Anti-hyperlipidemic and Anti-atherosclerotic Actions of Shosaikoto
(Kampo Medicine)

Yi Rong Shen,a Makoto Inoue,a Yuko Nagatsu,a Yukio Ogihara,a, b and
Masaki Aburada

Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nagoya City University,a 3-1 Tanabe-dori,
Mizuho-ku, Nagoya 467, Japan and Tsumura Co., Ltd., a 6-2 Rokuban-cho, Chiyoda-ku, Tokyo 102, Japan.
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We investigated the anti-atherosclerotic action shown by Shosaikoto, a Kampo medicine, using hypercho-
lesterolemic mice. Oral administration of Shosaikoto significantly suppressed the elevation of serum cholesterol in C57BL/6 mice fed a 1.25% cholesterol-enriched diet for four weeks and improved the T cell ratio in peripheral blood, which decreased with the increase of the serum cholesterol level. In addition, Shosaikoto reduced the accu-
mulation of cholesteryl olate, which alters macrophages into foam cells, after the treatment of macrophages with oxidized or acetylated low density lipoprotein (LDL). Enzymatic study revealed that the treatment of macrophages with oxidized LDL enhanced acyl-coenzyme A: cholesterol acyltransferase (ACAT) activity and markedly reduced neutral cholesteryl ester hydrolase (NCEase) activity. Shosaikoto treatment prevented a decrease in the NCEase activity, however due to the oxidized LDL treatment, although it slightly augmented ACAT activity. Thus, Shosaikoto, which is known to modulate the immune system, improves macrophage and lymphocyte functions diminished by hypercholesterolemia, resulting in an anti-atherosclerotic action.

Key words Shosaikoto; atherosclerosis; macrophage; hypercholesterolemia; neutral cholesteryl ester hydrolase

Atherosclerosis is a major cause of coronary artery and cerebrovascular diseases, and hyperlipidemia, hyperten-
sion, diabetes mellitus and fatness are considered to be the risk factors in the development of atherosclerosis. Regardless of the causes of atherosclerosis, plural cells such as macrophages, T lymphocytes, smooth muscle cells and endothelial cells have been shown to contribute to its development.1,2) Macrophage-derived foam cell formation is a characteristic feature of atherosclerotic lesion, and T lymphocytes are found to be constituents of athero-
sclerotic lesion at all stages of development, suggesting that atherosclerosis is not only an inflammatory but also an immune disorder in a sense.3)

Macrophages are well known to play a pivotal role both in the lipid metabolism and immune response. Actually, they scavenge oxidatively modified low density lipoprotein (LDL), which is thought to be oxidized under endothe-
lithum, and protect blood vessels from the toxicity of oxidized LDL.4) The accumulation of massive amounts of cholesteryl ester which forms foam cells is determined by the balance between the activities of acyl-coenzyme A: cholesterol acyltransferase (ACAT) and neutral cholesteryl ester hydrolase (NCEase). Although ACAT is a rate-limiting enzyme in the esterification phase of the cholesteryl ester cycle and NCEase is believed to be the enzyme responsible for the hydrolytic phase of chole-
sterol ester, the regulation of hydrolysis of cytoplasmic cholesteryl ester also plays an important role in control-
ing the net efflux of cholesterol in macrophages. Further, the accumulation of cholesteryl ester is determined by the influx of cholesterol and cholesteryl ester as oxidized LDL and the efflux of extra cholesterol by high density lipoprotein (HDL). On the other hand, T lymphocytes found in atherosclerotic lesion are reported to be im-
munologically activated, as evidenced by the expression of MHC class II antigen.5) Furthermore, both CD4+ and

