Bofutsushosan, a Traditional Chinese Formulation, Prevents Intimal Thickening and Vascular Smooth Muscle Cell Proliferation Induced by Balloon Endothelial Denudation in Rats

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Bofutsushosan (BOF), a traditional Chinese formulation (Kampo formulation in Japanese), is widely used for patients with obesity and hyperlipidemia resulting from long-term inappropriate lifestyles. Since atherosclerosis, a lifestyle-related disease, is accompanied by an abnormal accumulation of vascular smooth muscle cells (VSMCs) in the intimal area of the artery, we investigated the preventive effect of BOF on intimal thickening. Oral administration of BOF extracts 3 d before and 7 d after balloon endothelial denudation dose dependently suppressed the intimal thickening and proliferation of VSMCs in the intimal area in rat carotid arteries. This model has a similar pathologic process to atherosclerosis and is considered to be an "accelerated atherosclerosis" model. BOF extract also dose dependently inhibited the migration of cultured VSMCs. BOF extract suppressed serum lipid levels, which are a major risk factor for atherosclerosis. These findings clarified the usefulness of BOF in cardiovascular risk-reduction therapy.

Key words atherosclerosis; Bofutsushosan; vascular smooth muscle cell; balloon endothelial denudation; intimal thickening; lifestyle-related disease

Atherosclerosis is a basic pathologic lesion of ischemic heart disease and brain infarction, and its preventive therapy for lifestyle-related diseases is important. Vascular endothelial cell injury and subsequent migration and proliferation of vascular smooth muscle cells (VSMCs) are closely involved in the initiation and progression of atherosclerosis.[3] This pathology can be reproduced by evaluation of intimal thickening after stripping endothelial cells with a balloon catheter in animal carotid arteries. This animal experimental system involves endothelial cell injury, and VSMC migration and proliferation induced by various growth factors, and is known as an "accelerated atherosclerosis" model[4] because of its similarity to atherosclerosis in humans.

Using this experimental model, we have been investigating the efficacy of traditional Chinese drugs[5] and formulations (Kampo formulations)[6,7] in the prevention of atherosclerosis. In this study, we investigated the preventive effects of Bofutsushosan (BOF, *Fanfengtongshengsan* in Chinese) on intimal thickening and VSMC proliferation. BOF is the major Kampo formulation used for the prevention of obesity,[8] hypertension, and insulin resistance.[9,10] Several pharmacologic studies of BOF have focused on antiobesity,[11] and antihyperglycemia in diabetes mellitus mice,[12] although information regarding the preventive effects of BOF on atherosclerosis is lacking.

MATERIALS AND METHODS

Samples and Reagents Freeze-dried extract of BOF (lot no. ONO31AO) was supplied by Kanebo Ltd. (Tokyo, Japan). The common human (60 kg) daily dose of the preparation is 5700 mg. The crude drug composition and HPLC profile of BOF are shown in the legend to Fig. 1. The sources of the hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitor simvastatin (SV, positive control compound), anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (PC-10), biotinylated anti-mouse second antibody, and streptavidin-conjugated peroxidase were the same as in our previous report.[6] The same Dullbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd.), fetal bovine serum (FBS, JRH Bioscience), penicillin (Gibco BRL), and streptomycin (Gibco BRL) were used as in our previous study.[13]

Animal Experiments Male Wistar rats (13 weeks old, 340—360 g body weight, Sankyo Lab. Service, Tokyo, Japan) were anesthetized with pentobarbital and balloon endothelial denudation in the left carotid artery was performed according to our previously described method.[7] Briefly, the rats ($n=8$) were fed a normal diet containing 1% cholesterol and BOF (three doses as shown in Fig. 2, $n=8$) for 3 d before and then for 7 d after the injury. SV (0.83 mg/kg daily, $n=8$) was administered orally during the same period as the BOF treatment. The doses of BOF 950 mg/kg and SV 0.83 mg/kg are 10-fold higher than the common human daily dose.

The intimal thickening and proliferation of VSMCs in the intimal area in left carotid artery sections 7 d after denudation were evaluated histologically and immunohistochemically according to our previous method.[7] The stenosis ratio, which is an index of the increase in intimal area and decrease in luminal area, was assessed by the equation shown in the legend to Fig. 2.

