Restorative Effect of Shosaikoto (Kampo Medicine) on Diminution of Nitric Oxide Synthesis in Murine Peritoneal Macrophages Induced by Hypercholesterolemia

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Macrophages play important roles both in immune response and in lipid metabolism and contribute to the development of atherosclerosis. To clarify the mechanism by which Shosaikoto, a Kampo medicine, shows anti-atherosclerotic action, we studied its effect on macrophage function. The production of nitric oxide (NO), prostaglandin E₂ and interleukin 1 by macrophages in mice was reduced by feeding of a cholesterol-enriched diet, and the reduced production was observed 1 week after the beginning of cholesterol feeding. Furthermore, although oxidized low density lipoprotein (LDL) and lysophosphatidylcholine (LPC) reduced NO production, macrophages prepared from mice treated with Shosaikoto at a dose of 1.2 g/kg/d restored the reduced NO production by them as well as by hypercholesterolemia. When the content of LPC was measured, no difference was observed between mice fed a cholesterol-enriched diet in the presence or absence of Shosaikoto treatment, suggesting that the restorative effect of Shosaikoto is not due to the inhibition of LPC production or accumulation. Conclusively, Shosaikoto prevents the modification of macrophage function induced by atherogenic factors, which is probably linked to its displayed anti-atherosclerotic action.

Key words Shosaikoto; atherosclerosis; macrophage; nitric oxide; lysophosphatidylcholine; prostaglandin

Nitric oxide (NO) was identified as an endothelium-derived relaxing factor, a potent endogenous vasodilator released from the endothelium and well known to play important roles in the physiological or pathological process.¹⁾ In addition to the regulation of vasomotion, NO is known to suppress monocyte adherence,²⁾ platelet aggregation,³⁾ vascular smooth muscle proliferation,⁴⁾ and neutrophil adhesion.⁵⁾ Hypercholesterolemia inhibits NO synthesis in endothelial and smooth muscle cells and further decreases prostaglandin synthesis in macrophages.⁶⁾ Furthermore, long-term inhibition of NO synthesis promotes atherosclerosis in the hypercholesterolemic rabbit thoracic aorta⁷⁾ and the attenuated NO-mediated platelet inhibition produced by increased reactive oxygen species or impaired antioxidant defense may cause a thrombotic disorder in human.⁸⁾ In the search for causal substances to suppress NO synthesis, an endogenous inhibitor of NO synthesis was reported to exist in serum of rabbits fed a cholesterol-enriched diet.⁹⁾ In addition, oxidatively modified low density lipoprotein (LDL), which is present in human and animal atherosclerotic lesions,^{10,11)} is known to suppress NO synthesis in macrophages,¹²⁾ whereas the effect on endothelial cells was controversial.^{13,14)} Long-term oral administration of L-arginine, the precursor of NO, however, prevents atherogenesis in the coronary artery of the hypercholesterolemic rabbit.^{15,16)} These lines of evidence would suggest that NO contributes to suppression of arteriosclerosis.

NO is produced in various cells, for example, endothelial cells,¹⁷⁾ smooth muscle cells,¹⁸⁾ macrophages,¹⁹⁾ neurons²⁰⁾ and hepatocytes.²¹⁾ Macrophage-derived NO is believed to play an important role in host defense against viruses, mycobacteria, protozoa, and helminths. However, excessive NO may cause tissue damage in arthritis, glomerulonephritis, diabetes, stroke, septic shock, viral and autoimmune encephalitis. Although the roles of NO

released from macrophages in the development of atherosclerosis have not been defined, NO inhibits the oxidative modification of LDL with cytokine-stimulated macrophages by neutralizing superoxide.²²⁾ Furthermore, the earliest characteristic of atherosclerosis is the adherence of monocytes to the arterial endothelium, and a nitric oxide synthetase (NOS) inhibitor, *N*-methyl-arginine, increases the adhesion of neutrophils to the endothelium by upregulating of CD11/18 expression.⁵⁾ This suggests the possibility that the adhesion of monocytes to the endothelium in early atherogenesis is stimulated or mediated by the reduction of NO synthesis in monocytes and endothelial cells. Shosaikoto, a Kampo medicine, which is used to treat chronic hepatitis and cirrhosis, showed anti-atherosclerotic activity in an atherosclerotic rabbit fed a cholesterol-enriched diet²³⁾ and suppressed the enhanced adherence of monocytes by hypercholesterolemia to endothelial cells.²⁴⁾ Shosaikoto also modified the lipid metabolism in macrophages resulting in protection from cholesteryl ester accumulation,²⁵⁾ enhanced phagocytic activity and interleukin secretion and also diminished prostaglandin production in macrophages.²⁶⁾

