Anti-atherosclerotic Effect of the Methanol Extract of *Sorbus commixta* Cortex in the High Cholesterol-Diet Rats

Dae Gill Kang, Eun Jin Sohn[1], Jin Sook Kim[1], Yun Jung Lee, Mi Kyoung Moon, An Sook Lee, Jun Seok An, Ho Sub Lee*

Professional Graduate School of Oriental Medicine and Medicinal Resources Research Institute (MeRRI), Wonkwang University, 1:Korea Institute of Oriental Medicine (KIOM)

Hypercholesterolemia is a pivotal pathogenic factor for the development and maintenance of atherosclerosis. The present study was designed to evaluate whether the methanol extract of *Sorbus commixta* cortex (MSC) restores vascular dysfunction in association with the aortic expressions of proinflammatory and adhesion molecules in high cholesterol (HC) diet-rats. Chronic treatment with low (100 mg/kg/day) or high doses (200 mg/kg/day) of MSC lowered the increase in plasma levels of triglyceride (TG) and low-density lipoprotein (LDL) cholesterol induced by a cholesterol-enriched diet without affecting on the plasma level of high density lipoprotein (HDL)-cholesterol. Vascular tone attenuated in the HC-diet rats was restored by administration with MSC. Treatment with MSC also suppressed the HC-induced increase in the monocyte chemoattractant protein-1 (MCP-1) and nuclear factor-κB (NF-κB) p65 expressions as well as expressions levels of adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin in aorta. The present study also showed that MSC inhibited the HC-mediated induction of ET-1 and ACE expression. In histopathological examination, aortic segments in the HC-diet rat revealed thickening intima and media, which were blocked by administration with MSC. Taken together, MSC could suppress the development of atherosclerosis in the HC-diet rat model through the inhibition of the aortic expression levels of pro-inflammatory and adhesion molecules.

Key words : methanol extract of *Sorbus commixta* cortex (MSC), hypercholesterolemia, atherosclerosis

Introduction

Hypercholesterolemia is one of the most important risk factors for the development of atherosclerosis and related to occlusive vascular disease[1]. The pathogenesis of atherosclerosis induced by hypercholesterolemia involves endothelium dysfunction, infiltration of monocytes, activation of monocytes into macrophages, and smooth muscle cell proliferation[2]. Studies in animal and humans have shown that hypercholesterolemia-induced vascular dysfunction are associated with the abnormal regulation of vasoactive systems such as nitric oxide (NO)-cGMP, endothelin-1 (ET-1), and/or local rennin angiotensin system (RAS) in the vascular tissues[3,4]. In animal models with chronic administration with high cholesterol, vascular function was deteriorated with down-regulation of NOS/NO/cGMP system[5]. On the other hand, local RAS and ET-1 systems were augmented in the vascular tissue of high cholesterol-diet animal model, which also cause in vascular dysfunction[6,7]. Moreover, inflammatory and adhesion molecules are regulated by vasoactive system as a proinflammatory action. Under hypercholesterolemic conditions, numerous leukocytes adhere to the vascular endothelium and transmigrate, thus potentiating endothelial dysfunction and tissue injury[8]. The adhesion was mediated by adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), which are regulated by vasoactive system via nuclear factor-κB (NF-κB)[9].

Elevated plasma cholesterol level is another risk factor for atherosclerosis induced by high cholesterol-diet. Oxidized LDL, one of the factors thought to affect vessel wall integrity, can lead to an inflammatory response. Such responses induce endothelial cell activation, in sequence, a series of adhesion molecules and powerful cofactors such as growth factors, cytokines, initiate gene transcription, and allow the extravasation of monocytes or T lymphocytes, and then induce vascular inflammation[10].

* To whom correspondence should be addressed at : Ho Sub Lee, Professional Graduate School of Oriental Medicine, Wonkwang University, 344-2, Shinyong-dong, Iksan, Chonbuk, Korea

· E-mail : host@wonkwang.ac.kr, · Tel : 063-850-6841

· Received : 2006/07/19 · Revised : 2006/08/29 · Accepted : 2006/09/22

In the previous study, the methanol extract of Sorbus commixta cortex (MSC) dilates vascular smooth muscle via up-regulation of endothelieum-dependent NO-cGMP signaling and protects L-NAME-induced atherosclerosis[11,12]. Because NO-GMP system inhibit vascular inflammatory process, this pharmacological action of MSC on vascular tissue could be useful for the treatment of high cholesterol-induced atherosclerosis.

Therefore, the present study was designed to determine whether MSC ameliorates vascular function with restoration of vasoactive systems, improves plasma lipid profile, and/or suppresses expression levels of inflammatory and adhesion molecules in rat diet with high cholesterol.

Methods & Materials

1. Animal

All animal procedures for animal experiments 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 rats (weighing 170 to 200 g) were purchased from Korean Experimental Animals Co. (Daejeon, Korea) were housed in metabolic cages to collect 24-h urine samples in the animal room with an automatic temperature (22 ℃) 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 7 weeks of experiments were planned. The rats were allocated to normal rat chow diet with water alone (control group) or diet supplemented with 2% cholesterol (ICN, pharmaceuticals Inc, Irvine, CA, USA) during the first three weeks of experiments. Then, rats were further divided into four groups and treated for 4 more weeks as follows: 1) control group, 2) HC group; high cholesterol diet group, 3) HC/MSC1 group; high cholesterol diet with MSC-containing water (100 mg/kg/day), 4) HC/MSC2 group; high cholesterol diet with MSC-containing water (200 mg/kg/day) (n=8, each group).

