Antihypercholesterolemic Action of a Traditional Chinese Medicine (Kampo Medicine), Ogi-Keishi-Gomotsu-To-Ka-Kojin

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The effect of Ogi-Keishi-Gomotsu-To-Ka-Kojin (OKKG), a traditional Chinese herbal medicine (Kampo medicine), on cholesterol metabolism was studied in male Sprague-Dawley rats. Intake of OKKG at doses of 1.38 g/kg for 4 weeks significantly reduced total cholesterol levels in the serum and liver of hypercholesterolemia rats fed a cholesterol-enriched diet. OKKG suppressed cholesterol absorption through the intestine and stimulated excretion of cholesterol into feces as bile acids. Biochemical study indicated that OKKG treatment enhanced cholesterol 7α-hydroxylase activity the rate limiting enzyme of cholic acid synthesis, in the liver without any effect on the rate limiting enzyme of cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Further, cholesterol-enriched diet containing cholic acid suppressed cholesterol 7α-hydroxylase activity, whereas OKKG administration reversed the suppression. In conclusion, these results supported the idea that OKKG may be an effective agent for treatment of patients with hypercholesterolemia.

Key words: Kampo medicine; hypercholesterolemia; cholesterol 7α-hydroxylase activity; cholic acid; cholesterol absorption; red ginseng

Atherosclerosis is a major cause of coronary arterial and cerebrovascular diseases, and hyperlipidemia, hypertension, diabetes mellitus and obesity are considered to be risk factors in the development of atherosclerosis. Patients with plural risk factors have a tendency to develop cardiovascular diseases easily. Accumulating evidence indicates that plural cells such as macrophages, T lymphocytes, smooth muscle cells and endothelial cells are involved in development of atherosclerosis.1,2 This evidence suggests that treatment other than reduction of serum cholesterol is required to prevent atherosclerosis. However, hypercholesterolemia is known to modify several cell functions, for example, attenuation of endothelial response to acetylsalicylic acid and suppression of endothelium-derived relaxing factor production by endothelial cells.3–5 When other factors such as local inflammation, oxidant stress, and immunosuppression exist in combination with hypercholesterolemia, the latter is always the first risk factor of atherosclerosis.6,7 The source of body cholesterol is dietary cholesterol absorbed in the small intestine and de novo synthesis in the liver and extrahepatic organs. Whole body cholesterol homeostasis is controlled by the supply and removal pathways. The liver is the only organ capable of excreting cholesterol, either by biliary secretion or by prior conversion of cholesterol into bile acid, and plays a crucial role in maintaining cholesterol homeostasis.8 Cholesterol homeostasis in the liver is regulated by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme of cholesterol synthesis, cholesterol 7α-hydroxylase, the rate limiting enzyme of bile acid synthesis, and receptor-mediated uptake of lipoproteins. When plasma cholesterol levels are increased, low density lipoprotein (LDL) elimination from blood through the hepatic LDL receptor is stimulated, resulting in the suppression of cholesterol synthesis from HMG-CoA reductase inhibition and the enhancement of cholesterol excretion from cholesterol 7α-hydroxylase stimulation. Modifying the enzyme activities related to cholesterol metabolism in liver in addition to absorption of cholesterol in the intestine is believed to be the best way to control body cholesterol. Ogi-Keishi-Gomotsu-To-Ka-Kojin (OKKG) is a Kampo medicine that is composed of six medicinal plants and has been used clinically to improve sensory disturbance of the limbs such as palsy and cold sensation, especially that originating from subacute myelo-opticneuropathy. In our attempt to determine its usefulness and pharmacological action, we first speculated that it might result from improving disorder of the circulatory system and the nervous system. In fact, we found in a previous study that OKKG showed antihypertriglyceridermic action against a hypertriglycerideremia rat model induced by giving 15% glycerol and 15% fructose solution. This resulted from the inhibition of triglyceride and phospholipid syntheses in the liver and lipoprotein lipase and hepatic triglyceride lipase activation.9 The pharmacological and biochemical effects have not been fully investigated, however, and in this study we therefore focused on cholesterol metabolism and studied the effect of OKKG on hypercholesterolemia.

MATERIALS AND METHODS

Animals Male Sprague-Dawley (SD) rats weighing 170—190 g were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). They were housed in a temperature-controlled room (at 23±1°C) with lighting from 7 a.m. to 7 p.m. The humidity was automatically maintained at 50—70%.

