

Effects of Shosaikoto (Kampo Medicine) on Lipid Metabolism in Macrophages

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We investigated effects of Shosaikoto treatment on cholesterol metabolism in macrophages. Although macrophages, harvested from mice treated with Shosaikoto, took up a small amount of control low density lipoprotein (LDL) (thiobarbituric acid-reactive substance (TBA-RS) value was 0.27 pmol/mg of protein) as control macrophages, they took up more LDL modified with CuSO_4 (TBA-RS value was 6.12 pmol/mg of protein) than control macrophages. Degradation of both control LDL and oxidized LDL was enhanced in Shosaikoto treated macrophages. In the presence of control LDL or in the absence of LDL, incorporation of [^3H]oleic acid into cholesteryl oleate was significantly reduced in Shosaikoto treated macrophages. This suggests that acyl-coenzyme A:cholesterol acyltransferase (ACAT) activity in macrophages was partly inhibited by Shosaikoto treatment. On the other hand, in the presence of oxidized LDL, cholesteryl ester accumulated in Shosaikoto treated macrophages as much as in controls. However, cholesteryl oleate efflux from macrophages in the presence of high density lipoprotein (HDL) was enhanced in Shosaikoto treated macrophages. These results indicate that Shosaikoto facilitates oxidized LDL catabolism in macrophages, resulting in the augmentation of oxidized LDL uptake and the elimination of cholesterol from macrophages by HDL. These Shosaikoto effects may prevent foam cell formation and the progression of atherosclerotic lesions.

Keywords macrophage; atherosclerosis; oxidized LDL; Shosaikoto

Atherosclerosis is a major cause of coronary artery and cerebrovascular diseases and macrophage-derived foam cells are an important feature of early atherosclerotic lesions. Native low density lipoprotein (LDL) does not provoke foam cell formation because effective accumulation of cholesterol or cholesteryl ester is prevented by down-regulation of the native LDL receptor. On the other hand, oxidatively modified LDL, detected in atherosclerotic plaques,¹⁾ acetylated LDL, and other chemically modified LDL are avidly taken up by macrophages, mediated by scavenger receptors, resulting in foam cell formation.²⁾ The accumulation of massive amounts of cholesteryl ester, which forms foam cells is due to the activation of acyl-coenzyme A:cholesterol acyltransferase (ACAT) resulting from increased cholesterol pools available to ACAT.³⁾ Recent studies have shown that Probucol, an antioxidant, retards atherosclerosis in Watanabe heritable hyperlipidemic rabbits^{4,5)} At present, the relationship between the manifestation of atherosclerosis and oxidized-LDL (ox-LDL) formation merits investigation. Shosaikoto is a Kampo medicine, widely used for therapy of chronic diseases in Japan. Shosaikoto is effective therapy for chronic hepatitis and inflammation,^{6,7)} however the mechanism by which it works is not well understood. Shosaikoto administration reduces prostaglandin formation and also augments phagocytosis⁸⁾ and interleukin-1 release. These actions are thought to result from modification of macrophage functions. Although Shosaikoto doesn't exhibit anti-hypercholesterolemic action in the rabbit hypercholesterolemic model, it reduces atherosclerotic plaque area, and suppresses collagen synthesis in thoracic aorta.⁹⁾ Thus the influence of Shosaikoto on macrophage function was studied to determine the mechanisms responsible for its antiatherosclerotic actions.

Materials and Methods

Materials RPMI1640, Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Nippon Bio. Supp. Center Co., Ltd., Tokyo, Japan. [^{125}I]Iodine was obtained from Amersham Japan.

[^3H]Oleic acid was from Du Pont-New England Nuclear. Chloroquine was obtained from Sigma.

Animals Male ICR mice (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were used at 6 weeks age. New Zealand White Rabbits (Nippon Bio. Supp. Center Co., Ltd., Tokyo, Japan) were fed with commercial chow containing 1% cholesterol. They were kept in an air-conditioned room (24°C) and given commercial diet and water *ad libitum*.

Preparation of Serum Lipoprotein Low and high density lipoprotein (LDL and HDL, respectively) were isolated from the serum of rabbits fed a 1% cholesterol diet, following sequential ultracentrifugal flotation using a RP80AT rotor of a Hitachi HC100 ultracentrifuge for 5 and 8 h respectively at 80000 rpm. LDL was dialyzed against 150 mM NaCl, 0.3 mM ethylenediaminetetraacetic acid (EDTA).