2. Plant material and Extraction

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

3. Monitoring of Renal function

Each group of rats was maintained in separate metabolic cages for 2 day, allowing quantitative urine collections and measurements of water intake. 24-hr urine samples were collected (between 09:00 and 10:00 a.m) for the determination of the levels of creatinine, sodium, potassium, osmolality, and other parameters of renal function. Plasma levels of sodium, potassium, osmolality, and creatinine were also measured. The concentrations of ion were measured using an electrolyte analyzer (NOVA 5, Biochemical, Waltmam, MA, USA). Osmolality was measured using an Advanced CRYOMATICTM osmometer (Model 3900, Advanced Instruments Inc., Norwood, MS, USA). Creatinine concentrations (Ccr) of plasma and urine were measured by colorimetric method using a spectrophotometer (Milton Roy, Rochester, NY, USA). Solute-free water reabsorption (T^cH_2O) was calculated by the following formula: $T^cH_2O = V$ (Uosmol/Posmol - 1), where V is urine volume, Uosmol is urinary osmolality, and Posmol is plasma osmolality.

4. Plasma cholesterol, triglycerides, and cholesterol assay

Plasma triglycerides and cholesterol levels were assayed enzymatically by an automated commercial method (Behringer Mannheim, Marburg, Germany).

5. Nitrite assay

The nitrite concentration in the aorta 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 reaction (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). The absorbance of the mixture at 550 nm was determined using an ELISA plate reader (Bio-Rad, Hercules, CA, USA).

6. Protein preparation and Western blot analysis

The thoracic aorta were homogenized with Polytron homogenizer at 3,000 rpm in a solution containing 250 mmol/L sucrose, 1 mmol/L Ethylenediamine tetraacetic acid (EDTA), 0.1 mmo/L phenylmethyl sulfonyl 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 (1,000 g, 5 min; 10,000 g 10 min) at 4 ℃. Supernatants from these low speed spins were ultracentrifuged at 100,000 g for 1 h at 4 ℃. 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 gel and 5% stacking gel, followed by transfer to a nitrocellulose membrane at 20 V and 100 mA (current constant) overnight. The membrane was washed, blocked, incubated with primary antibodies (1:2000 dilution) against ICAM-1 (Zymed Lab, San Fransico, USA), VCAM-1(Biosource, Nivelles, Belgiem), E-selectin (Zymed Lab, San Fransico, USA), ACE (Alpha Diagostic, San Antonio, USA), ecNOS (Transduction Laboratories, Lexington, KY, USA), NF-κB p65, MCP-1, and actin proteins (Santa Cruz Biotechnology, USA). The bound horseradish peroxidase-conjugated secondary antibody was detected by enhanced chemiluminescence (Amersham, Buckingghamshire, England) procedure. The protein expression levels were detemined by analysing the signals captured on nitrocellulose membrane (Amersham, Buckinghamshire, England) using a Chemi-doc image analyzer (Bio-Rad, Hercules, CA, USA).

7. Reverse Transcription - Polymerase Chain Reaction

Total RNA was isolated using a Trizol reagent (Sigma, Missouri, USA) as a suggested by the manufacturer. RNA concentrations were determined using spectrophotometer (Shimadzu, Tokyo, Japan). 5 μg of RNA was used for reverse transcription-polymerase chain reaction (PCR) (MJ Reaserch INC, Watertown, USA) according to the manufacturers instruction. The following sequence steps wrer performed for each PCR reaction: 94 ℃ for 1 min (1 cycle); 94 ℃ for 1 min, 60 ℃ for 1 min, and 72 ℃for 2 min (with variable number of cycle); and a final extension phase at 72 ℃ for 7 min. The numbers of cycles for ET-1 and actin were 30. Primer sequence for analysis of ET-A and GAPDH mRNA were 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 TTG 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 mg/ml ethidium bromide (EtBr). The lengths of the amplicons were 230 and 460 base pairs for ET-1 and GAPDH, respectively.

8. Preparation of aorta and record of isometric vascular tone

Each group of rats was sacrificed by decapitation. The thoracic aortas of these rats were rapidly and carefully dissected and placed into ice-cold Krebs solution (pH 7.4) containing 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.1 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 1.5 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, and 10 mmol/L Glucose. The aorta were removed free of connective tissue and fat, and then cut into rings of approximately 3 mm wide. 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 ℃, while being continuously bubbled with 95% O_2-5% CO_2 (pH 7.4). The baseline load placed on the aortic rings was 1.0 g, and the changes in isometric tension were recorded using a force-displacement transducer (Grass FT 03, Quincy, MA, USA) connected to a Grass polygraph recording system (Model 7E). In the first set of experiments, the aortic rings were contracted with phenylephrine (1×10^{-6} M) to obtain maximal response. Once the maximal response to phenylephrine had been obtained, the aortic rings were 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 acetycholin chloride (ACh). After each test, the aortic rings should be washed three times with fresh Krebs solution and allowed for 30 min to equilibrate.

9. Histological examination

The aortas isolated from all groups were fixed in 10% (v/v) formalin in 50 mM potassium phosphate buffer (pH 7.0) for 24 hrs at 4 ℃. The tissues were subsequently embeded in paraffin, sectioned (4 μm) and stained with hematoxylin and eosin. Slides were examined under the light microscope for histopathological changes. Histological change in vessel were examined using light microscopy (Olympus, Tokyo, Japan)

10. Statistical analysis

Results were expressed as means ± SEM. The statistical significance of difference between the group means determined using one-way ANOVA and Student's t-test.