In this study we attempted to determine the effect of Shosaikoto on the function of macrophage, mainly NO synthesis, which was affected by atherogenic factors.

MATERIALS AND METHODS

Materials RPMI1640 and fetal bovine serum (FBS) were obtained from Irvine Scientific Co. (Santa Ana, CA). Griess reagent was from Sigma Chemical Co. (St. Louis, MO). Lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4) was purchased from Difco Laboratory (Detroit, MI). Lysophosphatidylcholine (LPC) was obtained from Sigma Chemical Co. (St. Louis, MO).

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Animals and Diet Male ICR mice at 6 weeks of age (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were fed a commercial chow or a 1.25% cholesterol-enriched diet containing 20% milk casein, 50% sucrose, 15% coconut milk, 4.95% crystallized cellulose, 1% corn oil, 5% mineral mixture, 1% vitamin mixture, 1% choline chloride, 0.3% methionine (Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum*.

Preparation of Shosaikoto Shosaikoto (dose per person per day) consists of 7 crude drugs and 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 700 ml water, decocted for 1 h and concentrated to 300 ml. This decoction was lyophilized to give 7.2 g of powdered extract.

Preparation of LDL and Modified LDLs LDL was 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 h at 80000 rpm (at a density of 1.019–1.063). LDL was dialyzed against 150 mM NaCl, 0.3 mM ethylenediamine-tetraacetic acid (EDTA), as described previously.²⁵⁾ Resultant LDL was dialyzed against Dulbecco's phosphate-buffered saline (PBS) prior to oxidation. For preparation of oxidized-LDL, LDL was incubated with 5 μ M CuSO₄ at 37 °C for 6 h in 60 mm culture plate in 5% CO₂ in air and dialyzed against 150 mM NaCl, 0.3 mM EDTA. Acetyl LDL was prepared by the method of Fraenkel-Conrat.²⁷⁾

NO Production by Macrophages Thioglycolate-elicited peritoneal macrophages were harvested from ICR mice 4 d after intraperitoneal injection of 2 ml of 3% thioglycolate broth (Difco Laboratories, Detroit, MI) and washed twice with PBS. The cells were seeded in 24-well tissue culture plates at 2×10^6 cells/well in RPMI1640 supplemented 10% FBS and cultured for 2 h at 37 °C in 5% CO₂ in air. Non-adherent cells were removed by thorough washing with PBS and then the monolayers were placed in fresh RPMI1640 medium supplemented 10% FBS. LPS at a concentration of 10 μ g/ml was added to

macrophages and cultured for 24 h. Macrophage NO synthesis after LPS challenge was measured indirectly as nitrite concentration in the culture medium according to the method of Stuehr and Nathan.²⁸⁾

Prostaglandin E₂ (PGE₂) and Interleukin 1 (IL-1) Assay Production of PGE₂ and IL-1 was assayed 24 h after LPS challenge (10 μ g/ml) using Eliza kits, Neogen Co., Lexington, KY and PerSeptive Diagnostics, Cambridge, MA, respectively.

Determination of LPC in Macrophages Lipid was extracted from macrophages according to the method of Bligh and Dyer²⁹⁾ and separated on TLC using CHCl₃-MeOH-H₂O (65:35:5 v/v) as a solvent. The area corresponding to the standard of LPC was scraped off and extracted three times with 2 ml of CHCl₃-MeOH (2:1). After the extracted LPC was dried under N₂ gas, LPC content was determined as phosphorous amount.³⁰⁾

Other Statistical significance was determined by Student's *t*-test and each value was given as the mean $\pm$ S.E.M.