CD8+ T lymphocytes are detected in human lesions from the early stage of atherosclerosis,6) while in rabbits fed a high cholesterol diet the CD4+ T lymphocytes pre-
dominate.7) However, the specific role of the immune system in atherosclerosis has not been defined and T lymphocytes seem likely to produce several cytokines and growth factors that interact with cells in blood vessels, resulting in various effects on the development of athero-
sclerosis, for example cell proliferation, migration, the expression of adhesion molecule, and cholesteryl ester deposition.8) In fact, interferon-γ inhibits the expression of scavenger receptor and suppresses atherosclerosis in cholesterol-fed rabbits.9)–11) In contrast, the immuno-
suppressor, cyclosporin A, enhances atherosclerosis in hyperlipidemic mice12) and immune deficient mice de-
tvelop typical atherosclerotic fatty streaks when fed an atherogenic diet.13) In short, as atherosclerotic plaques are regarded as foci of chronic inflammation, agents that modulate the immune response seem likely to be effective for prevention of atherosclerosis. We have so far de-
termined that Shosaikoto, a Kampo medicine, which is used to treat chronic hepatitis and cirrhosis, shows anti-atherosclerotic activity in an atherosclerotic rabbit fed a cholesterol-enriched diet,14) and also that Shosaikoto modifies the function of the lipid metabolism in macrophages, resulting in protection from cholesteryl ester accumulation.15) In addition, the treatment of mice with Shosaikoto enhanced phagocytic activity and interleukin secretion in macrophages.16) We further reported that the monocyte number in blood of hypercholesterolemic rabbits decreases with the progress of hypercholester-
olemia. Moreover, the degree of progression of atherosclerosis is inversely correlated with the monocyte number,17) whereas oral Shosaikoto administration to rabbit prevented the reduction of monocyte number.18) Therefore, in order to identify the mechanism by which

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Shosaikoto shows anti-atherosclerotic activity, we attempted in this study to determine what kind of effect Shosaikoto shows in hyperlipidemic mice.

MATERIALS AND METHODS

Materials Cholesteryl [1-14C]oleate and [1-14C]oleyl-CoA were purchased from Amersham Japan (Tokyo, Japan). RPMI1640 and fetal bovine serum (FBS) were obtained from Irvine Scientific Co. (Santa Ana, CA).

Animals and Diet Twenty male CS7Bl/6j mice (Charles River Japan, Ltd.) weighing 20–24g at 6 weeks of age were assigned to one of four treatment groups: 5 were fed a cholesterol-free diet; 5 were received a cholesterol-enriched diet (Oriental Yeast Co., Ltd., Tokyo, Japan); 5 were treated with an oral administration of Shosaikoto at a dose of 1.2g/kg; 5 were received a cholesterol-enriched diet with oral administration of Shosaikoto. Cholesterol-enriched diet consisted of 1.25% cholesterol, 20% milk casein, 50% sucrose, 15% coconut milk, 4.95% crystallized cellulose, 1% corn oil, 5% mineral mixture, 1% vitamin mixture, 1% choline chloride and 0.3% methionine. Male New Zealand white rabbits were from Charles River Japan (Tokyo, Japan).

Preparation of Shosaikoto Shosaikoto (dose per person per day) was prepared as follows. Bupleuri radix (7g), Pinelliae tuber (5g), Scutellariae radix (3g), Ginseng radix (3g), Zingiberis rhizoma (4g), Zizyphi fructus (3g) and Glycyrrhizae radix (3g) were added to 700ml water, decocted for 1 h and concentrated to 300ml. This decoction was lyophilized to give 7.2g of powdered extract.

Preparation of Serum Lipoprotein LDL and HDL were prepared from the serum of rabbits fed 1% cholesterol diet by sequential ultracentrifugal flotation using an RPS80AT rotor of a Hitachi HC100 ultracentrifuge for 5 h and 8 h at 80000 rpm, respectively. LDL was dialyzed against 150mm NaCl, 0.3mm EDTA.

Modification of LDL For the preparation of oxidized LDL, LDL was first dialyzed against Dulbecco’s phosphate-buffered saline (PBS) prior to oxidation. LDL was incubated at 37°C with 5μm CuSO4 for 24 h and then dialyzed against 150mm NaCl, 0.3mm EDTA. To prepare acetylated LDL, 16mg of LDL protein in saline was added to 1ml of saturated solution of sodium acetate with continuous stirring on ice. Then, acetic anhydride was added in multiple small aliquots (2μl) over a period of 1 h with continuous stirring. After the addition of a total mass of acetic anhydride equal to 1.5 times the mass of protein, the mixture was stirred for another 30 min without further addition. The resultant solution was then dialyzed for 24 h at 4°C against 150mm NaCl and 0.3mm EDTA, pH 7.4.