Modification and Labeling of Isolated Serum Lipoprotein LDL was dialyzed against Dulbecco's phosphate buffer saline (PBS) prior to oxidation. The LDL was incubated at 37°C with 5 μM CuSO_4 for 6 h and dialyzed against 150 mM NaCl, 0.3 mM EDTA. Iodination of LDL or ox-LDL was carried out using the iodine monochloride method.¹⁰⁾

Isolation of Macrophages Resident peritoneal macrophages harvested from mice were plated at 1.5×10^6 cells per 16-mm well in RPMI1640 containing 10% fetal bovine serum (FBS). After a 2 h incubation, nonadherent cells were removed by washing with PBS, and used for the following experiments.

Binding and Degradation of [^{125}I]LDL To measure binding at 4°C and uptake at 37°C, macrophages were incubated in RPMI1640 containing [^{125}I]LDL or [^{125}I]ox-LDL (0.1 mg of protein per ml, 100–200 cpm/ng of protein) at 4°C or 37°C (with or without 50 μM chloroquine) for 5 h. Media were subsequently used for measuring of degradation. The macrophages were washed with PBS and solubilized with 1 N NaOH. [^{125}I]LDL degradation was measured by assaying the amount of [^{125}I]labeled trichloroacetic acid-soluble material in the medium.¹¹⁾

Cellular Cholesterol Esterification The formation of cholesteryl ester by macrophages was measured from the incorporation of [^3H]oleate into cellular [^3H]cholesteryl oleate. Macrophages were incubated in Dulbecco's minimum essential medium (DMEM) containing 0.1 mM [^3H]oleate-albumin (190 dpm/pmol) in the presence of 0.1 mg/ml of LDL or ox-LDL at 37°C for 5 h. Cells were washed with PBS and their lipids were extracted with 0.5 ml of hexane-isopropyl alcohol (3:2, v/v) at room temperature for 30 min. After additional extraction with the same solvent, the pooled lipid extract was dried under nitrogen and separated by thin layer chromatography in a solvent of hexane-diethyl ester-acetic acid (80:20:1).

Preparation of Shosaikoto Shosaikoto (dose per person per day) was prepared as follows. Bupleuri Radix (7 g), Pinelliae Tuber (5 g), Scutellariae Radix (3 g), Ginseng Radix (3 g), Zingiberis Rhizoma (4 g), Zizyphi Fructus (3 g) and Glycyrrhizae Radix (2 g) were added to 700 ml of water, boiled for 1 h and concentrated to 300 ml. This decoction was

lyophilized to give 7.2 g of powdered extract.

Others Thiobarbituric acid reactive substance was measured by the method of Yagi.¹²⁾ Statistical significance was determined by Student's *t* test and each value was given as mean ± S.E.M.

Results and Discussion

We've already shown that phagocytic ability of macrophages is stimulated by Shosaikoto treatment.⁸⁾ And it is known that macrophage scavenger receptors recognize ox-LDL, acethyl-LDL and negatively charged macromolecules. Therefore, we first examined effects of Shosaikoto on uptake and degradation of Cu²⁺-ox-LDL (Fig. 1). Oral Shosaikoto treatment at 10 times the human daily dose didn't influence the uptake of control LDL. However, ox-LDL was taken up by macrophages treated with Shosaikoto about 1.6 times more than by control macrophages. Uptake was markedly enhanced compared with control LDL. Shosaikoto treatment stimulated both the degradation of control LDL and ox-LDL significantly as shown in Fig. 1. In Fig. 2, no difference was observed in the uptake of ox-LDL between the control and Shosaikoto treatment group at 4°C. However, the uptake at 37°C was significantly enhanced by Shosaikoto treatment compared with controls. Furthermore, uptake following complete inhibition of LDL degradation by chloroquine (a known

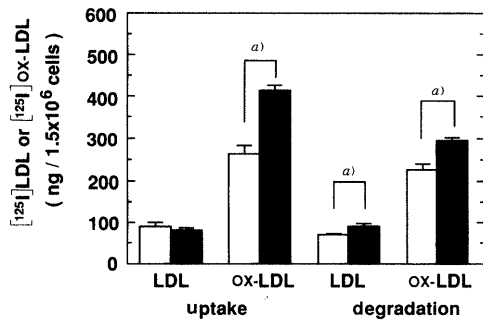


Fig. 1. Effects of Shosaikoto on Uptake or Degradation of LDLs by Mouse Peritoneal Macrophages

Control [¹²⁵I]LDL and Cu²⁺-ox-[¹²⁵I]LDL (100 µg/ml each) were incubated for 5 h with control macrophages (open column) or with macrophages prepared from 9 mice treated with Shosaikoto (1.2 g/kg of body weight) orally for 5 consecutive days (closed column). The amount of LDLs taken up or degraded was determined as described under Materials and Methods. Values represent means ± S.E. from 6 cultures. *a)* *p* < 0.01 vs. control group.