RESULTS

The production of NO, PGE₂, and (IL-1) was determined in thioglycolate-elicited macrophages prepared from ICR mice fed a cholesterol-enriched diet for 2 weeks to determine the effect of hypercholesterolemia on macrophage function. Shosaikoto was orally administered at a dose of 1.2 g/kg/d. Figure 1 shows that the production of NO, PGE₂ and IL-1 after LPS challenge was markedly reduced by feeding of a cholesterol-enriched diet. On the other hand, although the serum cholesterol concentration between cholesterol-fed mice and those treated with Shosaikoto was not significantly different (245 and 240 mg/dl, respectively), the reduced production of NO and IL-1 was partially restored and that of PGE₂ was completely restored by Shosaikoto treatment. When the effect of the feeding duration of cholesterol-enriched diet on the suppression of NO production was evaluated, 1 week feeding was enough to diminish the response of macrophages to LPS, and Shosaikoto restored the di-

Fig. 1. Effect of Shosaikoto on the Production of (a) NO, (b) PGE₂ and (c) IL-1 by Macrophages Prepared from Mice Fed a Normal or Cholesterol-Enriched Diet for 2 Weeks in the Presence or Absence of Oral Shosaikoto Treatment at a Dose of 1.2 g/kg/d

□, normal group; ■, cholesterol group; ▨, Shosaikoto group; ▤, cholesterol/Shosaikoto group. Each point represents the mean $\pm$ S.E. of 6 mice. * *p* < 0.05, ** *p* < 0.01.

inished response dose-dependently (Fig. 2). The reduced production of NO in macrophages was noted during the feeding of cholesterol-enriched diet for 4 weeks and its restoration by Shosaikoto treatment was similarly observed for the entire period (data not shown). Oxidized LDL, which is considered to induce foam cells and to play a causal role in atherogenesis, is known to suppress NO synthesis in endothelial cells and macrophages. We therefore studied the effect of oxidized LDL and acetyl LDL as modified LDL on NO production by macrophages and the effect of oral administration of Shosaikoto. Macrophages that were prepared from normal or Shosaikoto-treated mice were incubated with oxidized LDL for 6 h and then with LPS for 24 h after being thoroughly washed with PBS. NO production in macrophages from hypercholesterolemic mice was reduced by oxidized LDL or acetyl LDL treatment, whereas Shosaikoto

treatment prevented its reduction (Fig. 3). The oxidative modification of LDL is associated with many changes in its chemical composition, such as degradation of apolipoprotein B-100, generation of oxysterols, and conversion of phosphatidylcholine to LPC. To analyze the substance in oxidized LDL or acetyl LDL to reduce NO production, we extracted lipids from both modified LDLs according to the method of Bligh and Dyer²⁹⁾ and then fractionated them by acetone. When an acetone insoluble fraction was added to macrophages, NO production was dose-dependently reduced, whereas its reduction was suppressed in macrophages prepared from mice treated with Shosaikoto (Fig. 4). Furthermore, LPC, which is known to show several atherogenic activities such as chemotactic activity for monocytes,³¹⁾ induction of cell adhesion molecules³²⁾ and mitogenic activity for macrophages,³³⁾ inhibited NO production dose-dependently (Fig. 5) and its inhibition was not due to its cytotoxic activity (data not shown). We measured LPC content in macrophages prepared from hypercholesterolemic mice

Fig. 2. Dose-Dependent Restoration of NO Production by Shosaikoto

Macrophages were prepared from mice fed a normal or cholesterol-enriched diet for 1 week in the presence or absence of oral Shosaikoto treatment at the dose indicated. ■, normal group; □, cholesterol-fed group. Each point represents the mean $\pm$ S.E. of 5 mice. ** $p < 0.01$. a) $p < 0.01$ vs. cholesterol group.