Results

1. Body weight, blood pressure, and renal functions

As shown in table 1, no significant differences were found among four groups with regards to Ccr, blood pressure, body weight, and T^cH_2O, respectively. However, urinary electrolytes excretion rates including UNaV, UkV, and UClV were increased in rats with HC-diet rats but did not changed significantly by treatment with MSC (Table 1).

Table 1. Effect of the MSC on body weight, blood pressure, and renal functions in rats with high cholesterol diet

	Experimental groups			
	Control	HC	HC/MSC1	HC/MSC2
BW (g)	377.1±1.4	371.4±7.2	376.5±7.2	373.9±9.1
SBP(mmHg)	110.7±1.4	119.5±1.9	115.3±1.7	113.8±1.5
UV(ml/kg/day)	34.47±2.38	35.58±2.82	36.18±2.79	31.60±1.65
UNaV(mEq/min/kg)	1.96±0.05	2.34±0.13*	2.43±0.11*	2.27±0.05*
UKV(mEq/min/kg)	5.87±0.16	7.02±0.39*	7.29±0.34*	6.82±0.16*
UClV(mEq/min/kg)	9.63±0.32	13.86±0.85*	14.06±0.38*	12.66±0.54*
Ccr(ml/min/kg)	2.41±0.18	2.20±0.21	2.47±0.10	2.08±0.14
T^cH_2O(μl/min/kg)	236.6±4.9	239.2±15.3	240.9±9.8	236.3±6.5

Control: normal diet group, HC: high cholesterol diet group, HC/MSC1: HC-diet group administered with MSC (100 mg/kg/day), HC/MSC2: HC- diet group administered with MSC (200 mg/kg/day). *p<0.05 compared with control. #p<0.05 compared with HC. BW: body weight, SBP: systolic blood pressure, UV: urinary volume, UNaV: urinary sodium excretion, UKV: urinary potasium excretion, UClV: urinary chloride excretion, Ccr: creatinine clearance, T^cH_2O: solute-free water reabsorption. Values are mean ± SEM (n=8, each group).

2. Plasma cholesterol and lipoprotein analysis

Table 2 shows the plasma levels of triglycerides, HDL-cholesterol, and LDL-cholesterol in the experimental rats. The increases in triglyceride and LDL-cholesterol were observed in rats with HC-diet. which were significantly attenuated by treatment low or high doses of MSC, respectively (p<0.05 vs HC diet group, respectively).

Table 2. Effect of the MSC on plasma cholesterols and triacylglycerol in rats with high cholesterol diet

	Experimental Groups			
	Control	HC	HC/MSC1	HC/MSC2
HDL-cholesterol(mg/dL)	24.20±0.51	21.70±0.59	19.18±0.50	19.00±0.68
LDL-cholesterol(mg/dL)	10.50±0.74	46.00±3.53*	31.27±1.28*#	35.30±2.43*#
Triacylglycerol(mg/dL)	117.60±14.20	197.60±18.90*	126.63±8.25#	107.91±4.75#

Control: normal group, HC: high cholesterol diet group, HC/MSC1: HC-diet group administered with MSC (100 mg/kg/day), HC/MSC2: HC-diet group administered with MSC (200 mg/kg/day).*p<0.05 compared with control. #p<0.05 compared with HC-diet group. Values are mean ± SEM (n=8, each group).

3. Nitrite level in plasma and aorta

The plasma concentration of nitrate did not differ among the all experimental groups. However, the aortic nitrate content was decreased in HC rats compared with the control group (p<0.05 vs control), which was significantly restored by treatment low or high doses of MSC, respectively (p<0.05 vs HC diet group, respectively)(Table 3).

Table 3. Effect of MSC on the nitrite level in plasma and aorta in rats with high cholesterol-diet.

	Experimental Group			
	Control	HC	HC/MSC1	HC/MSC2
Plasma(μM)	8.23±0.81	10.82±0.92	9.82±0.82	8.28±0.65
Aorta(μmol/mg protein)	16.52±0.26	13.47±0.41*	14.57±0.27#	14.48±0.16#

Values are mean ± SEM (n=8, each group). *p<0.05 compared with control. Control, #p<0.05 compared with HC. Control normal group, HC HC-diet group, HC/MSC1: HC-diet group administered with MSC (100 mg/kg/day), HC/MSC2: HC-diet group administered with MSC (200 mg/kg/day).

4. ecNOS expression in aorta

The expression level of ecNOS in aortic tissue was significantly attenuated in HC group compared with that of the control group (p<0.01). However, the decreased expression level of ecNOS was significantly restored by treatment with the low or high doses of MSC (p<0.05, p<0.01 vs HC diet group, respectively) (Fig. 1A & B)

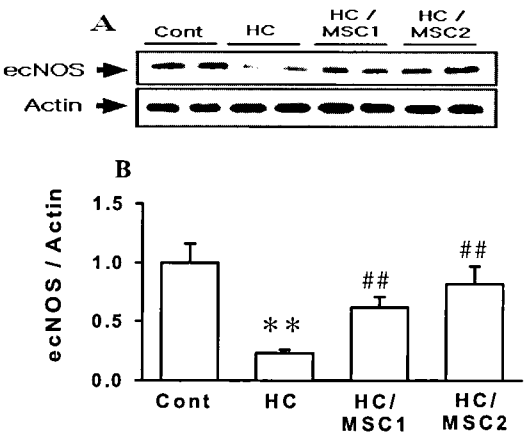

Fig. 1. Effects of MSC on expression level of ecNOS in aorta. Representative Western blotting analysis (A) and densitometric quantification (B) of ecNOS protein level in aortic tissue. See Table 1 for other legends. **p<0.01, compared with control. #p<0.05, ##p<0.01, compared with HC-diet group. Values are mean ± SEM (n=8, each group).