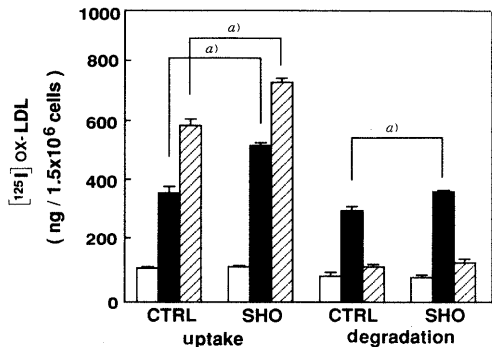


Fig. 2. Effects of Shosaikoto on Uptake or Degradation of Oxidized LDL by Mouse Peritoneal Macrophages

Cu²⁺-ox-[¹²⁵I]LDL (100 µg/ml) was incubated for 5 h at 4°C (open column), at 37°C (closed column) and in the presence of chloroquine (50 µM) at 37°C (hatched column). CTRL: control macrophages, SHO: macrophages prepared from 9 mice treated with Shosaikoto. Values represent means ± S.E. from 6 cultures. *a)* *p* < 0.01 vs. control group.

inhibitor of lysosomal enzymes) was significantly facilitated in the Shosaikoto treatment group. In brief, Shosaikoto enhances an ability of macrophages to endocytose ox-LDL, but not control LDL. These results suggest that Shosaikoto treatment enhances ability of macrophages in atherosclerotic plaques to scavenge modified LDL, found in subendothelia of atherosclerotic plaque by Herttuala *et al.*¹³⁾ Usually, increased uptake of ox-LDL is considered to lead to accumulation of cholesteryl ester, resulting in formation of foam cells or expansion of atherosclerotic lesion. Therefore, we investigated effects of Shosaikoto on cholesteryl ester accumulation in macrophages. We determined cholesteryl ester production by measuring incorporation of [³H]oleic acid into cholesteryl oleate. As shown in Fig. 3, cholesteryl oleate formation was slightly but significantly suppressed in the absence or presence of LDL in the Shosaikoto treatment group. Furthermore, the incorporation of [³H]oleic acid into cholesteryl oleate was suppressed throughout the 7.5 h incubation (Fig. 4). The quantity of cholesterol in macrophage before the incubation with [³H]oleic acid was determined by high performance liquid chromatography (HPLC). The values were 4.2 nmol/ 1.5 × 10⁶ cells and 4.4 nmol/1.5 × 10⁶ cells in Shosaikoto treated macrophages and in control macrophages, respectively. A similar amount of [³H]oleic acid

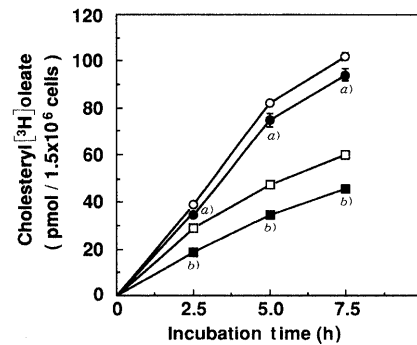


Fig. 3. Effects of Shosaikoto on Incorporation of [³H]Oleic Acid into Cholesteryl Oleate in Mouse Peritoneal Macrophages.

Macrophages were incubated for indicated times with control LDL (circle, 100 µg/ml) or with no LDL (square) in DMEM, 0.1% BSA containing 0.1 mM [³H]oleate (190 dpm/pmol). Other details are shown in Materials and Methods. Open symbols: control macrophages. Closed symbols: Shosaikoto treated macrophages. Values represent means ± S.E. from 6 cultures. *a)* *p* < 0.05, *b)* *p* < 0.01 vs. control group.

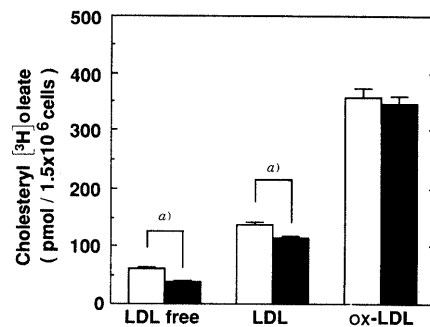


Fig. 4. Effects of Shosaikoto on Incorporation of [³H]Oleic Acid into Cholesteryl Oleate in the Presence of Control LDL or Cu²⁺-ox-LDL

Macrophages were incubated for 5 h with control LDL (100 µg/ml) or Cu²⁺-ox-LDL (100 µg/ml) in DMEM, 0.1% BSA containing 0.1 mM [³H]oleate (190 dpm/pmol). Open column: control macrophages. Closed column: Shosaikoto treated macrophages. Values represent means ± S.E. from 6 cultures. *a)* *p* < 0.01 vs. control group.