Fig. 3. Effect of Shosaikoto on NO Production by Macrophages That Were Treated with Modified-LDLs

Macrophages were prepared from mice fed a normal diet for 2 weeks in the presence or absence of oral Shosaikoto treatment at a dose of 1.2 g/kg/d. □, none; ■, native LDL; ▨, oxidized LDL; ▩, acetyl LDL. Values represent mean $\pm$ S.E. of 5 mice. ** $p < 0.01$ vs. native LDL. a) $p < 0.01$ vs. native LDL in normal group, b) $p < 0.01$ vs. oxidized LDL in normal group, c) $p < 0.05$ vs. acetyl LDL in normal group.

Fig. 4. Effect of Shosaikoto on NO Production by Macrophages Treated with Acetone Insoluble Fraction Extracted from Modified LDLs

Macrophages were prepared from mice fed a normal diet for 2 weeks in the presence or absence of oral Shosaikoto treatment at a dose of 1.2 g/kg/d. □, none; ■, native LDL; ▨, oxidized LDL; ▩, acetyl LDL. Values represent mean $\pm$ S.E. of 5 mice. ** $p < 0.01$ vs. native LDL. a) $p < 0.01$ vs. oxidized LDL in normal group, b) $p < 0.01$ vs. acetyl LDL in normal group.

Fig. 5. Effect of Shosaikoto on NO Production by Macrophages Treated with LPC

Macrophages were prepared from mice fed a normal diet for 2 weeks in the presence or absence of oral Shosaikoto treatment at a dose of 1.2 g/kg/d. ■, normal macrophages; □, macrophages from Shosaikoto-treated mice. Values represent mean $\pm$ S.E. of 5 mice. * $p < 0.05$ ($n = 5$).

Fig. 6. LPC Content in Macrophages

Macrophages were prepared from mice fed a normal or cholesterol-enriched diet for 2 weeks in the presence or absence of Shosaikoto treatment at a dose of 1.2 g/kg/d. Values represent mean $\pm$ S.E. of 4 mice. ** $p < 0.01$ ($n = 4$).

and those treated with Shosaikoto to learn whether the restorative action of Shosaikoto on NO production resulted from decreasing LPC content in macrophage. Hypercholesterolemic mouse macrophages contained a higher amount of LPC than normal macrophages (Fig. 6), however, there was no difference between the content of LPC in macrophages from hypercholesterolemic mice and those treated with Shosaikoto. This could indicate that the restorative activity of Shosaikoto was not due to the inhibition of LPC production. Although data is not shown here, the content of cholesterol and cholesteryl ester in peritoneal macrophages prepared from mice fed a cholesterol-enriched diet for 2 weeks was not significantly different from those in normal macrophages and no effect of Shosaikoto was observed.

DISCUSSION

In this study the function of macrophage was modified in hypercholesterolemic mice fed a cholesterol-enriched diet from an early age, as evidenced by the production of NO, PGE₂, and IL-1 as macrophage functions being reduced by the hypercholesterolemia. NO produced by endothelial cells would function as an anti-atherosclerotic substance,^{2,3)} whereas the implication of macrophage-producing NO in atherosclerosis has not yet been defined. However, NO scavenges superoxide anion, which is thought to impair endothelium and cause the contraction of blood vessels,²²⁾ and modulates the expression of adhesion molecules of various cells resulting in decreased adherence to endothelium. Furthermore, administration of a NOS inhibitor for a long period stimulates the development of atherosclerosis⁷⁾ and the administration of L-arginine, which is the substrate of NOS, suppresses the development of this condition.^{15,16)} These facts seem enough to consider that macrophage-derived NO may share in an anti-atherosclerotic action with endothelium-derived NO. On the other hand, PGE₂ shows a selective effect on cytokines, such as the inhibition of secretion of IL-2 and interferon- γ (IFN- γ) by T helper 1 cells and of IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) by T helper 1 and 2 cells without an effect on secretion of IL-4 and IL-5 by T helper 2 cells.³⁴⁾ PGE₂ also evokes T cell migration³⁵⁾ and protects CD4⁺CD8⁺ thymocytes from apoptosis,³⁶⁾ indicating that it is a diverse potent immunological mediator. Furthermore, IL-1 shows various biological activities, such as the activation of T and B lymphocytes,^{37,38)} and induction of acute phase proteins by hepatocytes.³⁹⁾ With regard to atherogenesis, IL-1 stimulates a monocyte and a leukocyte binding to endothelium by induction of the vascular cell adhesion molecule,⁴⁰⁾ increases endothelium procoagulant activity⁴¹⁾ and production of a plasminogen activator inhibitor,⁴²⁾ and stimulates cholesterol esterification.⁴³⁾ Although these various effects of PGE₂ and IL-1 are not directly correlated with the cause of development of atherogenesis, the balance of their activities would be critical to regulate immunity and atherogenicity. In other words, their decreased production presumably attenuates the immune response. Interferon- γ suppresses the expression of scavenger receptor of macrophages and the development of atherosclerosis in hypercholesterolemic rabbit fed a high cholesterol diet.^{44,45)} Cyclosporin A, an immunosuppressor, facilitates atherosclerosis in hypercholesterolemic mice,⁴⁶⁾ and immuno-deficient mice easily develop atherosclerosis by feeding of an atherogenic diet.⁴⁷⁾ These lines of evidence suggest that the suppression of immunity develops or aggravates atherosclerosis. In our study, hypercholesterolemia would reduce the production of PGE₂ and IL-1 in macrophages to lower immune response, resulting in the acceleration of atherosclerosis. The suppression of macrophage function was observed 1 week after cholesterol-enriched diet feeding, indicating that immune response and lipid metabolism concerned with macrophages may not function normally. If this suppression is brought about by oxidized LDL, or LPC contained in oxidized LDL, oxidation of LDL is thought