5. Vascular tone of aorta in rat with cholesterol diet

Fig. 2 shows that effect of MSC on vasodilatory response to ACh in aortic strip with endothelium from HC-diet rat. The relative relaxation induced by ACh was attenuated in the aorta derived from the HC rats compared with that of the control group rats (p<0.05). ACh-induced relaxation curve in the aorta from MSC treated HC diet rat was partially similar to that of control rats (Fig. 2A & B).

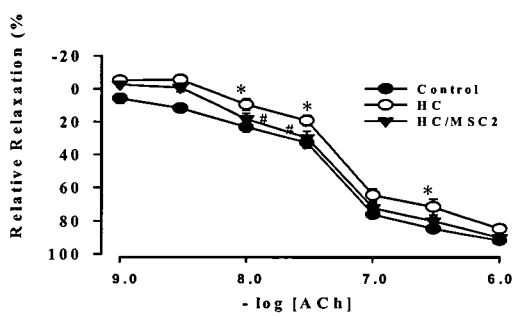

Fig. 2. Effect of MSC on vascular relaxation induced by ACh in aortic ring from experimental groups. *p<0.05 compared with control. #p<0.05 compared with HC-diet group. Values are mean ± SEM (n=8, each group).

6. Endothelin-1 mRNA expression in aorta

To evaluate mRNA expression level of vascular ET-1, RT-PCR analysis of thoracic aorta was performed. As shown in Fig. 3, the aortic expression level of ET-1 was enhanced in the HC-diet group (p<0.01). However, Treatment HC-rats with MSC significantly inhibited the aortic expression level of ET-1 (p<0.01 vs HC-diet group) (Fig. 3A & B).

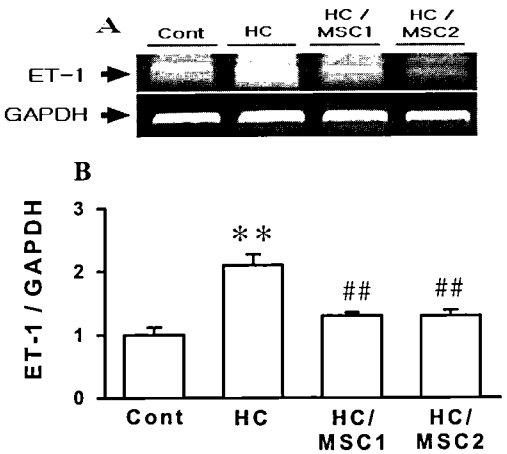

Fig. 3. Effects of MSC on ET-1 and GAPDH mRNA expression in the aorta from HC-diet rats. Representative RT-PCR products (A) and quantification (B) of ET-1 mRNA expression in aortic tissues. See Table 1 for other legends. **p(0.05, compared with control. #p(0.05, ##p(0.01, compared with HC diet group. Values are mean ± SEM (n=8, each group).

7. Aortic ACE expression and plasma ACE activity

Compared with the control group, plasma ACE activity was significantly higher in the HC-diet group ($p<0.05$, Fig. 4A & B). Treatment with low and high doses of MSC prevented increasing in ACE activity in plasma and expression in aorta ($p<0.05$, vs. HC-diet group) (Fig. 5A & B)

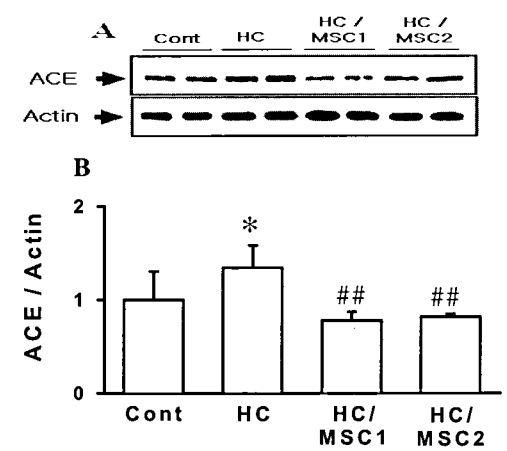

Fig. 4. Effect of MSC on aortic ACE expression. Representative Western blot analysis (A) and densitometric quantification (B) of ACE protein levels in aortic tissue. See Table 1 for other legends. *p(0.05, vs. control, ##p(0.01, vs. HC diet group. Values are mean ± SEM (n=8, each group).

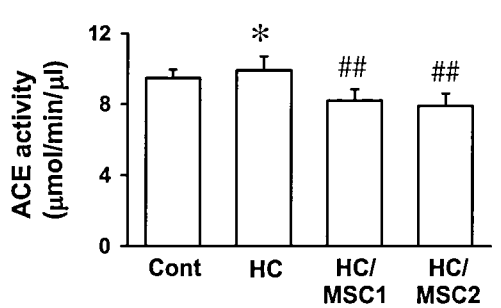

Fig. 5. Effect of MSC on ACE activity in plasma. See Table 1 for other legends. *p(0.05 compared with control. ##p(0.01 compared with HC diet group. Values are mean ± SEM (n=8, each group).

8. NF-κB p65 and MCP-1 expressions

By Western blot analysis, effects of MSC on the protein expression levels of MCP-1 and NF-κB p65 in the aorta were examined. Both MCP-1 and NF-κB p65 expression levels were significantly increased in HC-diet group compared with those of control group ($p<0.01$ for MCP-1 and NF-κB p65, respectively). However, such increased protein expressions were significantly reduced in the HC/MSC1 and HC/MSC2 groups, respectively ($p<0.05$, $p<0.01$ vs HC diet group, respectively) (Fig. 6A & B).

