It is well recognized that vascular inflammation is involved in the pathogenesis of atherosclerosis. Infiltration of circulating leukocytes into the inflamed vascular endothelium requires the up-regulation of adhesion molecules of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The components of bee venom (BV) used in the current study were carefully scrutinized with chromatography. Despite of its well known anti-inflammatory properties, there are no reports regarding the influence of BV on the expression of cellular adhesion molecules to vascular endothelium. Much information does exist about the effect of atherogenic diet on atherosclerotic changes in the artery. However little is known about how BV affects the molecular mechanisms and the levels of gene expression involved in the anti-inflammatory process. Experimental atherosclerosis was induced by lipopolysaccharide (LPS) injection to mice maintained on an atherogenic diet. Animals were divided into 3 groups: (a) control group; (b) a LPS/fat group maintained on an atherogenic diet along with intraperitoneal injections of LPS; (c) a LPS/fat + BV group that was maintained on an atherogenic diet, and given LPS and intraperitoneal BV injections. At the end of each treatment period, serum levels of total cholesterol (TC) and triglyceride (TG) were significantly higher in LPS/fat mice than in the control mice (p &lt; 0.05).


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Bee venom reduces atherosclerotic lesion formation via anti-inflammatory mechanisms

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Abstract

It is well recognized that vascular inflammation is involved in the pathogenesis of atherosclerosis. Infiltration of circulating leukocytes into the inflamed vascular endothelium requires the up-regulation of adhesion molecules of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The components of bee venom (BV) used in the current study were carefully scrutinized with chromatography. Despite of its well known anti-inflammatory properties, there are no reports regarding the influence of BV on the expression of cellular adhesion molecules to vascular endothelium. Much information does exist about the effect of atherogenic diet on atherosclerotic changes in the artery. However little is known about how BV affects the molecular mechanisms and the levels of gene expression involved in the anti-inflammatory process. Experimental atherosclerosis was induced by lipopolysaccharide (LPS) injection to mice maintained on an atherogenic diet. Animals were divided into 3 groups: (a) control group; (b) a LPS/fat group maintained on an atherogenic diet along with intraperitoneal injections of LPS; (c) a LPS/fat + BV group that was maintained on an atherogenic diet, and given LPS and intraperitoneal BV injections. At the end of each treatment period, serum levels of total cholesterol (TC) and triglyceride (TG) were significantly higher in LPS/fat mice than in the control mice (p < 0.05). At the same time, the serum high-density lipoprotein-cholesterol (HDL-C) was slightly lower in LPS/fat mice than non LPS/fat mice. However, the productions of TC and TG in the BV treated mice were conversely decreased compared to mice with atherosclerotic lesions. Furthermore, BV treatment longer than 8 weeks boosted HDL-C concentration compared with other groups. LPS and atherosclerotic diet induced a significant expression of tumor necrosis factor (TNF)-α and interleukin (IL)-1β from the serum compared with the normal control (p < 0.05). The contents of cytokines reduced consistently in the BV treatment groups compared with those in LPS/fat group. BV significantly reduced ICAM-1, VCAM-1, transforming growth factor-
β1 (TGF-β1) and fibronectin in aorta compared with LPS/fat treated animals (p < 0.05). A similar pattern was also observed in the heart. The reduction of TGF-β1 and fibronectin gene levels induced by BV was striking. The alleviating effect of BV on atherosclerotic changes in the artery and heart was further supported by histopathological and immunohistochemical approaches. In conclusion, BV has antiatherogenic property via its lipid-lowering and anti-inflammatory mechanisms.