Chemicals [4,14C]Cholesterol (51.3 mCi/mmol) and [1,2-3H]cholesterol (50.0 Ci/mmol) were purchased from Dupont NEN Research Products (Boston, MA, U.S.A.). 7α-Hydroxycholesterol was from Steraloids Inc. (Wilton, NH, U.S.A.). Glucose-6-phosphate, NADP⁺, diithiothreitol (DTT), and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co.

Preparation of OKKG9 The crude drug composition of OKKG is described in Table 1. Plant materials used in this study, the grades of which conformed to Japanese pharmacopoeial standards, were authenticated and provided by Tsumura Co., Ltd., Tokyo, Japan. Six crude drugs were added to 700 ml of distilled water, boiled for 1 h using an
electric heater and concentrated to 300 ml. This decoction was filtered and lyophilized to give 7.5 g of powdered extract.

The main pharmacological components were astragalin, cinnamic aldehyde, paoniflorin, gingerol, and oleanolic acid. Ogi-Keishi-Go-Matsu-To (OKG) consists of the same crude drugs as OKG with the exception of red ginseng (RG). In this study, human daily doses of OKG and RG were 5.8 and 0.06 g.

**Hypercholesterolemia Rat Model**

Twenty five rats were randomized into five groups to evaluate the antihypercholesterolemic effect of the drug. Group 1 was fed a normal chow (CE-2, Nippon Clea Co., Ltd., Shizuoka, Japan). Group 2 was fed a cholesterol- and fat-enriched diet (C diet), which was CE-2 fortified with 1% cholesterol, 0.2% cholic acid and 2.5% olive oil. Groups 3, 4, and 5 received OKG, OKG, and RG, which were dissolved in 20 ml of water at doses of 1.38, 1.08, and 0.01 g/kg body weight/d, respectively, while also receiving the C diet. These diets and water were given ad libitum.

**Assay of Serum and Liver Total Cholesterol**

Rats were fasted for 16 h and blood was drawn from the hearts every 2 weeks. The serum samples were obtained by centrifugation at 15000 × g for 15 min and the livers were homogenized in phosphate-buffered saline at the end of experiment. Total lipids were extracted with chloroform/methanol (2:1 v/v) according to the method of Folch et al. Total cholesterol (TC) and free cholesterol (FC) levels of the serum and liver were determined by cholesterol oxidase method using an Iatron TC assay kit (Iatron Laboratory, Inc., Tokyo). Cholesterol ester (CE) was calculated from the value of (TC-FC).

**Measurement of Cholesterol Absorption**

Cholesterol absorption was studied using the dual isotope ratio method. Rats were administered OKG (1.38 g/kg body weight) for 2 weeks. After fasting for 16 h, [3H]cholesterol (2.2 μCi) suspended in 0.5 ml of 0.9% NaCl was injected into the femoral vein and [14C]cholesterol (2 μCi), which consisted of 6 mg of cholesterol dissolved in 2 ml of 156 mg of triolein and 7.5 mg of cholic acid in water, was orally administered simultaneously. After administration of radioactive cholesterol, the diet and water were resumed ad libitum. Twenty four, 48, and 96 h later, 0.4 ml of blood was drawn from the heart, added to 1 ml of 15% KOH/ethanol and then incubated at 80°C for 2 h. Sterol was extracted with petroleum ether three times and the extract was dried under nitrogen gas. [1H] and [14C]cholesterol were determined by liquid scintillation spectrometry.

**Measurement of Cholesterol Excretion into Feces**

Eight rats were randomly divided into 2 groups. One group was administered OKG (1.38 g/kg body weight) for 2 weeks and the other group was given water instead as control. On the last day, [4-14C]cholesterol (0.25 μCi per 100 g of body weight) was injected into the femoral vein and then feces were collected for 48 h and for another 48 h. An equal weight of feces and water were homogenized and refluxed in a 10-fold volume of ethanol for 12 h at 85°C using Soxhlet’s apparatus. An aliquot of the ethanol extract was used for determination of the total [14C] radioactivity. Another aliquot was evaporated, dissolved in a small amount of chloroform, and then applied to thin-layer chromatography (silica gel 60, Merck) using toluene–ethylacetate (2:3) as a developing solvent. The spot indicating cholesterol was scratched off after exposure to iodine vapor, and radioactivity was determined by liquid scintillation spectrometry. The excretory rate of total [14C] radioactivity and [14C]cholesterol into feces was calculated by the following formula: dpm in feces/dpm of [4-14C]cholesterol injected.