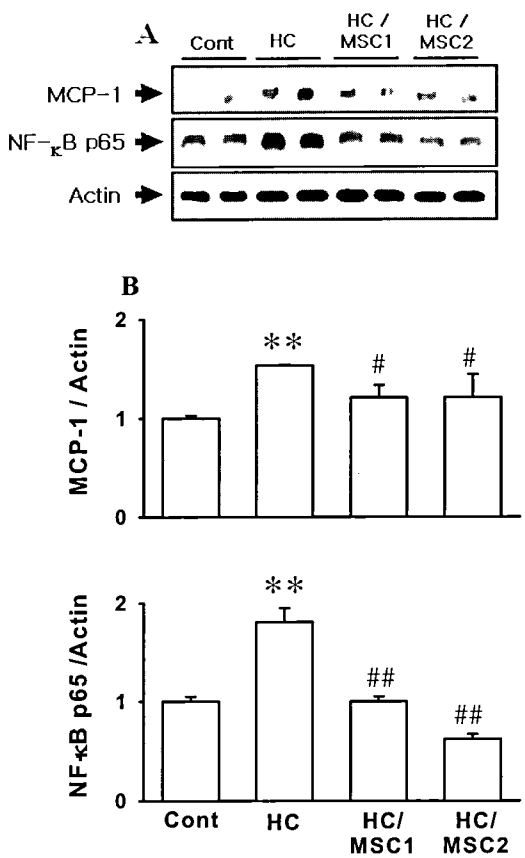

Fig. 6. Effect of MSC on expression level of NF-κB p65 and MCP-1. Representative western blot (A) and densitometric quantification (B) of NF-κB p65 and MCP-1 protein expressions in aortic tissues. See Table 1 for other legends. **p(0.01 vs. control. #p(0.05, ##p(0.01 vs. HC diet group. Values are mean ± SEM (n=8, each group).

9. Adhesion molecules expression

Expression of endothelial VCAM-1, ICAM-1, and E-selectin of the thoracic aorta were determined by Western blot analysis using actin as an internal standard. Expression levels of VCAM-1, ICAM-1, and E-selectin were significantly increased in HC-diet group compared with control group ($p<0.01$). However, down-regulation of adhesion molecules expression by MSC supplementation of the aorta of rats fed the HC did reach statistical significance ($p<0.05$, $p<0.01$ vs HC diet group, respectively) (Fig. 7A & B)

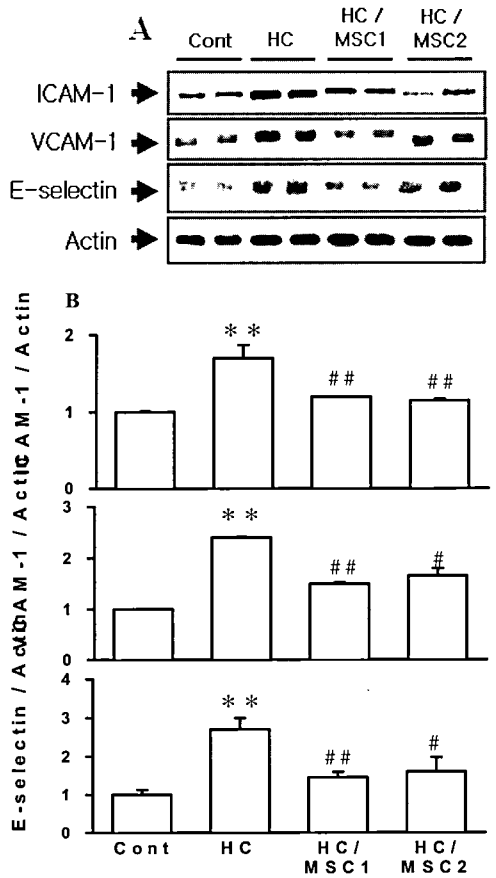

Fig. 7. Effects of MSC on aortic adhesion molecules expression in rat with high cholesterol diet. Representative Western blot (A) and densitometric quantification (B) of ICAM-1, VCAM-1, and E-selectin protein expression in arotic tissue. See Table 1 for other legends. *p<0.05, vs. control. #p<0.05, ##p<0.01, vs. HC diet group. Values are mean ± SEM (n=8, each group).

10. Histological finding

To examine histopathological changes in aorta segments were stained with hematoxylin and eosin.

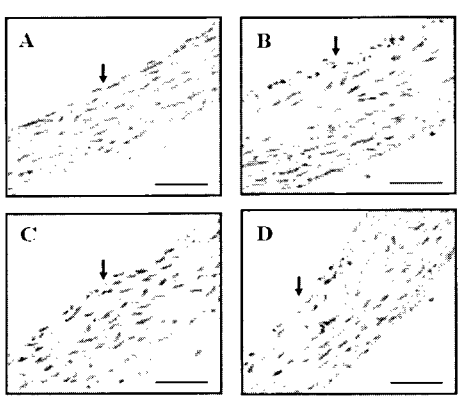

Fig. 8. Representative microscopic photographs of aorta stained with hematoxylin and eosin (magnification × 400). Aorta obtained from control (A), HC-diet group (B), MSC1-treated HC group (C), and MSC2-treated HC group (D). Bars represent 10 mm. Arrows indicate endothelial layer.

Although, obvious atheromatous lesions were not detectable in aorta of HC-diet rat (Fig. 8B). On the other hand, aorta segment in the HC-diet group had significantly thickened intima and media (Fig. 8B). However, intima thickening of aorta in the MSC treated HC-diet groups were observed as low levels compared to the HC-diet group (Fig. 8C & D).