to occur at a very early time. Oxidized LDL is generally detected only in atheroma plaque or its adjacent area, but not in normal blood vessels.^{10,11} When the contents of cholesterol and cholesterylester in macrophages were measured, no difference was noted between normal mice and mice fed a cholesterol-enriched diet for 2 weeks, nor was there any effect of Shosaikoto. This probably excluded the possibility that peritoneal macrophages ingest oxidized LDL, which might be produced for a short time following the beginning of cholesterol feeding, and are modified by it. However, the content of LPC was markedly increased in mice fed a cholesterol-enriched diet, although no difference in content between control and Shosaikoto-treated groups was seen, indicating that macrophage function of mice in the cholesterol group may be suppressed by the increased LPC, but the restorative effect of Shosaikoto is not mediated by inhibition of this production. LPC is known to induce several endothelial genes expressed in early atherosclerosis, for example, vascular adhesion molecule-1, intercellular adhesion molecule-1, platelet-derived growth factor, and cyclooxygenase II,^{32,48,49} and these inductions seem likely to be mediated by protein kinase C,⁵⁰ elevated intracellular Ca^{2+} ,⁵¹ and uncoupling of cell surface receptors from G protein,⁵² suggesting that Shosaikoto may inhibit the signal transduction of LPC. At present, whether the LPC in macrophages prepared from mice fed a cholesterol-enriched diet is derived from oxidized LDL that was ingested *via* scavenger receptor or resulted from the activation of phospholipase A₂ by an unknown mechanism has not been definitely determined. Besides LPC, oxidized LDL contained oxysterols, lipidperoxides and degraded apo B. Among them, 7 β -hydroxycholesterol and 25-hydroxycholesterol as oxysterols show such strong biological activities as: inhibition of LDL receptor function,⁵³ activation of acyl-coenzyme A: cholesterol acyltransferase (ACAT),^{54,55} inhibition of prostacyclin⁵⁶ and suppression of lipoprotein lipase (LPL) expression.⁵⁷ However, in our study, the suppression of NO production in macrophages was detected by adding acetone insoluble lipid fraction which consists of phospholipids, but not acetone soluble fraction, indicating that the implication of oxysterol may be disregarded.

Conclusively, we have found that hypercholesterolemia markedly suppresses macrophage function, and also that oxidized LDL and LPC are candidates as its suppressors. Shosaikoto, which shows immunoregulatory and anti-atherosclerotic actions, prevents the reduction of macrophage function by hypercholesterolemia, oxidized LDL and LPC, suggesting that it maintains macrophage function to protect blood vessels from atherosclerosis.

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