**Preparation of Rat Liver Microsomes**

Rats (6 weeks of age) were administered OKG (1.38 g/kg/d) for 2 weeks and killed by decapitation at 01:00 a.m. for HMG-CoA reductase and cholesterol 7α-hydroxylase assay, taking into account the diurnal rhythm of these enzymes. The livers were rapidly removed to ice-cold buffer containing 0.1 M sucrose, 50 mM KCl, 40 mM potassium phosphate (pH 7.4), 30 mM EDTA. After homogenization with four strokes, the homogenate was centrifuged at 700 × g for 5 min. The supernatant was then centrifuged at 12000 × g for 30 min. The microsomal fraction was obtained by centrifugation of 12000 × g supernatant fluid at 105000 × g for 1 h. The microsomal pellet was suspended in the homogenizing buffer containing 10 mM DTT and stored immediately at −80°C until use.

**Assay of HMG-CoA Reductase**

HMG-CoA reductase activity was assayed as described by Kuroda and Endo. The assay mixture contained 2.55 mM dl-[3-14C] HMG-CoA (144 MBq/mmol), 10 mM NADPH, 100 mM DTT, 100 mM EDTA-2Na, 0.5 mM potassium phosphate buffer (pH 7.4), and 50 μg of liver microsomal protein in a total volume of 40 μl. After the reaction mixture was prewarmed at 37°C for 3 min, the reaction was started by the addition of microsomal protein. Incubation followed at 37°C for 15 min with shaking, the reaction was terminated by addition of 10 μl of 2N HCl, and then the mixture was further incubated at 37°C for 15 min and applied to a silica gel plate (Merck). The reaction mixture was developed in benzene/aceton (1:1 v/v) and the spot of mevalonolactone (Rf: 0.5—0.7) was scratched directly into scintillation vials and [14C] mevalonolactone radioactivity measured by scintillation spectrometry. Activity was expressed in units of nmol mevalonolactone per min per mg protein.

**Assay of Cholesterol 7α-Hydroxylase Activity**

Liver microsomes containing 0.2—0.8 mg of protein were preincubated for 10 min at 37°C in a medium containing 50 mM potassium phosphate (pH 7.4), 2.5 mM DTT, 105 μM [4-14C] cholesterol and 1.5 mg/ml Tween 80. Assay was started by addition of a NADPH regenerating system containing 20 mM glucose 6-phosphate, 2 mM NADP, 4 mM MgCl2, and
1.4 IU glucose 6-phosphate dehydrogenase to a final volume of 1.0 ml. After incubation for 30 min at 37 °C in the dark, the reaction was stopped by addition of 10 ml chloroform/methanol (2:3) containing 0.01% butylated hydroxytoluene. Following mixing and centrifugation, the organic layer was washed with 2 ml of saline. The chloroform phase was evaporated under N₂ gas and spotted on a silica gel plate. Samples were developed in benzene/ethyl acetate (2:3) using 7α-hydroxycholesterol as a standard and the spot corresponding to 7α-hydroxycholesterol was visualized by fluorometry, BAS 2000 (Fuji Film Co., Japan). The radioactivity of 7α-hydroxycholesterol was counted using liquid scintillation spectrometry. Results are expressed as counts incorporated into 7α-hydroxycholesterol per min per mg of microsomal protein. Microsomal protein was determined according to the method of Lowry et al. using bovine serum albumin as a standard.

**Statistical Analysis** Results were represented as mean±S.E. with the number of animals or experiments in parentheses. Statistical analysis was performed by Tukey-Kramer's test on a Stat Light software program or Student's t-test. p values less than 0.05 were considered significant.