TABLE I. Effects of Shosaikoto on Ratio of Degradation to Synthesis of Cholesteryl Oleate

		LDL	ox-LDL
Degradation synthesis	Control	0.740 ± 0.052	0.068 ± 0.032
	Shosaikoto	0.868 ± 0.013 ^{a)}	0.235 ± 0.040 ^{b)}

Cholesteryl oleate in macrophages was pre-labeled for 5 h with 0.1 mM [³H]oleate (190 dpm/pmol)/0.1% BSA in DMEM. Macrophages were then washed 3 times with PBS to remove [³H]oleate. After washing, macrophages were incubated with HDL (400 µg/ml) for another 3 h. Ratios indicated were calculated as follows. (Degradation/synthesis) = ([³H]cholesteryl oleate decreased in the presence of HDL per hour/[³H]cholesteryl oleate pre-labeled per hour). Values represent means ± S.E. from 6 cultures. a) $p < 0.02$, b) $p < 0.01$ vs. control group.

was incorporated into phospholipids and triacylglycerol in Shosaikoto and control groups (1.15 ± 0.05 nmol and 178.0 ± 6.6 pmol per 1.5×10^6 cells in the Shosaikoto group; 1.10 ± 0.04 nmol and 125.0 ± 3.6 pmol per 1.5×10^6 cells in controls). These results show that the size of cholesterol and fatty acid pools in Shosaikoto treated macrophages and control macrophages are not different and suggest that Shosaikoto partially inhibits ACAT activity in macrophages. However, in the presence of ox-LDL, cholesteryl ester was accumulated to the same degree in both groups. That differences in cholesteryl oleate formation were not seen despite the enlargement of ox-LDL uptake by Shosaikoto treatment suggests that Shosaikoto partly suppresses ACAT activity even in the presence of ox-LDL or enhances elimination of cholesteryl ester by stimulating cholesteryl esterase or by promoting cholesterol efflux to HDL. Tabas *et al.* have shown that a pentacyclin triterpene ester derived from a dietary plant triterpene was found in rabbit and human liver and inhibited ACAT activity.¹⁴⁾ Such compounds may contribute to the ACAT inhibitory activity shown by Shosaikoto. Therefore, we evaluated the degradation of cholesteryl oleate by macrophages. Cholesteryl oleate was pre-labeled with [³H]oleic acid in the presence of control LDL or ox-LDL for 5 h. After washing LDLs off, macrophages were further incubated for 3 h in the presence of HDL.

Values shown in Table I represents ratios of decreased [³H]cholesteryl oleate per hour in the presence of HDL divided by increased [³H]cholesteryl oleate per hour in the presence of LDL. In incubations with control LDL, the ratio was larger in Shosaikoto treatment groups than in controls, indicating that the degradation of cholesteryl oleate in the presence of HDL and the release of cholesterol by HDL was stimulated in Shosaikoto treated macrophages. On the other hand, in the case of incubations with ox-LDL, the ratio values in controls was much less than that in the Shosaikoto treatment group. When incubated with ox-LDL, much ox-LDL was taken up by macrophages and macrophage ACAT activity was most likely activated. Due to increased ACAT activity, cholesteryl oleate degradation apparently might look small. However, when Shosaikoto treated macrophages were incubated with ox-LDL, hydrolysis of cholesteryl oleate in the presence of HDL was actually enhanced compared with control macrophages, even though the value was markedly smaller than that in the

case of incubations with control LDL. In this paper, we have partly demonstrated the mechanism of Shosaikoto's antiatherosclerosis activity. Macrophages treated with Shosaikoto took up more ox-LDL than control macrophages. Since oxidatively modified LDL is cytotoxic to endothelial cells, it's necessary to remove it from atherosclerotic plaques to protect arteries from atherosclerosis. Shosaikoto protects arteries from ox-LDL by stimulating the ability of macrophage to take it up. However, cholesterol and cholesteryl ester taken up as ox-LDL induce macrophages to form foam cells. Therefore, reasonable ways to reduce the accumulation of cholesteryl ester in macrophages are to inhibit ACAT, to stimulate cholesteryl esterase and to enhance cholesterol release by HDL or by the augmentation of apoprotein synthesis in macrophages. Data presented in this paper suggest that Shosaikoto treatment reduces the accumulation of cholesteryl ester through several of the mechanisms indicated above. We have demonstrated one of the mechanism by which Shosaikoto shows anti-atherosclerosis action is to modulate macrophage functions, namely, the activation of catabolism of ox-LDL. Other actions not shown in this paper may contribute to Shosaikoto's antiatherosclerotic action, for instance, reduction of LDL oxidation in blood by an antioxidant action, or reduction of monocytes recruitment in the artery. Due to its versatile actions, Shosaikoto may effective therapy for atherosclerosis.

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