSC-untreatment, and maintained for additional 3 weeks. Thus, L-NAME was administered for 6 weeks to the appropriate groups. The five experimental groups are as follows: 1) control group; 2) MSC-treated (200 mg/kg/d) control group; 3) L-NAME group; 4) low dose MSC-treated (100 mg/kg/d) L-NAME group; 5) high dose MSC-treated (200 mg/kg/d) L-NAME group. 

Protein Preparation and Western Blot Analyses The thoracic aortae were homogenized with Polytron homogenizer at 3000 rpm in a solution containing 250 mmol/l sucrose, 1 mmol/l Ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/l phenylmethylsulfonyl fluoride (PMSF) and 20 mmol/l potassium phosphate buffer, at pH 7.6. Large tissue debris and nuclear fragments were removed by two low speed spins in succession (1000 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 pellet was resuspended for protein blotting and the protein concentration was determined by the method of Bradford with bovine serum albumin as a standard. Protein samples (50 μg) were electrophoretically fractionated with a discontinuous system consisting of a 10% or 13% polyacrylamide resolving gels and 5% stacking gel, followed by transfer to a nitrocellulose filter. The membrane was washed, blocked, and then incubated with primary antibodies and 100 mA (current constant) overnight. The membrane was dried, exposed to X-ray film for 1 day, and then analyzed and quantified by densitometry. 

RESULTS

Aortic Expressions of NF-κB p65 and MCP-1 Aortic MCP-1 and NF-κB p65 expression levels, examined by Western blot analyses, were significantly greater in the L-NAME group than in the control group (p < 0.05 for MCP-1; p < 0.01 for NF-κB p65) (Fig. 1). The increased MCP-1 and NF-κB p65 expressions were prevented by both low dose and high dose MSC-treatments (p < 0.01 vs. L-NAME group for MCP-1, each; p < 0.05 vs. L-NAME group for NF-κB p65, each).

Aortic Expressions of Adhesion Molecules Expression levels of VCAM-1, ICAM-1, and E-selectin in the thoracic aorta were determined by Western blots using actin as an internal standard. Results were expressed as ratios between VCAM-1, ICAM-1, and E-selectin and actin. Expression levels of VCAM-1, ICAM-1, and E-selectin in the aorta were higher in the L-NAME treated group than in the control group (p < 0.05 for ICAM-1 and VCAM-1, each; p < 0.01 for

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 and eosin. Slides were examined under the light microscope for histopathological changes. Representative sections were photographed using Olympus automatic photo micrographic system (Tokyo, Japan).

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

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E-selectin) (Fig. 2). L-NAME-induced expressions of adhesion molecules were significantly inhibited in both low dose and high dose MSC-treated L-NAME group ($p < 0.05$ vs. L-NAME group for each of the adhesion molecules). Moreover, MSC lowered aortic ICAM-1 and E-selectin even in the vehicle-treated control group ($p < 0.05$, each).

**Aortic Expressions of ACE and ecNOS** Compared with the control group, the expression level of ACE in aortic tissue was significantly greater in the L-NAME group ($p < 0.01$) (Fig. 3). Treatment with the low or high doses of MSC prevented the increases in aortic ACE expressions ($p < 0.05$ and $p < 0.01$ vs. L-NAME group, respectively). On the other hand, the expression level of ecNOS in aortic tissue was significantly decreased in the L-NAME group compared with that of control group ($p < 0.01$) (Fig. 4). The decreased ecNOS expression was restored by treatment with the low or high doses of MSC with more significant improvement in high dose MSC-treated L-NAME group ($p < 0.05$ and $p < 0.01$ vs. L-NAME group, respectively).

**Aortic ET-1 mRNA Expression** As shown in Figure 4, the expression level of aortic ET-1 in the L-NAME-group was greater than in the control group ($p < 0.01$), but the increased level was attenuated by treatment with low and high doses of MSC ($p < 0.01$ vs. L-NAME group).

**Histological Examination** Figure 5 shows H-E stained aortic segments from the experimental groups. In the aortic segment of the L-NAME group, there was pathologic change that included the thickening of intima and media (Fig. 5B). These changes were ameliorated by the low or high doses of MSC treatment (Figs. 5C, D).