*Keywords*: Atherosclerosis; Inflammation; Bee Venom; Intercellular Adhesion Molecule-1; Vascular Cell Adhesion Molecule-1.
Introduction

The involvement of vascular inflammation in the pathogenesis of atherosclerosis is well established (Hansson et al., 2006). The early stage of atherosclerosis involves the activation of vascular endothelium in response to many stimuli such as low-density lipoproteins, free radicals, infectious microorganisms, shear stress, hypertension and toxins from smoking (Stoll and Bendszus, 2006). This leads to a radical turnover of relatively non-adhesive and non-thrombogenic endothelial cell surface into activated state allowing the expression of adhesion molecules (Hansson et al., 2006). These adhesion molecules include cellular adhesion molecules of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Upregulation of endothelial adhesion molecules promotes the development of atherosclerotic lesions in rabbit (Li et al., 1993), subhuman primate (Shi et al., 2005) and human (Poston et al., 1992; Hwang et al., 1997). The expression of adhesion molecules is followed by attachment of circulating macrophages, T-cells, smooth muscle and undefined mesenchymal-appearing cells to the endothelium to render atherosclerotic plaques heterogeneous which is facilitated by inflammation associated proteins such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β (Wilcox et al., 1994). Therefore, suppression of cell adhesion molecule expression and macrophage accumulation at the level of endothelium is of particular significance in the management of vascular inflammatory process.

The induction of endothelial adhesion molecules by inflammatory cytokines depends on activation of fibronectin and transforming growth factor-β1 (TGF-β1). Due to its role in cell proliferation, adhesion and migration, increased deposition of fibronectin-rich matrix has been associated with atherosclerosis (Kolachala et al., 2007). Similarly TGF-β1 as profibrotic cytokine associates transiently with upregulation of vascular extracellular matrix to elicit the pathological process in diabetes-associated atherosclerosis (Pham et al., 2010).
Bee venom (BV) therapy is the therapeutic application of honeybee venom to the treatment of various diseases. BV therapy has been used as a traditional medicine to treat a variety of conditions, such as arthritis, rheumatism, back pain, cancerous tumors, and skin diseases (Hider, 1988). The aqueous fraction of BV, less than 20 kDa, showed the anti-inflammatory property by decreasing cyclooxygenase (COX)-2 and phospholipase (PL) A2 expression and blocking the production of TNF-α, IL-1, IL-6, nitric oxide (NO) and reactive oxygen species (ROS) (Son et al., 2007). The components of BV utilized in the current study were carefully scrutinized with chromatography. Despite of its well published anti-inflammatory property, there are no reports regarding the influence of BV on the expression of cellular adhesion molecules to vascular endothelium. Much information exist about the effect of atherogenic diet on atherosclerotic changes in the aorta, but little is known about the molecular mechanisms and the levels of gene regulation involved in the anti-inflammatory process by BV. We summarize experiments which demonstrate the involvement of BV in transcriptional and posttranscriptional mechanism of ICAM-1, VCAM-1, TGF-β1, and fibronectin endothelial expression in wild type mice.
Materials and Methods

BV Analysis

The BV was collected from Asian honeybee workers (*Apis mellifera* L.) that are maintained at the National Institute of Agricultural Science and Technology (NIAST), Suwon, Korea. It was then dissolved in distilled water and centrifuged at 12,000 g for 10 minutes to remove insoluble materials. The BV was lyophilised by freeze dryer and refrigerated at 4 °C for later use. The amount of 100 mg frozen and dried BV was dissolved in 0.1 M ammonium formate (pH 4.5). Particles were removed by centrifugation and filtration (0.2 μm membrane filter, Millipore, MA, USA) prior to sample application to a Sephadex TM200 column (AKTAexplorer, MN, USA) equilibrated in 0.1 M ammonium formate (pH 4.5). Bee venom standard proteins were purchased commercially (Sigma, MO, USA). All fractions collected were examined for total protein, hyaluronidase, phospholipase A₂, melittin and apamin. Protein concentration was determined by the Bradford method (BioRad, CA, USA). The purity of proteins and peptides was assessed by SDS-PAGE on 10-20% gradient tricine gels (Novex, CA, USA). Proteins and peptides were stained with Coomassie blue R-250. Proteins from BV were separated by size exclusion gel chromatography. The verification of the main components of BV was confirmed by comparison with venom protein standards from Sigma. The comparison between peaks of BV standards and whole BV showed that all major venom proteins and peptides were found to be similar in terms of compositions, although with a slight difference in proportions.