Serum lipids were examined in blood samples collected from the abdominal aorta 7 d after denudation. The total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and lipid peroxides (LPO) were also determined using commercial assay kits according to our previous method.[7]

All animal experiments and care were conducted in conformity with the Guidelines of the Animal Care and Use Committee of Toyama Medical and Pharmaceutical Univer-

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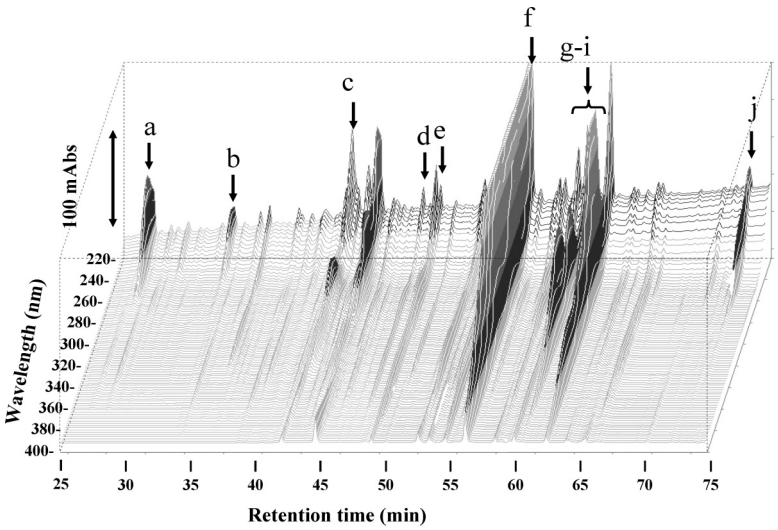

Fig. 1. HPLC Profile of MeOH-Soluble Portion of Freeze-Dried Extract of Bofutsushosan (BOF)

BOF contains 18 crude drugs: Scutellariae Radix (2.0), Glycyrrhizae Radix (2.0), Platycodi Radix (2.0), Gypsum Fibrosum (2.0), Atractylodis Rhizoma (2.0), Rhei Rhizoma (1.5), Schizonepetae Spica (1.2), Gardeniae Fructus (1.2), Paeoniae Radix (1.2), Cnidii Rhizoma (1.2), Angelicae Radix (1.2), Menthae Herba (1.2), Saposhnikoviae Radix (1.2), Ephedrae Herba (1.2), Forsythiae Fructus (1.2), Zingiberis Rhizoma (0.4), Kadinum (3.0), and Natrium Sulfuricum (0.75). Each figure in parentheses represents the ratio in the formulation (g/d). In the HPLC profile, a: geniposide (36 mg/5.7 g extract), b: paeoniflorin (16 mg/5.7 g extract), c: liquiritin, d: sennoside B, e: sennoside A (7.0 mg/5.7 g extract), f: baicalin (141 mg/5.7 g extract), g: isoliquiritin, h: oroxylin A-7-*O*-glucuronide, i: wogonin-7-*O*-glucuronide, j: glycyrrhizin (27 mg/5.7 g extract). These peaks were identified by cochromatography using authentic compounds. HPLC analysis: The 50% MeOH extract of BOF was analyzed using HPLC (LCMS-2010 with SPD-M10A and CLASS M10A Ver. 1.64, Shimadzu), with a YMC-Pack ProC18 AS-3C2 (YMC, 2.0 mm×15 cm, 20°C) and UV spectrometer (detection at 220—400 nm) using the mobile phase, A: CH_3CN (0.1% HCOOH) and B: H_2O (0.1% HCOOH). A : B (5 : 95)→(70 : 30) in 100 min. Flow rate, 0.2 ml/min.

sity, as approved by the Japanese Association of Laboratory Animal Care.

Cultured VSMC Experiments VSMCs (rat thoracic aorta SMCs: A7r5, Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan) were grown in DMEM supplemented with an antibiotic mixture (penicillin G 100 unit/ml and streptomycin 100 μg/ml) with 10% FBS and incubated at 37 °C in a humidified atmosphere with 5% CO_2. VSMC migration was assayed according to our previously reported method[13] using a microchemotaxis chamber (Neuro Probe Inc.) and polycarbonate filters (8 μm in diameter, Nucleopore Corp.). Briefly, a VSMC suspension (200 μl, 1.5×10^5 cells/ml) was placed in the upper compartment and FBS-DMEM (600 μl) containing BOF extracts (25, 50, 100, 250 μg/ml) was placed in the lower compartment. After 4 h incubation, the number of VSMCs that had migrated to the lower surface of the filter was determined microscopically. Migration activity was calculated as the mean number of migrated cells observed (five measurements).

VSMC viability was assessed using trypan blue dye exclusion.[14] VSMCs (2.0×10^5 cells) were seeded in 35-mm dishes and incubated in 10% FBS-DMEM containing BOF extract (100, 250, 500, 750 μg/ml) for 24 h.