Uptake of Modified LDL by Murine Peritoneal Macrophages and Efflux of Cholesteryl Ester from Macrophages by HDL Peritoneal macrophages were harvested from CS7Bl/6j mice 4d after the injection of 2ml of 3% thioglycolate broth (Difco Laboratories, Detroit, MI) in the peritoneal cavity and plated at 2×106 cells per 16mm well in RPMI1640 medium supplemented with 10% FBS. After a 2h incubation at 37°C, nonadherent cells were removed by washing with PBS. The macrophages were incubated with native, copper-oxidized, or acetylated LDL at a concentration of 50μg protein for 6h at 37°C. After the incubation, cholesterol and cholesteryl ester taken up by macrophages were extracted two times with 2ml of a solvent (isopropyl alcohol–hexane, 2:3) at room temperature. The extract was dried under N2 gas and total cholesterol and free cholesterol contents were determined by the cholesterol oxidase method. Foamed macrophages derived from macrophages after incubation with modified LDL were then incubated with HDL for another 3h to measure the efflux of cholesterol by determining cholesteryl ester and cholesterol contents as described above.

Immunofluorescent Flow Cytometry Immunofluorescent flow cytometric analysis was performed using antibodies against mouse lymphocyte surface antigens. Peripheral blood was treated with FACS lysing solution (Becton Dickinson Co., Mountain View, CA), washed with PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-Thy-1.2 antibody, FITC-conjugated anti-Lyt-2 antibody, phycoerythrin (PE)-conjugated anti-L3T4 antibody or phycoerythrin-conjugated anti-B220/CD45 antibody (Pharmaning, San Diego, CA) for 30 min at 4°C. The assay was carried out with FACSscan (Becton Dickinson Co., Mountain View, CA).

ACAT Assay ACAT activity was measured by a slightly modified method of Tabas et al.190 Mouse peritoneal macrophages (2×107 cells) were preincubated for 2 h in RPMI1640 and then treated for 5 h in RPMI1640 containing 10% lipoprotein deficient serum in the absence or presence of 0.1mg/ml oxidized LDL. The monolayers were then washed two times with ice-cold PBS, and the cells were scraped with a rubber policeman into PBS and collected by centrifugation. After washing once with 10ml PBS, the cell pellet was stored at -80°C until use. For preparation of microsome, the cell pellet was thawed in 2ml of 100mm potassium phosphate, pH 7.4, containing 1mm dithiothreitol and homogenized at 4°C. The cell homogenate was then depleted of whole cells and nuclei by centrifugation at 800g for 10 min. The post-nuclear supernatant was then centrifuged at 100000g for 1h, and the microsomal pellet was resuspended in 0.5ml of 100mm potassium phosphate, pH 7.4, containing 2mm dithiothreitol. For ACAT assay, aliquots (25μg of protein) of the microsome fraction were incubated for 30 min (within the linear range of the reaction for both protein and time) at 37°C in a final volume of 0.1ml containing 100mm potassium phosphate, pH 7.4, 1mm dithiothreitol, 0.15mg of fatty acid-free bovine serum albumin (BSA) and the reaction was started with 25μm [1-14C]oleoyl-CoA (30cpm/pmol). The reaction was stopped and assayed for cholesteryl [1-14C]oleate radioactivity as follows. The lipid of macrophages was extracted by the method of Bligh and Dyer20 and analyzed for cholesteryl [1-14C]oleate content by thin-layer chromatography (hexane–diethyl ether–glacial acetic acid, 70:30:1).