Animals and Experimental Design

Eight weeks of age male C57BL/6 mice (n=45, Orient, Korea) weighing 20-25 g were housed individually in polycarbonate cages maintained at a constant temperature (22±2°C) and humidity (55%). The mice had free access to food, water, and were subjected to a 12:12hr
light:dark cycle. The average weight and behavior during this experiment did not differ significantly among groups at the end of the study. All surgical and experimental procedures used in the present study were approved by the Institutional Review Board Committee at Daegu Catholic University Medical Center and conform to the US National Institutes of Health guidelines for the care and use of laboratory animals. Mice were randomly assigned into three groups. Normal control group (NC, n=15) had free access to standard mouse laboratory chow diet. Mice in lipopolysaccharide (LPS, Sigma, MO, USA)/fat group (n=15) received a total of three intraperitoneal injections of LPS at a concentration of 2 mg/kg administered every other day during the first week. The mice were then fed ad libitum an atherogenic diet containing 1.25% cholesterol, 15% fat, and 0.5% cholic acid for 2, 4, 8, and 12 weeks. Animals in LPS/fat+BV group (n=15) were given LPS, atherogenic diet and intraperitoneal BV injections (0.1 mg/kg, twice per week) for 2, 4, 8, and 12 weeks. A total of 15 mice from each group were further subdivided into 3, 4, 4 and 4 mice for each treatment week. At the end of each treatment period, the animals were sacrificed by cervical dislocation, and the heart and aorta were collected. After perfusion with PBS (pH 7.4), the heart and aorta with its main branches were rapidly dissected entirely to the iliac bifurcation for protein isolation and histopathological analyses of atherosclerotic lesions. Blood was immediately collected by cardiac puncture from the right ventricle. After clotting for 30 minutes at room temperature, serum was separated by centrifugation at 1000g for 10 minutes and stored at -80°C for lipid analysis and cytokine assay.

**Serum Lipid Analysis and Proinflammatory Cytokine Assay**

Serum triglycerides (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) were measured enzymatically using an automated analyzer (Type 7170A, Hitachi, Japan). The serum levels of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) were
measured by enzyme-linked immunosorbent assay (ELISA) commercial plate (R&D system, MN, USA). Briefly, 100 µl binding solution (0.05 M Na₂CO₃ buffer, pH 9.6) containing the capture antibody, anti-mouse TNF-α and IL-1β antibody (2 µg/ml) was added to each well of an ELISA plate. The plate was then incubated overnight at 4°C. After three washes using 0.05% Tween 20 in phosphate buffered saline, the plate was blocked with 1% bovine serum albumin for two h at room temperature. The plate was washed twice and then 100 µl of each sample or recombinant mouse TNF-α and IL-1β at different concentrations were added in triplicate to corresponding wells for two h at room temperature. The plate was then washed three times and 100 µl of the detection antibody, biotinylated mouse TNF-α and IL-1β antibody (0.2 µg/ml), was added and the plate was incubated for two h at room temperature. After washing, 100 µl of streptavidin-horseradish peroxidase at a dilution of 1:200 was added to each well and the plate was incubated for 20 minutes at 37°C. After further four washings, 100 µl of a 1:1 mixture of reagents A (H₂O₂) and B (tetramethylbenzidine) were added and the plate was incubated for 20 minutes at 37°C. Finally, the reaction was terminated by adding 50 µl of H₂SO₄. Optical density was read using a microtitre plate reader set at 450 nm. A standard curve was generated by correlating the original concentrations of the recombinant mouse TNF-α and IL-1β and the corresponding optical densities. TNF-α and IL-1β concentrations in plasma samples were then calculated according to the standard curve.