Discussion

The purpose of the present study is to determine whether improvements on vascular endothelial function with MSC administration are associated with the potentially beneficial effects of MSC treatment on the restoration of proinflammatory and adhesion molecules expression by modulation of vasoactive systems in the aorta of rats while they fed on hypercholesterolemic diet. The results of this study demonstrate that HC feeding (a) increased plasma LDL-cholesterol formation together with up-regulation of endothelial adhesion molecules in aortic tissues; (b) impaired endothelium-dependent relaxation in response to ACh along with impairment of ecNOS-NO system and up-regulation of both aortic ET-1 and ACE; (c) up-regulated aortic expression levels of NF-κB and MCP-1.

In the present study, treatment HC-diet rats with low (100 mg/kg/day) or high (200 mg/kg/day) doses of MSC improved endothelium-dependent relaxation together with restoration of vasoactive systems such as NOS-NO, ACE, and ET-1 in the thoracic aorta. Adhesion and proinflammation molecules induced by HC-diet were also restored by administration with MSC.

It has been well documented that hypercholesterolemia is one of the most important pathogenic factors for atherosclerosis[1]. Hypercholesterolemia alters endothelial cell function and increases permeability to low density lipoprotein (LDL)[13]. The plasma cholesterol levels of high cholesterol-diet rat were significantly increased compared with those of control group rats, suggesting that hyperlipidemia and development of atherosclerotic plaque in the aorta had been induced in the HC fed rat[13]. Considerable experimental and clinical data suggest that elevated serum levels of total and LDL cholesterol are associated with impaired endothelial function[14-16]. Lipid lowering is a major priority for patients or experimental animals with atherosclerosis, because hyperlipidemia or hypercholesterolemia is one of the major risk factors associated with the development of this condition. The present study shows that increases in total cholesterol, triglyceride, and LDL-cholesterol in the cholesterol-enriched diet rats were attenuated by administration with MSC. These findings, at least in part, indicate that MSC may play role in inhibiting the initiation and development of atherosclerosis by improving lipid metabolism.

In the present study, HC-diet leads to endothelial dysfunction as evidenced by decrease of ACh-induced vascular tone. These findings suggest explanation in the molecular level for impairments in endothelium-dependent vascular relaxation in the vessels. In fact, it has been well documented that endothelium-dependent vascular relaxation is abnormal in both hypercholesterolemia and atherosclerosis because the ability of nitric oxide (NO) to maintain vascular tone is deficient in this condition[17,18]. Nitric oxide (NO), which is synthesized from L-arginine by endothelial constitutive NO synthase (ecNOS), is permanently released from vascular endothelium. NO plays a key role in the relaxation of vascular smooth muscle cells (VSMCs) and the inhibition of vascular inflammatory process such as VSMC proliferation, adhesion of platelets and leukocytes, endothelial permeability, and extracellular matrix collagen synthesis[19,20]. A broader understanding of endothelial dysfunction would include not only reduced vasodilation but also a proinflammatory and prothrombic state associated with dysfunction of the endothelium. In animal models and humans, abnormalities in the endothelial NO pathway is a main pathogenic factor in the initiation and development stages of atherosclerosis[21]. The present study shows that ACh-induced vascular tone was decreased together with decrease in aortic ecNOS expression in the HC-diet rats, which is in line with previous findings that ecNOS and NO were decreased in high cholesterolemic animal models[22]. Improvement of the impaired endothelium-dependent relaxation was observed by administration of MSC along with restoration of aortic ecNOS expression. We suggest that MSC dilates vascular smooth muscle via endothelium-dependent NO/cGMP pathway. Taken together, administration with MSC may improve vascular abnormality in rats HC-diet via increment of ecNOS expression in aorta.

ET-1, a potent vasoconstrictor peptide, is primarily released from endothelial cell and exerts its biological effect through the activation of specific ET receptor. ET-1 production is increased in systemic cardiovascular disease like early atherosclerosis[23,24] and hypercholesterolemia[25], which can disrupt the delicate balance between endothelium-derived vasoactive factors. Indeed, coronary and peripheral endothelial function was restored in experimental hypercholesterolemia during ET-1 blockade[26], supporting the role of ET-1 as an early contributor to endothelial dysfunction in hypercholesterolemia. Our study showed that the expression level of ET-1 was enhanced by hypercholesterolemic diet group. Thus, it was suggested that the increase in ET-1 seen with hypercholesterolemia might be one of the causes of impairing endothelium-dependent relaxation[27,28]. However, MSC co-administration in HC group significantly inhibited the HC-mediated induction of ET-1 expression.

Furthermore, the present study shows that treatment with MSC attenuates increase of aortic ACE expression in the high cholesterol-diet rat. Angiotensin II, which is produced by activation of ACE, plays critical role in the development of atherosclerosis induced by hypercholesterolemia[29]. An increase in angiotensin II activity mediated via type 1 receptors has been shown to cause vascular inflammation, oxidative stress, and then atherosclerosis. The present study also showed that the expression level of ACE was decreased by administration with MSC in the HC-diet rats. Taken together, the beneficial effects of MSC on vascular tissues seen in the present study may be explained by decrease in ACE and ET-1 and increase in ecNOS expressions.