NCEase Assay NCEase activity was measured by a slightly modified method of Khoo et al.211 Mouse peritoneal macrophages (2×107 cells) treated with oxidized LDL were obtained by the identical method described for ACAT assay, except for the use of 10μm Tris–HCl, pH 7.0, 1mm EDTA, 0.25m sucrose instead
of 100 mM potassium phosphate, pH 7.4, containing 1 mM dithiothreitol. For preparation of homogenate, the cell pellet was thawed in 0.2 ml of 10 mM Tris–HCl, pH 7.0, 1 mM EDTA, 0.25 M sucrose and homogenized at 4°C. The homogenate was then depleted of whole cells and nuclei by centrifugation at 800 × g for 10 min and the post-nuclear supernatant was used for the following assay. For NCEase assay, the homogenate (25 μg of protein) was added to 0.4 ml of substrate mixture containing 62.5 μM cholesteryl [1-14C]oleate (20 dpm/pmol), 0.5% BSA, 0.15 M NaCl, 10% glycerol, 2 mM EDTA, 50 mM potassium phosphate, pH 7.0. The assay was carried out at 30°C for 1 h. The activity was linear with respect to time and enzyme concentration. The reaction was terminated by the addition of chloroform-methanol-benzene (1:2.4:2, v/v) and 0.1 N NaOH. Free [14C]oleic acid was extracted and the radioactivity counted.

Other Statistical significance was determined by Student’s t-test, and each value was given as the mean ± S.E.M.

RESULTS

To study the effect of Shosaikoto on hypercholesterolemia, C57BL/6 mice were fed a cholesterol-enriched diet and the medicine’s effect on serum cholesterol level was investigated. Figure 1 reveals that oral administration of Shosaikoto to these mice at a dose of 1.2 g/kg for 4 weeks significantly reduced the serum cholesterol level, whereas its administration to normal mice did not affect this level. Hypercholesterolemia is known to enhance the adhesion of macrophages and lymphocytes to endothelium and to start early atherosclerosis. We therefore measured the variation of lymphocytes in peripheral blood with the increased serum cholesterol level using a flow cytometer, after staining with FITC- or PE-conjugated antibody.

![Fig. 1. Effect of Shosaikoto on Serum Cholesterol in C57BL/6J Mice Fed a Cholesterol-Enriched Diet for 4 Weeks](image)

Mice were fed a normal chow in normal and Shosaikoto groups and a cholesterol-enriched diet in cholesterol and cholesterol/Shosaikoto groups. Shosaikoto was orally administered at a dose of 1.2 g/kg of body weight per day. ○, normal group; ■, cholesterol group; ●, Shosaikoto group; ■, cholesterol/Shosaikoto group. Values represent mean ± S.E. of 5 mice. *p<0.05 vs. cholesterol group.

![Fig. 2. Effect of Shosaikoto on Variation in Number of Peripheral T or B Cells in Cholesterol-Fed Mice](image)

Mice were fed as described in Fig. 1. Blood collected in tubes containing 10 mM EDTA in PBS was stained with FITC-conjugated anti-Thy1.2 antibody and PE-conjugated anti-B20/C4D5 antibody, after erythrocytes were lysed by lysing solution. □, normal group; ■, cholesterol group; ●, Shosaikoto group; ■, cholesterol/Shosaikoto group. Values represent mean ± S.E. of 5 mice. *p<0.05, **p<0.01.

![Fig. 3. Effect of Shosaikoto on Variation in Number of T4 or T8 Cells in Cholesterol-Fed Mice](image)

Procedure for staining was identical to that in Fig. 2 except for use of FITC-conjugated anti-Lyt 2 and PE-conjugated anti-1:3T4 antibodies. □, normal group; ■, cholesterol group; ●, Shosaikoto group; ■, cholesterol/Shosaikoto group. Values represent mean ± S.E. of 5 mice. *p<0.05, **p<0.01.
against Thy-1,2, Lyt-1, L3T4, and B220/CD45. Feeding of a cholesterol-enriched diet decreased the ratio of T and B lymphocytes to whole white blood cells at 4 weeks (Fig. 2), although antibody production was not significantly altered after mice injection with sheep red blood cells (data not shown). Shosaikoto administration suppressed the decrease of T lymphocyte ratio in high cholesterol diet-fed mice, whereas no effect was observed in B lymphocytes. Investigation of variation in subtypes of T lymphocytes showed that both T4 and T8 lymphocyte ratios were decreased in cholesterol-fed mice but not in cholesterol-fed mice treated with Shosaikoto. In addition, when normal mice were treated with Shosaikoto, the T lymphocyte ratio was increased slightly but significantly compared with the normal group (Fig. 3).