**Protein Isolation and Western Blot Analysis**

Frozen tissues were homogenized in a lysis buffer (50 mM pH 8.0 Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 100 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, and 1 M DTT). After incubation for 30 min on the ice, the samples were centrifuged at 8,000 g, at 4°C for 30 min and the supernatant was transferred to a new tube. For western blots, total protein concentration was determined with a Bio-Rad Bradford kit (Bio-Rad Laboratories, CA, USA),
then 50 μg of total protein was used concentration from 10% to 12% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA) which was blocked with 5% skim milk in TBS-T (10 mM Tris, 150 mM NaCl and 0.1% Tween-20) for 1 h at room temperature. The membrane was probed with the primary antibody for 3 h and was washed several times with TBS-T and then incubated with a horseradish peroxidase (HRPO)-conjugated secondary antibody. Finally, the membrane was washed, and developed with an enhanced chemiluminescence detection system (Amersham, NJ, USA). The primary antibodies used in this study were anti-TGF-β1 (R&D system, MA, USA), anti-fibronectin (Abcam, MA, USA), anti-VCAM-1 (R&D system, MN, USA), and anti-ICAM-1 (R&D system, MN, USA). Signal intensity was quantified by image analyzer (Las 3000, Fuji, Japan).

Histological Analysis

All tissue specimens were fixed in 10% formalin for at least 24 h at room temperature. After fixation, perpendicular sections to the anterior–posterior axis of the wound were dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Thin sections (3 μm) were mounted on glass slides, dewaxed, rehydrated to distilled water, and stained with hematoxylin, and eosin. As part of the histological evaluation, all slides were examined by a pathologist, without knowledge of the previous treatment, under a light microscope.

Immunohistochemical Staining

Paraffin-embedded sections were deparaffinized with xylene, dehydrated in decreasing concentrations of ethanol, and then treated with 3% hydrogen peroxidase in methanol for 10 min to block endogenous peroxidase activity. Tissue sections were processed in 10 mM citrate buffer (pH 6.0) and heated to 100°C for 10 min for antigen retrieval. Sections were
incubated with antibodies against anti-TGF-β1 (R&D system, MA, USA), anti-fibronectin (Abcam, MA, USA), anti-ICAM-1 (R&D system, MN, USA), and anti-VACM-1 (R&D system, MN, USA) for 1 h at 37°C. The sections were incubated in an Envision system (DAKO, CA, USA) for 30 min at 37°C with washing in PBS before incubation. DAB (3, 3′-diaminobenzidine tetrahydrochloride) was used as the color reagent and hematoxylin was used as a counterstain.

**Statistical Analysis**

The data were analyzed using one-way ANOVA and expressed as means ± standard error, significant difference between groups was detected by SPSS 12.0 software. Student’s t-test was used for comparison between two groups. A value of p < 0.05 was statistically considered to be significant. All experiments were performed at least three times.
Results

Serum Lipid Concentration

To test whether BV had lipid-lowering properties, three lipid parameters in the serum of mice from all groups were determined (Fig. 1). At the end of each treatment period, serum levels of TC and TG were significantly higher in LPS/fat mice than in the control mice (p < 0.05). At the same time, the serum HDL-C was slightly lower in LPS/fat mice than non LPS/fat mice. However, the productions of TC and TG in the BV treated mice were conversely decreased compared to mice with atherosclerotic lesions. Furthermore, BV treatment longer than 8 weeks boosted HDL-C concentration compared with other groups. These data indicate that BV treatment decreased total cholesterol and triglycerides content while increasing HDL-C.

Levels of Proinflammatory Cytokines

LPS and atherosclerotic diet induced a significant expression of TNF-α and IL-1β from the serum compared with the normal control (p < 0.05, Fig. 2). The contents of cytokines reduced consistently in the BV treatment groups compared with those in LPS/fat group. These results show that BV suppresses the expression of proinflammatory cytokines in the blood.

Expression of Adhesion Molecules and Fibrotic Cytokines

To investigate whether BV reduced vascular inflammation, ICAM-1, VCAM-1, TGF-β1 and fibronectin protein expression in the heart and aorta was assessed. BV significantly reduced ICAM-1, VCAM-1, TGF-β1 and fibronectin in aorta compared with LPS/fat treated animals (p < 0.05, Fig. 3). It is noteworthy that the most prominent reduction was seen at weeks 12 and 8 for ICAM-1/VCAM-1 and TGF-β1/fibronectin, respectively. A similar pattern was also observed in the heart (Fig. 4). The reduction of TGF-β1 and fibronectin gene levels induced
by BV was striking. These results illustrate that BV effectively blocks the adhesion molecules and fibrotic factors in atherosclerotic animal model.