Data Analysis Data are expressed as the mean±S.D. of the indicated number (n) of experiments. The statistical significances within a parameter were evaluated by one-way and multiple analysis of variation (ANOVA). A p value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Body Weight and Food Intake No rats ($n=8$) died in any experimental group over the 10 d. No abnormality of body weight or food intake was recognized. The body weight ratio on the 10th day in the BOF group (maximum dose 1425 mg/kg; 15-fold higher than the common human daily dose) was 98.1±2.4% of that on the first day.

Intimal Formation and VSMC Proliferation *in Vivo* In the cholesterol-fed control rats, the intimal area (0.046±0.004 mm^2) induced by denudation was more extensive ($p<0.05$) than that (0.040±0.002 mm^2) in the normal diet-fed control rats (denuded). The results are similar to those in our previous report.[7] Figure 2 shows that the increase in the intimal area and the stenosis ratio in the cholesterol-fed rats were dose dependently reduced by oral administration of BOF extract for 10 d. Oral administration of SV, the positive control compound, also significantly reduced the increase in the intimal area and the stenosis ratios.

Seven days after denudation, the number of VSMCs immunoreactive to anti-PCNA antibody in the intimal area was reduced by oral administration of BOF extract in a dose dependent manner. The PCNA labeling index (Fig. 2) is used as an index of VSMC proliferation.[15] At 10-fold higher than the human dose, the inhibitory effects of BOF extract (950 mg/kg) on intimal thickening and VSMC proliferation were comparable with those of SV (0.83 mg/kg), for which the inhibitory effects have been clarified in a similar balloon injury model.[16] These results suggest that the inhibitory effects of BOF on intimal thickening depend on its inhibition of VSMC proliferation, which is considered to be a major factor in the pathogenesis of intimal thickening after endothelial injury.[17]

Serum Lipids As shown in Table 1, the serum total cholesterol and LDL cholesterol levels in the cholesterol-fed control (denuded) group after a total of 10 d of feeding were significantly ($p<0.05$) increased compared with those in the normal diet-fed control group. There were no significant differences in serum LPO levels between the two control groups (data not shown).

Oral administration of BOF extract reduced the serum

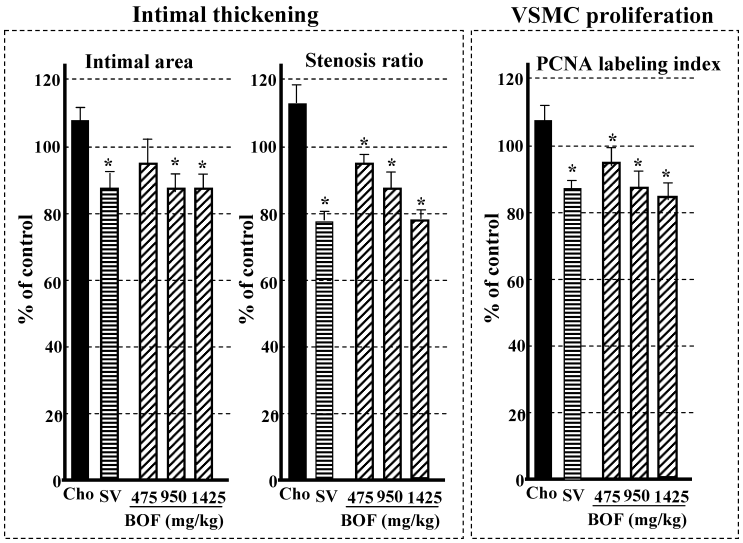

Fig. 2. Effects of BOF Extract on Intimal Area, Stenosis Ratio and PCNA Labeling Index in Rat Carotid Artery 7 d after Balloon Endothelial Denudation

Each value represents the percentage (mean±S.D., $n=8$) of the control group denuded normal diet-fed rats. Stenosis ratio (%)=(intimal area)×100/(intimal area+luminal area). PCNA labeling index (%)=(number of PCNA-positive VSMCs in intimal area)×100/(number of total VSMCs in intimal area). BOF extract (3 doses) was administered 3 d before and 7 d after denudation. The dose of BOF 950 mg/kg/d is 10-fold higher than the common human daily dose. SV, Simvastation (0.83 mg/kg/d; 10-fold higher than the common human daily dose).* Significantly different from the Cho group (denuded cholesterol diet-fed rats) at $p<0.05$.