We next examined the effect of Shosaikoto on the cholesterol metabolism in macrophages. Macrophages from Shosaikoto-treated mice incubated with oxidized LDL and acetyl LDL as modified LDLs accumulated more cholesterol ester than normal macrophages similarly incubated. However, when macrophages which avidly took up oxidized LDL were treated with HDL, the cholesterol ester content in Shosaikoto-treated macrophages was reduced much more than in normal macrophages, in which 3 h incubation was not enough to detect cholesterol ester degradation or cholesterol efflux (Fig. 4). When the net reduction of cholesterol ester was calculated from the data shown in Fig. 4a, b, Shosaikoto treatment was found to reduce cholesterol ester, as a result of the stimulated cholesterol efflux by HDL (Fig. 4c). The mechanism by which Shosaikoto stimulates cholesterol ester efflux was elucidated by measuring the enzyme activities involved in cholesterol ester accumulation, that is, ACAT and NCease. As shown in Fig. 5, ACAT activity in macrophages from Shosaikoto-treated mice was enhanced in either the presence or absence of oxidized LDL. On the other hand, NCease activity was markedly decreased in normal macrophages after treatment with oxidized LDL, whereas Shosaikoto treatment retained the activity at a similar level to normal macrophages without oxidized LDL treatment (Table 1). These results may suggest that Shosaikoto prevents the accumulation of cholesterol ester by increasing its net hydrolysis in macrophages.

**DISCUSSION**

We have examined the anti-atherosclerotic and anti-hyperlipidemic activities shown by some Kampo medicines. One of them, Shosaikoto, was found to show anti-hyperlipidemic action in rats fed a cholesterol-enriched diet, whereas no such action was observed in a rabbit hypercholesterolemia model. However, obvious anti-atherosclerotic action was revealed by the formation of
fatty streak or fibrous plaques being significantly reduced by Shosaikoto administration. In this study we further examined in detail the anti-atherosclerotic or anti-hyperlipidemic action shown by Shosaikoto. C57BL/6 mice are known to develop typical atherosclerosis in the aorta after 15 weeks on a high fat, high cholesterol diet, and they are considered good subjects for the study of atherosclerosis. Shosaikoto significantly reduced the serum cholesterol level in mice after 2 and 4 weeks of feeding in this study, although earlier we demonstrated it had no effect on serum cholesterol in hypercholesterolemic rabbits. In this model, the serum cholesterol level slightly declined at 4 weeks, that is why we used a cholesterol-enriched diet containing 1.25% cholesterol, which was not enough to maintain a high serum cholesterol level over a long period.

Devery et al. reported that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and cholesterol 7α-hydroxylase activities in liver in rabbits is much higher in rabbits than in mice, while intestinal HMG-CoA reductase activity in the two species was comparable. Conversely, ACAT activity in both sites in rabbits is considerably greater than in rabbits. These results suggest that the anti-hyperlipidemic action by Shosaikoto likely depends on the inherent lipid metabolism in the study, and the effect is divergent even though the target of Shosaikoto is the same in different animals. In hypercholesterolemia, lymphocytes and monocytes in peripheral blood were considered to be severely influenced by a high concentration of lipids, resulting in augmented adherence and chemotaxis of monocytes or lymphocytes. Endothelial cells expressed adhesion molecules and monocyte chemotactic protein-1 after exposure to minimally modified LDL, resulting in the facilitation of monocyte or T lymphocyte adhesion. Our data in this study have indicated that T and B lymphocyte ratios in peripheral blood are reduced in mice with highly sustained serum cholesterol, although the reduction did not influence the immunity which was determined by antibody production. However, the reduction of the lymphocyte ratio by hypercholesterolemia was not necessarily reflected by high serum cholesterol, because T or B cell reduction was definitely observed at 4 weeks of feeding, but not at 2 weeks, although serum cholesterol was higher at 2 weeks than at 4 weeks.