**DISCUSSION**

The results of the present study indicate that MSC treatment could suppress the L-NAME-induced inflammatory processes in the aorta from rats. MSC prevented the L-NAME-induced increase in the expressions of MCP-1 and
NF-κB p65 as well as various adhesion molecules. The present study also showed that the L-NAME-mediated induction of ET-1 and ACE could be prevented by MSC treatment, whereas down-regulation of aortic ecNOS in the L-NAME group could be restored by treatment with MSC. In accordance with the molecular changes of the aortic segments from the L-NAME group, histological examinations revealed a thickening of intima and media with increased foam cells and smooth muscle cell proliferation which are compatible with the processes of atherosclerosis. Again, even these morphologic changes could be prevented by treatment with MSC. These data suggest novel anti-inflammatory effects of MSC beyond the salutary effects on endothelial dysfunction.

NO could inhibit the expression of cell adhesion molecules on the vascular endothelium. 9,12 And, this inhibition of adhesion molecules by NO may represent one of its important anti-inflammatory and anti-atherosclerotic actions. Accordingly, it has been reported that chronic inhibition of endothelial NO synthesis by L-NAME in rats induces early vascular inflammatory changes, including monocyte infiltration into coronary vessels, NF-κB activation, and MCP-1 expression, as well as subsequent atherosclerosis. 17—19 Vascular pathophysiologic and pathobiological events occurring after high dose of L-NAME administration to rats are similar to those seen in the course of human atherosclerosis.

After we recently observed that MSC activates endothelium-dependent NO-cyclic GMP signaling pathway, 5 we aimed to investigate if MSC treatment could prevent various vascular inflammatory and pathophysiological changes induced by high dose of L-NAME. In the L-NAME-treated rats, the expression of aortic ecNOS was significantly decreased in the present study. But, as expected, the decreased expression of aortic ecNOS in the L-NAME-treated rats was restored by co-administration of MSC.

Furthermore, we revealed that treatment with MSC attenuates the increase of ACE expression in the aorta from the L-NAME-treated rats. An increase in angiotensin II (Ang II) action mediated via type 1 receptor has been shown to cause vascular inflammation, oxidative stress, and then atherosclerosis. 20,21 Therefore, the beneficial effects of MSC seen in the present study may be explained by the suppressions of vascular inflammation and ACE expression and by the upregulation of ecNOS.

ET-1 is also a potent vasoconstrictor peptide involved in homeostatic regulation of vascular smooth muscle tone, 22 and increased circulating ET-1 is associated with many cardiovascular disorders, including congestive heart failure, hypertension, and atherosclerosis. 23 Here we show that the co-administration of MSC is able to attenuate the enhanced expression of ET-1 in aorta from the L-NAME-treated rats.

An important link between vasoactive molecules and inflammation is NF-κB. NF-κB is a critical signal molecule in various inflammatory processes and in pathophysiologic responses to a variety of stimuli that include growth factors, lymphokines, UV irradiation, pharmacological agents, and oxidative stress. 24 The vasoactive molecules including Ang II and ET-1 could stimulate NF-κB in monocytes/macrophages and vascular smooth muscle cells (VSMCs), resulting in up-regulation of the proinflammatory molecules such as MCP-1. 25 In the present study, the expression levels of ACE and ET-1 in the aorta were augmented in the L-NAME-treated rats, but restored by treatment with low or high dose of MSC. These pathophysiologic changes by chronic L-NAME administration and therapeutic responses to MSC were paralleled by changes with similar trend in the expressions of NF-κB p65 and MCP-1. 25 In rats with L-NAME-treated atherosclerosis, the expression levels of MCP-1 as well as NF-κB p65 were higher than in the control group. The up-regulations of MCP-1 and NF-κB p65 were restored to normal levels in responses to both low and high dose MSC treatments.

In addition, NF-κB is a main transcription factor that up-regulates the expression of adhesion molecules in various inflammatory disorders. The inflammatory reactions involve the complex interactions between inflammatory cells and vascular cells. 9,19 In the present study, the increased levels of aortic adhesion molecules including ICAM-1, VCAM-1, and E-selectin in rats with L-NAME-induced atherosclerosis were normalized by treatment with MSC. Unexpectedly, high dose of MSC treatment lowered aortic ICAM-1 and E-selectin even in the vehicle-treated control group. Nonetheless, the exact mechanism of the inhibitory effect of MSC on vascular inflammation is not completely clear in the present study, the results suggest that MSC treatment decreases vascular adhesion molecules through suppression of NF-κB expression in this experiment model.

In conclusion, our results suggest that MSC treatment can prevent the atherosclerosis induced by L-NAME by suppressing the vascular over-expression of ET-1 and ACE, pro-inflammatory transcription factor, proinflammatory cytokines, and adhesion molecules as well as by augmenting vascular ecNOS.

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