**Histopathological Findings**

By the end of 8-12 week treatment period, mice fed the high-fat and high-cholesterol diet had developed widespread atherosclerotic lesions in the aorta (Fig. 5A) and heart (Fig. 5B). Luminal surface of aorta riddled with smooth muscle cells and fatty streaks (week 2) was advanced to stable plaque formation with lipidic-necrotic core containing extracellular lipid (week 4). Numerous macrophage foam cells and T cells were visible with increased intimal thickening and atheroma (week 8 and 12). Similarly increase of extracellular matrix, cholesterol clefts, build-up of fibroblasts and hemorrhage from atherogenic diet were observed in the heart. However, BV treatment significantly reduced lesion formation in the heart and aorta.

**Immunohistochemical Findings**

To assess whether BV modulated expression of adhesion molecules and fibrotic cytokines, we examined the effect of BV on the expressions of ICAM, VCAM, TGF-β1 and fibronectin in the aorta (Fig. 6A) and heart (Fig. 6B) by immunostaining assay. As demonstrated with representative photomicrograph of the assay at week 12, there was almost no staining in the controls. However strong stainings of adhesion molecules and fibrotic cytokines were observed in atherosclerosis induced mice, indicating increased expression of those proteins at the single cell level. BV treated groups showed a tendency to inhibit those expressions.
Discussion

In the present study, we demonstrated that BV, administered intraperitoneally, decreased the formation of atherosclerotic plaques and the level of serum lipids in the LPS treated and atherogenic diet fed mice. In addition, BV suppressed the expression of proinflammatory cytokines such as TNF-α and IL-1β. Furthermore, BV discouraged the expression of endothelial adhesion molecules of ICAM-1 and VCAM-1 implying that BV inhibited monocyte adhesion to activated endothelial cell. This study also found that the induction of endothelial adhesion molecules by inflammatory cytokines depends on activation of fibronectin and TGF-β1.

In experimental animals with diet-induced (Shi et al., 2005; Matsumoto et al., 2010) or genetically determined atherosclerotic lesions (Tikellis et al., 2008; Casós et al., 2008; Wan et al., 2009), the earliest morphological changes in affected arteries include the activation of endothelium by proinflammatory cytokines, focal adherence of mononuclear leukocytes to the endothelium and accumulation of monocyte-derived foam cells in the intima which was proven by our histopathological findings. This early process is self-maintaining and based on our understanding of the pivotal position of inflammation in the pathogenesis of atherosclerosis, therapeutic agents that target a smothering effect on the activation of endothelium opens opportunities in prevention and therapy of this disease (Moon et al., 2009). The interaction of leukocytes with the arterial intima is mediated by chemokines such as monocyte chemoattractant protein-1 (MCP-1) which chemoattract circulating monocytes and adhesion molecules of ICAM-1 and VCAM-1. Previous publications using mutant mice lacking MCP-1 or its receptor CCR2 and susceptible to atherosclerosis with the absence of genes encoding low density lipoprotein receptor and apolipoprotein E (apoE) have demonstrated striking decreases in local lipid deposition and lesion formation (Gu et al., 1998; Boring et al., 1998). A series of processes of adhesion molecule expression, monocyte...
adhesion and chemokine release are ultimately related with the release of several inflammatory cytokines. Eventually lipid-laden phagocyte accumulation is a major component of human atheromas and of atherosclerotic lesions in hyperlipidemic animals.