Shosaikoto treatment prevented the reduction of T lymphocytes in hypercholesterolemia mice, but not B lymphocytes. Moreover, the treatment of normal mice also enhanced the T lymphocyte number, indicating that the protective effect of Shosaikoto from the decrease of lymphocyte ratio by hypercholesterolemia may be due to not only an indirect action by the reduction of the serum cholesterol level but also to the direct action by which Shosaikoto itself increased the lymphocytes. We did not clarify, however whether the reduction of lymphocyte ratio resulted from the increased adhesion to endothelium or from the decreased generation.

Another effect of Shosaikoto was the reduction of cholesterol ester in macrophages by the enhanced cholesterol efflux by HDL, although the uptake of modified LDL was increased by Shosaikoto treatment. Usually ACAT activity is regulated by cholesterol pools available to ACAT, and the enhanced activity in Shosaikoto-treated macrophages would be due to the increased cholesterol taken up by the activated pathway through scavenger receptors, as the expression of scavenger receptor is known to be upregulated by the differentiation of monocytes to macrophages, and to be markedly and selectively increased by macrophage colony-stimulating factor (M-CSF). In addition, α-interferon and interleukin-4 have differential effects on scavenger receptor glycosylation in vitro, and Shosaikoto may well show such activity by enhancing scavenger receptor expression by certain cytokines.

Ishii et al. suggested the possibility that the reduced

![Chart 1](chart1.png)

**Chart 1.** Effect of Shosaikoto on the Relationship between Lipid Metabolism and Cytokine Network in Macrophages

This chart indicates the two-compartment model for cholesterol ester metabolism in macrophages and its regulation by various cytokines. Open arrows indicate stimulation by Shosaikoto and broken arrows do implication of cytokines.
NCEase activity in macrophages participates in the progression of atherosclerosis. Furthermore, acetyl LDL is reported to reduce NCEase in macrophages. In contrast, NCEase activity is activated by cyclic AMP-dependent protein kinase and is enhanced 4-fold by M-CSF. However, in this study we demonstrated that oxidized LDL markedly suppresses NCEase activity and that Shosaiokato prevents its suppression, although the mechanism remains to be identified. Also, it was not determined whether the enhanced efflux of cholesterol by HDL from macrophages in Shosaiokato-treated mice was due to the increased intracellular free cholesterol that was produced by NCEase which was not suppressed in cholesterol-fed mice treated with Shosaiokato, or to the increased endogenous apoE production which was shown to affect macrophage cholesterol efflux by regulating the rate of cellular cholesterol clearance by LDL. ApoE expression was regulated by cholesterol, protein kinase C activators and endotoxin. TGF-β, IL-1β, TNF-α and M-CSF have also been shown to stimulate apoE expression. Shosaiokato has been found to stimulate several cytokines such as interleukin-1, interleukin-6, interferon γ/β, GM-CSF, and G-CSF, suggesting that apoE synthesis in macrophages may be enhanced by this treatment. Furthermore, M-CSF was reported to prevent the progression of atherosclerosis in WHHL rabbits by increasing net hydrolysis of cholesteryl ester in macrophages. We summarized the action of Shosaiokato on lipid metabolism in macrophages in Chart 1, which caused us to recognize that its effect was very similar to that of M-CSF. This suggest the possibility that Shosaiokato may stimulate M-CSF secretion by macrophages, resulting in the modulation of macrophage functions to protect against atherosclerosis. In conclusion, Shosaiokato, which is known to modulate the immune system, improves macrophage and lymphocyte functions diminished by hypercholesterolemia in addition to having anti-hypercholesterolemic action, resulting in an anti-atherosclerotic action.

REFERENCES