Inflammation is basic pathological mechanism that underlies a variety of disease. Atherosclerosis is also clearly a results of inflammatory response and then interruption of the function of inflammatory mediator can decrease atherosclerotic lesion size[30]. The vascular inflammatory reaction involves the complex interactions between inflammatory cells and vascular cells. Researcher in vascular biology has progressed remarkably in the last decade, resulting in a better understanding of the vascular cell responses to inflammatory stimuli and result in the identification of major intercellular inflammatory signaling pathways, particularly the I-κB/nuclear factor-κB (NF-κB) system. NF-κB is a ubiquitously expressed multiunit transcription factor that is activated by diverse signals, possibly via phosphorylation of the I-κB subunit and its dissociation from the inactive cytoplasmic complex, followed by translocation of the active dimmer, p50 and p65, to the nucleus[31]. Under normal conditions, NF-κB is retained in the cytosol by inhibitory proteins of the I-kB family. In response to an inflammatory insult, I-kB proteins are degraded and the free NF-κB translocated to the nucleus where it initiates gene transcription of proinflammatory molecules[32]. NF-κB activation triggers gene expression of adhesion molecules including VCAM-1, ICAM-1, and E-selectin. These molecules are responsible for leukocyte infiltration into the repairing tissues[33]. In our data, monocyte chemoattractant protein-1 (MCP-1) and NF-κB p65 expression level were significantly increased in HC-diet group compared with those in control group. As expected, aortic expression levels of vascular adhesion molecules including VCAM-1, ICAM-1, and E-selectin were also markedly upregulated. However, administration with MSC attenuated aortic expression levels of MCP-1 and NF-κB p65 as well as adhesion molecules in the HC-diet rats. It means that MSC restores a pathway regulating

transcriptional activators, which are specific for HC-stimulated induction of vascular adhesion molecules expression with affecting NF-κB. Furthermore, it suggest that the MSC treatment blocks vascular inflammatory process in the HC-diet rats, which may be causally related with modulation of vasoactive systems such as NOS/NO, ET-1, and ACE.

Atherosclerosis is characterized by a thickening of the vascular wall through an infiltration of macrophages or lymphocytes. In animals with diet-induced or genetically determined hyperlipidemia, the earliest morphological changes in arteries include focal adherence of mononuclear leukocytes to the endothelium and accumulation of monocyte-derived foam cells in the intima[34,35]. Production of proinflammatory cytokines are believed to be prerequisites for the activation of the endothelium to induce chemokines and adhesion molecules to attract and adhere to monocytes at the early stage of fatty streak formation. In histopathological examination of present study, aorta segment in the HC-diet rats had significantly thickened intima and media. But intima and media thickening of aorta in the MSC-treated with HC-diet group was observed as low levels compared to the HC-diet group.

In summary, our study demonstrates that restoring expressions of vasoactive molecules such as ecNOS/NO, ET-1, and ACE were observed in the HC-diet rat by administerd with MSC. Moreover, MSC attenuates not only vascular expressions of NF-κB p65 and MCP-1 as well as adhesion molecules, but also restores vascular tone impairment in the HC-diet rat, which might serve as a mechanism for endothelial protection. Therefore, the present data suggest that MSC protects atherosclerotic process in the HC-diet rat model through the inhibition of the aortic expression levels of pro-inflammatory and adhesion molecules, which may be causally related with decrease in plasma LDL-cholesterol.

Acknowledgement

This work was supported by the Korea Research Foundation Grant." (KRF-2004-005-E00038)

References

1. Ross, R., Harker, L. Hyperlipidemia and atherosclerosis: chronic hyperlipidemia initiates and maintain lesions by endothelial cell desquamation and lipid accumulation, Science pp 1094-1100, 1976.
2. Ross, R. The pathogenesis of atherosclerosis: a prospective for 1990s. Nature 362: 801-809, 1993.
3. John, S., Schmieder, R.E. Impaired endothelial function in arterial hypertension and hypercholesterolemia: potential mechanisms and differences. J Hypertens 18(4):363-374, 2000.
4. Fan, J., Unoki, H., Iwasa, S., Watanabe, T. Role of endothelin-1 in atherosclerosis. Ann N Y Acad Sci 902: 84-93, 2000.
5. Laight, D.W., Matz, J., Caesar, B., Carrier, M.J., Anggard, E.E. Investigation of endogenous nitric oxide vascular function in the carotid artery of cholesterol-fed rabbits. Br J Pharmacol 117(7):1471-1474, 1996.
6. Mathew, V., Cannan, C.R., Miller, V.M., Barber, D.A., Hasdai, D., Schwartz, R.S., Holmes, D.R. Jr, Lerman, A. Enhanced endothelin-mediated coronary vasoconstriction and attenuated basal nitric oxide activity in experimental hypercholesterolemia. Circulation 96(6):1930-1936, 1997.
7. Sugano, M., Makino, N., Yanaga, T. The effects of renin-angiotensin system inhibition on aortic cholesterol content in cholesterol-fed rabbits. Atherosclerosis 15: 123-129, 1996.
8. Joris, I., Zand, T., N unnai, J.J., Krolikowski, F.J., Majno, G. Studies on the pathogenesis of atherosclerosis. Adhesion and Emigration of mononuclear cells in the aorta of hypercholesterolemic rats. Am J Pathol 113: 341-358, 1983.
9. Munro, J.M., Cotran, R.S. Biology of the disease: the pathogenesis of atherosclerosis: atherogenesis and inflammation. Lab Invest 58: 249-261, 1988.
10. Springer, T.A. Adhesion receptors of the immune system. Nature 346: 425-434, 1990.
11. Kang, D.G., Lee, J.K., Choi, D.H., Sohn, E.J., Moon, M.K., Lee, H.S. Vascular relaxation by the methanol extract of Sorbus cortex via NO-cGMP pathway. Biol Pharm Bull 28(5):860-864, 2005.
12. Sohn, E.J., Kang, D.G., Choi, D.H., Lee, A.S., Mun, Y.J., Woo, W.H., Kim, J.S., Lee, H.S. Effect of methanol extract of Sorbus cortex in a rat model of L-NAME-induced atherosclerosis. Biol Pharm Bull 28(7):1239-1243, 2005.
13. Kurozumi, T., Imamura, T., Tanaka, K., Yae, Y., Koga, S. Effects of hypertension and hypercholesteremia on the permeability of fibrinogen and low density lipoprotein in the coronary artery of rabbits. Immunoelectron-microscopic study. Atherosclerosis 49: 267-276. 1983.
14. Gibbons, G.H., Dzau, V.J. The emerging concept of vascular remodeling. N Engl J Med 330: 1431-1438, 1994.
15. Kuhn, F.E., Mohler, E.R., Reagan, K. Effects of high-density lipoprotein on acetylcholine-induced coronary vasoreactivity. Am J Cardiol pp 1425-1430, 1991.
16. Shimokawa, A.H., Vanhoutte, P.M. Hypercholesterolemia causes generalized impairment of endothelium-dependent relaxation to aggregating platelets in porcine arteries. J Am Coll Cardiol 13: 1402-1408, 1989.