**RESULTS**

**Effect on Serum and Liver Cholesterol Levels in Cholesterol-Induced Hypercholesterolemia Rats** Feeding of C diet for 4 weeks led to about a 2-fold increase in serum TC (Fig.1). In TC, serum CE increased to 100 mg/dl but FC only to 10 mg/dl. Intake of OKGK at a dose of 1.38 g/kg, which corresponded to 10 times the human daily dose, markedly lowered serum TC and CE after 4 weeks of administration. In contrast, FC was significantly reduced 2 weeks after OKGK treatment. Panax ginseng, a crude drug of OKGK, was reported to show antihypercholesterolemic action against human, chicken and rat. Hence, to determine the contribution of RG to the antihypercholesterolemic action shown by OKGK, we determined the effect of OKGK, OKG, and RG on hypercholesterolemic rats. OKG and RG lowered serum TC, CE an FC in a similar way to OKGK, but their effects were less potent. In addition, C diet markedly increased liver TC at the end of 4 weeks administration (Fig. 2), while the administration of OKGK at a dose of 1.38 g/kg reduced liver TC most effectively among OKGK, OKG, and RG groups.

**Effect on Cholesterol Absorption in Rats** To identify the mechanism by which OKGK showed its antihypercholesterolemic effect, we investigated cholesterol absorption in rats. After fasting for 24 h, the daily absorbance of cholesterol was measured using the feces (24 h). The results showed that the absorbance was significantly reduced in the group treated with OKGK, while no significant changes were observed in the other groups.
terolemic action, cholesterol absorption in the intestine was studied using the dual isotope ratio method as described in Materials and Methods. Rats were administered OKGK at a dose of 1.38 g/kg for 2 weeks to examine the early effect of the medicine. Figure 3 shows the ratio of $^{13}$C to $^{3}$H cholesterol in plasma prepared from rats 24, 48, and 96 h after their administration. The ratio did not change in either control rats or OKGK-treated rats over 96 h, indicating that the rate of cholesterol exchange between cholesterol pools in the body was identical in the two groups. Under the conditions used, absorption of $^{13}$C cholesterol was significantly decreased in OKGK-treated rats by 33% as compared to control rats.

**Effect on Excretion of Intravenously Injected $^{[4,14]}$C-Cholesterol into Feces** Next, we examined the effect of OKGK on cholesterol excretion into feces. The weight of feces collected for 48 h and for another 48 h after $^{[4,14]}$C-cholesterol injection was not changed significantly by OKGK treatment (control rats: 11.0 g for first 48 h, 12.0 g for the second; OKGK-treated rats: 12.1 g for first 48 h, 12.9 g for the second). Total $^{14}$C radioactivity excreted into feces, which included free $^{14}$C cholesterol and $^{14}$C bile acids, was greatly increased by OKGK treatment (Table 2). This increase was about 1.7 fold relative to normal rats, whereas excreted $^{14}$C cholesterol was increased only about 1.3 fold, suggesting that OKGK enhanced bile acid synthesis, resulting in stimulation of cholesterol excretion. This stimulatory effect of OKGK on cholesterol excretion was observed for 96 h.

**Effect on Cholesterol 7α-Hydroxylase Activity** As cholesterol 7α-hydroxylase is the rate limiting enzyme in the bile acid synthesis pathway, we investigated the effect of OKGK on its activity. Intake of OKGK at a dose of 1.38 g/kg/d for 2 weeks significantly increased liver microsomal cholesterol 7α-hydroxylase activity (Fig. 4), suggesting the stimulation of bile acid synthesis. Cholesterol 7α-hydroxylase activity, however, was markedly suppressed by feeding of C diet which contains cholic acid that is reported to inhibit cholesterol 7α-hydroxylase activity. Administration of OKGK at a dose of 1.38 g/kg reversed this suppression and allowed a return to normal level (Fig. 5). Although data are not shown, HMG-CoA reductase activity in the liver was not influenced by OKGK treatment.