The involvement of IL-1β as a prototypic inflammatory cytokine in adhesion molecule expression is well documented (Galea et al., 1996; Dewberry et al., 2000; Olofsson et al., 2009). Several IL-1 family proteins were reported to be present in human atherosclerotic lesions (Galea et al., 1996; Dewberry et al., 2000). IL-1β and its natural antagonist called IL-1 receptor antagonist (IL-1Ra) have been suggested as an important pathogenic pair in adhesion molecule expression and their balance has been reported to influence the outcome of atherosclerosis development (Olofsson et al., 2009). Accordingly our study illustrated the high level of IL-1β to be relevant to high protein expression of adhesion molecules in atherosclerosis induced mice. It is possible that BV treatment might have changed the balance between IL-1β and IL-1Ra towards a less pro-inflammatory state. This theory is further supported by animal and human study because reduced IL-1Ra levels led to aggravation of atherosclerosis (Isoda et al., 2004) and IL-1β levels increased without a corresponding increase of IL-1Ra in coronary aorta disease patients (Waehre et al., 2004). Recruitment of monocytes to the arterial intima is dependent on TNF-α expression as well. Several lines of evidence suggest that TNF-α can stimulate the adhesion molecule expression (Mo et al., 2007; Nizamutdinova et al., 2007; Hung et al., 2008). Circulating markers of inflammation as prediction of atherosclerosis event is proven by our study since serum levels of TNF-α and IL-1β in mice fed the high-fat and high-cholesterol diet were higher than non-treated counterparts. Therefore apoE-knockout mice showed undetectable amount of cytokines in the circulation (Nashed et al., 2005; Wan et al., 2009). It was presumed that an inflammatory reaction occurred in the local arterial intima during atherosclerosis which could not induce enough amounts of cytokines in serum (Wan et al., 2009) at least in apoE-deficient mice.
which is not the case in our study. Only excessive cytokine production may induce atherogenesis from early fatty streaks to complicated plaques in genetically determined animal model of atherosclerosis.

The role of LPS on the development of atherosclerosis is via the activation of innate immunological system. It involves T lymphocytes since Th1 cells secrete the proatherogenic interferon at the early stage of disease process and Th2 cells provide interleukin secretion with further secretion of TNF-α by both cells at the later stage (Ostos et al., 2002). The involvement of hypercholesterolemia induced by feeding similar to our present study can accelerate the switch from Th1 to Th2 to result in the activation of the B cell response (Zhou et al., 1998). Therefore our repeated injections of LPS promoted atherosclerotic lesion formation in the arterial intima which concurs with observations from other study (Ostos et al., 2002).

The anti-inflammatory effect of BV came most likely from the studies of rheumatoid arthritis which is an inflammatory autoimmune disease. The decrease in COX-2 and PLA2 expression, and the decrease in the levels of TNF-α, IL-1, IL-6, NO and ROS were reported be associated with the anti-arthritis effect of BV which is suggested to be related to its anti-inflammatory effects (Son et al., 2007). In order to gain better insight into the action mechanism of BV, our group examined the anti-inflammatory effect of BV in LPS-stimulated murine macrophage cell line, Raw 264.7 cells. Similar to other studies (Pelletier et al., 1998; Amin et al., 1999; Park et al., 2004; Jang et al., 2005), we also found that BV has an anti-inflammatory effect by inhibiting inducible nitric oxide synthase (iNOS) and TNF-α expression (Han et al., 2006). In a further mechanism study, our group identified the anti-inflammatory property of BV and its molecular action mechanisms. BV at 0.1-2 µg/ml suppressed the LPS-induced NO/iNOS generation including COX-2 and IL-1β in rat C6 glioma cells (Lee et al., 2009). Moreover, we verified that the regulation of NO generation by
BV was dependent on nuclear factor kappa B/activator protein-1 through down-regulation of protein kinase C-α (PKC-α) related MEK/ERK signaling pathways which suggests that PKC-α and its downstream effectors constitute an excellent target for inflammatory response related glioma treatment. Therefore, an anti-inflammatory mechanism is now considered to be a target for the management of atherosclerosis.