Table 1. Effects of BOF Extract on Serum Lipid Levels

	TC (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)
Control (normal diet)	63.0±9.8	18.0±5.7	42.5±9.2
Cholesterol-fed group	99.4±9.1[a)]	58.7±6.0[a)]	31.1±3.8[a)]
SV 0.83 mg/kg	87.3±8.5[a,b)]	46.0±2.6[a,b)]	26.3±4.5[a)]
BOF extract 475 mg/kg	95.9±10.4[a)]	51.3±5.6[a)]	31.8±3.4[a)]
950	92.0±4.4[a)]	46.1±5.6[a,b)]	34.6±3.0[a)]
1425	87.8±6.8[a,b)]	44.0±5.5[a,b)]	36.5±6.1[b)]

Each value represents the mean±S.D. ($n=8$). TC, total cholesterol; LDL C, low-density lipoprotein cholesterol; HDL C, high-density lipoprotein cholesterol. BOF and SV were orally administered for 3 d before and 7 d after denudation. *a*) Significantly different from control normal diet-fed rats (denuded) and *b*) from cholesterol-fed rats (denuded) at $p<0.05$.

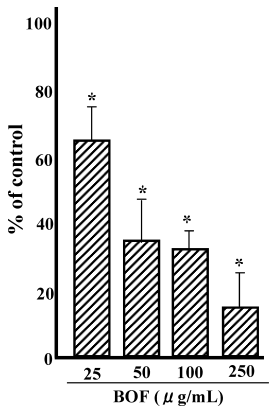

Fig. 3. Effects of BOF Extract on Cultured VSMC Migration

Each value represents the percentage (mean±S.D.) of the control value ($n=3$). * Significantly different from the control at $p<0.05$. The viability of VSMCs treated with BOF extract (100 and 250 μg/ml) was 97.4±1.7 and 96.6±0.7%, respectively. From these results, it is suggested that the inhibitory effects of BOF extract on VSMC migration are not directly involved in cell death.

total cholesterol and LDL cholesterol levels in a dose dependent manner, although its lipid-reducing effects were weaker than those of SV. BOF extract recovered the reduced HDL cholesterol levels. Since the intimal area increased with an increase in cholesterol load in the experimental system used (Fig. 2), the improvement of hyperlipidemia by BOF is one of mechanisms of inhibition of intimal thickening (atherosclerosis-preventive effect).

Since the Guidelines from the National Cholesterol Education Program recommended reduction of LDL cholesterol levels as the primary goal in cardiovascular risk-reduction therapy,[18] the LDL cholesterol-lowering effect of BOF may be useful for preventive therapy in atherosclerotic lifestyle-related diseases. Although no experiment to elucidate the mechanism of improvement of hyperlipidemia by BOF was performed in this study, BOF enhancement of thermogenesis in brown adipose tissue has been suggested,[19] and the adrenergic effects of the Ephedrae Herba ingredient (ephedrine) combined with BOF have been attracting attention.[20]

VSMC Migration *in Vitro* It has recently been considered that not only lipid deposition but also endothelial cell injury-induced VSMC migration and proliferation play important roles in the development of intimal thickening in atherosclerosis.[17] Inhibition of VSMC migration and proliferation may lead to the development of a preventive drug for atherosclerosis. Thus we investigated the effects of BOF on the migration of cultured VSMCs, and found that direct addition of BOF extract (25—250 μg/ml) inhibited VSMC migration in a dose dependent manner (Fig. 3). The concentration was rather high in this experiment, but the viability of VSMCs treated with BOF extract (100—750 μg/ml) was 95% or higher, as shown in the legend to Fig. 3. The inhibitory effects of BOF on the proliferation of cultured VSMCs have been reported,[21] suggesting that the inhibitory effects of BOF on VSMC migration (Fig. 3) and proliferation (Fig. 2) contribute to the preventive effect of BOF on intimal formation after balloon endothelial denudation.

There have been many reports of crude drugs combined with BOF with regard to the initiation and development of atherosclerosis. Atherosclerosis has been proposed to have

aspects of chronic inflammatory disease[22] associated with reactive oxygen species and various cytokines.[23] Thus BOF containing Rhei Rhizoma[24] and Scutellariae Radix[25] may have atherosclerosis-preventive effect. The inhibitory actions of a Rhei Rhizoma component combined with BOF on nitric oxide[26] and superoxide anion,[27,28] the free radical-scavenging action of Scutellariae Radix,[29] and the scavenging actions of Angelicae and Paeoniae Radix on the superoxide anion and hydroxyl radical[30] are also considered to be involved in the inhibitory effects of BOF on intimal thickening.

In conclusion, the effects of BOF, which is widely used in the prevention of lifestyle-related diseases, on intimal area formation associated with VSMC migration and proliferation were investigated. Continuous administration of BOF for 10 d prevented intimal thickening after balloon endothelial denudation in rat carotid arteries. This action was associated with inhibition of VSMC migration and proliferation and improvement of serum lipid levels (total cholesterol and LDL cholesterol). The details of the mechanism of action of BOF remain to be analyzed, but the findings of this study are pharmacologic evidence for the usefulness of BOF in the prevention of lifestyle-related diseases.

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