**DISCUSSION**

In this study, OKGK demonstrated anti hypercholesterolemic action by reducing serum and liver TC against rats fed a cholesterol- and fat-enriched diet. This cholesterol-reducing action is mainly due to the inhibition of cholesterol absorption in the intestine and the stimulation of cholesterol excretion from the liver. When enzymes responsible for cholesterol metabolism were studied, hepatic cholesterol 7α-hydroxylase activity was enhanced and HMG-CoA reductase activity was not altered in OKGK-treated rats. The liver is the key organ involved in adaptation to a high cholesterol diet, since it is not only the recipient of dietary cholesterol but also the major site through which cholesterol is eliminated from the body, either directly or after conversion to bile.
acids. Cholesterol 7α-hydroxylase is the rate-limiting enzyme in the bile acid biosynthesis pathway and is so far reported to be regulated by various factors including hormones, cytosolic factors, diurnal rhythm and phosphorylation/dephosphorylation in addition to a high level of hepatic cholesterol. Furthermore, bile acid biosynthesis is controlled by a negative feedback mechanism depending on the flux and composition of bile acids that undergo enterohepatic circulation. Rats infected with recombinant adenovirus carrying a gene encoding cholesterol 7α-hydroxylase transiently express the transgene at high levels in liver, and its overexpression is associated with a decline in total plasma and LDL cholesterol levels, indicating that cholesterol 7α-hydroxylase activity is closely linked to maintenance of cholesterol homeostasis. Intake of OKG increased cholesterol 7α-hydroxylase activity in normal rats, of which liver cholesterol is not affected by OKG, and further increased excretion of intravenously injected [4,14C]cholesterol into feces as bile acids. These results suggest that cholesterol 7α-hydroxylase activity enhanced by OKG may not be reflected by the change of liver cholesterol, and seem likely to result from modification of short-term regulation such as protein phosphorylation or of long-term regulation such as mRNA and protein induction.

The cholesterol-enriched diet used in this study contained 1% cholesterol, 0.2% cholic acid and 2.5% olive oil. Feeding of cholesterol in combination with a lipophilic bile acid like cholic acid is known to inhibit cholesterol 7α-hydroxylase activity in the liver, whereas hydrophobic bile acids such as ursodeoxycholate have the opposite effect. A recent study revealed identification of a bile acid response element in the cholesterol 7α-hydroxylase gene CYP7A, and found that both bile acid- and cholesterol-mediated regulation of cholesterol 7α-hydroxylase occurred at the transcriptional level in cultured hepatocytes and transgenic mice. OKG was found to reverse the suppression of cholesterol 7α-hydroxylase activity induced by cholesterol and fat-enriched diet. This result may suggest that OKG regulates cholesterol 7α-hydroxylase activity at the transcriptional level or that it changes the composition of the bile salt pool. Although HMG-CoA reductase is the rate-limiting enzyme for cholesterol synthesis, OKG did not show any inhibitory activity and the inhibition of cholesterol biosynthesis would not be involved in the cholesterol-lowering effect of OKG. Suppression of hepatic cholesterol synthesis is believed to be the primary response for increasing dietary cholesterol loads in most animal species. An additional compensatory mechanism is to down-regulate LDL receptor or to stimulate bile salt synthesis, and the latter is the case in the rat. In brief, OKG would stimulate the compensatory mechanism for adequate restoration of the cholesterol metabolism.

Panax ginseng was reported to show antihyperlipidemic action using rat and chicken hypercholesterolemia models. In the rat model, serum TC and TG were reduced 90% after oral administration at 1 g/kg of body weight, a dose much higher than that used in our study. Furthermore, they reported hepatic cholesterogenesis and lipogenesis were increased by ginseng administration. Serum TC and TG, however, were decreased in chicken model by ginseng root powder treatment, and further hepatic HMG-CoA and cholesterol 7α-hydroxylase activities were also lowered in the treatment group. Considering these reports together, although the antilipidemic action of ginseng was observed in different animal models, the mechanism by which it shows this action did not coincide among animal models or with that of OKG. In addition, OKG, which does not contain RG, showed similar antihypercholesterolemic activity to RG but not as strong as OKG, so that the action of OKG appeared to be in its combination with OKG and RG and not due only RG.

In this study, absorption of cholesterol through the intestine was also reduced by OKG treatment. Although plant sterol and saponins are known to inhibit cholesterol absorption in the intestine, the detailed mechanism whereby OKG reduces cholesterol absorption is under investigation.

In conclusion, OKG inhibited cholesterol absorption and stimulated cholesterol excretion by activating cholesterol 7α-hydroxylase, finally resulting in antihypercholesterolemic action in our model rats. In this respect, OKG would be an effective medicine to treat patients with hypercholesterolemia. Further detailed studies concerning the stimulatory activity of cholesterol 7α-hydroxylase, cholesterol excretion and the inhibitory activity of cholesterol absorption remain to be done.

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REFERENCES
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