The vascular endothelium serves as an important gate-keeper, regulating the movement of blood-borne mononuclear leukocytes into the intima (Wan et al., 2009). Under ordinary circumstances, the endothelial monolayer in contact with flowing blood resists firm adhesion of leukocytes. With an atherogenic diet as an inflammatory trigger, blood leukocytes start to attach to the endothelial cells which was revealed by light microscopy (Poole and Florey, 1958). At the same time, endothelial cells increase expression of ICAM-1 and VCAM-1. Circulating leukocytes enter inflamed tissue by binding its receptors to those adhesion molecules which is controlled largely by the interaction of complementary adhesion molecules and leukocytes (Wan et al., 2009). Thus up-regulation of these adhesion molecules on arterial endothelium is important in atheroma initiation. In this regard, our study clearly shows that BV has a potent inhibitory effect on cytokine-induced adhesion of monocytes to the tissues of aorta and heart. It is due to the ability of BV to inhibit cytokine-induced gene expression of ICAM-1 and VCAM-1 including TGF-β1 and fibronectin.

In conclusion, BV inhibits the development of atherosclerosis in C57BL/6 mice induced by injected LPS with feeding of atherogenic diet. It is likely through mechanisms involving anti-hypertriglyceridemic and anti-inflammatory effects of BV. The reduction of adhesion molecules and inflammatory factors by BV may be a protection against the atherosclerotic lesion formation. Given the multiple therapeutic effects, BV has the potential to be a useful adjunct as an antiatherogenic agent.
Acknowledgments

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Matsumoto, T., H. Watanabe, T. Ueno, A. Tsunemi, B. Hatano, Y. Kusumi, M. Mitsumata, N. Fukuda, K. Matsumoto, S. Saito and H. Mugishima. Appropriate doses of Granulocyte-Colony Stimulating Factor Reduced Atherosclerotic Plaque Formation and Increased Plaque


Figure Legends

Figure 1. Effects of BV on plasma total cholesterol (A), triglycerides (B) and high density lipoprotein-cholesterol (C) in normal mice, LPS treated and atherogenic diet fed mice, and BV treated mice. BV treatment decreased total cholesterol and triglycerides content while increasing high density lipoprotein. Data were expressed as means±SD. *p < 0.05 vs NC, †p < 0.05 vs LPS+fat group.

Figure 2. The contents of TNF-α (A) and IL-1β (B) reduced consistently in the BV treatment groups compared with those in LPS/fat group. LPS and atherosclerotic diet induced a significant expression of cytokines. Data were expressed as means±SD. *p < 0.05 vs NC, †p < 0.05 vs LPS+fat group.

Figure 3. Effects of BV on ICAM-1, VCAM-1, TGF-β1 and fibronectin expression in the aorta of mice. ICAM-1, VCAM-1, TGF-β1 and fibronectin expression levels was significantly decreased in the LPS/fat + BV group compared with LPS/fat group. Data were expressed as means±SD. *p < 0.05 vs NC, †p < 0.05 vs LPS+fat group.

Figure 4. Effects of BV on ICAM-1, VCAM-1, TGF-β1 and fibronectin expression in the heart of mice. ICAM-1, VCAM-1, TGF-β1 and fibronectin expression levels was significantly decreased in the LPS/fat + BV group compared with LPS/fat group. Data were expressed as means±SD. *p < 0.05 vs NC, †p < 0.05 vs LPS+fat group.

Figure 5. Histopathological lesions of atherosclerosis of the aorta (A) and heart (B) stained with hematoxylin and eosin (×200). They show the gradual inflammatory process of
atherosclerotic plaques with corresponding treatment weeks. No atherosclerotic lesion was observed in the control. Mice in LPS/fat group developed obvious atherosclerotic lesions which were markedly attenuated by BV treatment.

Figure 6. Representative photomicrograph of immunohistochemical staining for ICAM-1, VCAM-1, TGF-β1 and fibronectin expression in the aorta (A) and heart (B) at week 12 (× 200). While there was no staining in the controls, strong staining of adhesion molecules and fibrotic cytokines were observed in atherosclerosis induced mice. BV treated groups showed a tendency to inhibit those expressions.
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