17. Jayakody, R.L., Senaratne, M.P., Thomson, A.B., Kappagoda, C.T. Cholesterol feeding impairs endothelium-dependent relaxation of rabbit aorta. Can J Physiol Pharmacol 63: 1206-1209, 1985.
18. Freiman, P.C., Mitchell, G.G., Heistad, D.D., Armstrong, M.L., Harrison, D.G. Atherosclerosis impairs endothelium-dependent vascular relaxation to acetylcholine and thrombin in primates. Circ Res 58: 783-788, 1986.
19. Draijer, R., Atsma, D.E., van der Laarse, A., van Hinsbergh, V.W. cGMP and nitric oxide modulate thrombin-induced endothelial permeability: regulation via different pathways in human aortic and umbilical vein endothelial cells. Circ Res 76: 199-208, 1995.
20. Myers, P.R., Tanner, M.A. Vascular endothelial cell regulation of extracellular matrix collagen: role of nitric oxide. Arterioscler Thromb Vasc Biol 18: 717-722, 1998.
21. Wever, R.M., Luscher, T.F., Cosentino, F., Rabelink, T.J. Atherosclerosis and the two faces of endothelial nitric oxide synthase. Circulation 97: 108-112, 1998.
22. Aji, W., Ravalli, S., Szabolcs, M., Jiang, X.C., Sciacca, R.R., Michler, R.E., Cannon, P.J. L-Arginine prevents xanthoma development and inhibits atherosclerosis in LDL receptor knockout mice. Circulation 97: 430-437, 1997.
23. Barton, M., Haudenschild, C.C., d'Uscio, L.V., et al. Endothelin ET A receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. Proc Natl Acad Sci 95: 14367-14372, 1998.
24. d'Uscio, L.V., Barton, M., Shaw, S., et al. Endothelin in atherosclerosis: Importance of risk factors and therapeutic implications. J Cardiovasc Pharmacol 35: 55-59, 2000.
25. Best, P.J., McKenna, C.J., Hasdai, D., et al. Chronic endothelin receptor antagonism preserves coronary endothelial function in experimental hypercholesterolemia. Circulation 99: 1747-1752, 1999.
26. d'Uscio, L.V., Barton, M., Shaw, S., et al. Chronic ET(A) receptor blockade prevents endothelial dysfunction of small arteries in apolipoprotein E-deficient mice. Cardiovasc Res 53: 487-495, 2002.
27. Lerman, A., Webster, M.W., Chesebro, J.H., Edwards, W.D., Wei, C.M., Fuster, V., Burnett, J.C. Jr. Circulating and tissue endothelin immunoreactivity in hypercholesterolemic pigs. Circulation 88: 2923-2928, 1993.
28. Uyama, H., Haraoka, S., Shimokama, T., Goto, K., Dohi, K., Watanabe, T. Diet-induced hypercholesterolemia increases endothelin-1 release by aortic endothelial cells. Pathobiology 64: 328-332, 1996.
29. Jacobsson, L.S., Persson, K., Aberg, G., Andersson, R.G., Karlberg, B.E., Olsson, A.G. Antiatherosclerotic effects of the angiotensin-converting enzyme inhibitors captopril and fosinopril in hypercholesterolemic minipigs. J Cardiovasc Pharmacol 24(4):670-677, 1994.
30. Ross, R. Atherosclerosis: an inflammatory disease. N Engl J Med 340: 115-126, 1999.
31. Gosh, S., Baltimore, D. Activation in vitro of NFkB by phosphorylation of its inhibitor IkB. Nature 344: 678-682, 1990.
32. Bowie, A., O'Neill, L.A. Oxidative stress and nuclear factor kappa B activation: a reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol 59: 13-23, 2000.
33. Radi, Z.A., Kehrli, Jr M.E., Ackermann, M.R. Cell adhesion molecules, leukocyte trafficking, and strategies to reduce leukocyte infiltration, J Vet Intern Med 15: 516-529, 2001.
34. Klurfeld, D.M., Weber, M.M., Levine, E.M., Mueller, S.N., Kritchevsky, D. Increased atherosclerosis in rabbits immunized with endothelial cells. Atherosclerosis 55: 283-297, 1985.
35. Rogers, K.A., Hoover, R.L., Castellot, J.J. Jr, Robinson, J.M., Karnovsky, M.J. Dietary cholesterol-induced changes in macrophage characteristics. Relationship to atherosclerosis. Am J Pathol 125: 284-291, 1986.