Cholesterol Metabolism in Serum and Aorta of Inbred Mice Fed a High-Cholesterol Diet

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Abstract—Biochemical characterization of the serum and aorta in inbred C57BL/6Cr mice fed a high-cholesterol diet was investigated by determining the total cholesterol (TC) and free cholesterol (FC) levels in serum, high density lipoprotein (HDL) and aorta. Serum lecithin:cholesterol acyltransferase (LCAT) activity was also determined. A modified fluoroenzymatic method for microdetermination of cholesterol was successfully used. TC and FC levels of the aorta in the mice were significantly increased by the high-cholesterol diet. Serum TC and FC levels of mice fed the high-cholesterol diet were increased about 80% and 110%, respectively, compared with the control. On the other hand, both HDL-TC and HDL-FC levels were decreased about 50%. The HDL-TC/serum-TC ratio was markedly decreased, while the atherogenic index was markedly increased with the high-cholesterol diet. LCAT activity was also strikingly decreased. A positive correlation was observed between LCAT activity and HDL-cholesterol. These changes in the serum may facilitate cholesterol accumulation in the aorta. The results indicate that a biochemical approach using mice may be possible for drug evaluation.

Atherosclerosis has been induced in many animal species. However, there is no single convenient experimental model for primary screening of anti-atherosclerotic compounds. Variation in the incidence of atherosclerotic lesions makes drug evaluation difficult in practice. The genotype of the animal is known to be important in the control of serum cholesterol levels and the development of lipid deposits in the arterial wall (1–4). Wang et al. (5) have used C57 strain mice and induced atherosclerotic lesions by feeding them an atherogenic diet. Thompson (6) has found histologically that the male C57BL/6J inbred mouse is a more suitable animal to use for producing consistent atherosclerotic lesions in the wall of the aortic sinus.

This paper reports the biochemical examination of the aorta and serum in inbred C57BL/6Cr strain mice fed a high-cholesterol diet; a modified fluoroenzymatic method for microdetermination of tissue cholesterol was used. The relationship between high density lipoprotein (HDL)-cholesterol and lecithin: cholesterol acyltransferase (LCAT, EC 2.3.1.43) activity was also studied.

Materials and Methods

Animals: Six-week-old male C57BL/6Cr mice (Shizuoka Agricultural Cooperative Association for Laboratory Animals) were housed in wire net cages in an air-conditioned room (24±2°C, 60±10% humidity) and maintained on a purified basal diet or a high-cholesterol diet. The composition of the basal diet was 20% casein, 63.2% sucrose, 10% corn oil, 2% agar, 0.8% vitamin mixture (7) and 4% salt mixture (7). The high-cholesterol diet consisted of the basal diet supplemented with 5% cholesterol and 0.5% cholic acid. These supplements were added to the basal diet in place of an equal amount of sucrose. The basal and high-cholesterol diets were fed ad libitum for a period of 8 weeks. At the end of the feeding, the mice, after fasting for 12 hr, were sacrificed under
ether anesthesia by bleeding from the ophtalmic vessels, and then the aorta from the arch to the iliac bifurcation was excised and carefully freed of periaortal tissue. The aorta was freeze-dried to a constant weight and subsequently extracted at 50°C for 20 min with chloroform-methanol (2:1, v/v). The blood was centrifuged to obtain the serum. A sample of the serum (200 μl) was used for fractionation of HDL by the heparin-Mn²⁺ precipitation procedure (8).

Fluoroenzymatic microdetermination of cholesterol: Aorta cholesterol levels were determined by a modified method (9) originally developed by Carlson and Goldfarb (10) and Takano et al. (11). Aliquots (1/4–1/6) of the total aorta extract and standards, containing 0.5–5.0 μg cholesterol, were dissolved in acetone (100 μl) containing 2.5 mg of Triton X-100, and evaporated by a centrifugal evaporator (Yamato Scientific Co., Model RD-21). The residues of lipid and Triton X-100 were suspended in 200 μl of the enzymatic cholesterol reagent (Table 1), the contents vortexed and incubated at 37°C for 30 min. The blank consisted of 2.5 mg Triton X-100 and the enzymatic cholesterol reagent. Following incubation, 2.8 ml of 0.05 N NaOH was added to the reaction mixture, and the fluorescence of each solution was measured. Fluorescence spectra were obtained on a spectro-fluorophotometer (JASCO Model FP-550). The excitation wavelength was 323 nm, and the emission wavelength was 420 nm. For the determination of free cholesterol (FC) concentration, only cholesteryl ester hydrolase was removed from the enzymatic cholesterol reagent formulation.

Serum and HDL-cholesterol levels were determined as described above, except that the acetone solution of Triton X-100 was not added to each sample. Serum samples were diluted 1:20 with 0.1 M Na-Na phosphate buffer (pH 7.0). In order to prevent the inhibitory action of Mn²⁺ ion on the enzymatic reaction, HDL samples (10 μl) were added to 10 μl of 0.15 M disodium ethylenediaminetetraacetate (EDTA) solution. The enzymatic cholesterol reagent (200 μl) was then added directly to the diluted serum sample (20 μl) and the EDTA-treated HDL sample (20 μl). Standard solutions, containing 0.5–5.0 μg cholesterol, were made up in the phosphate buffer.

LCAT assay: The rate of FC esterification in the serum was assessed by measuring LCAT activity with a slight modification of the method of Dieplinger and Kostner (12). Freshly collected duplicate serum samples (20 μl) were pipetted into two test tubes, which were then closed with glass stoppers. The serum in one test tube was incubated at 37°C for 90 min and then the reaction was terminated by the addition of 10 μl of 0.3 M cholic acid solution. The cholic acid solution was added to the serum in the other test tube immediately before incubation. FC contents in the two test tubes were determined before and after 90 min incubation at 37°C as described above for the serum cholesterol assay. LCAT activity was shown by the difference in FC content between the two test tubes.

Chemicals: Cholesteryl ester hydrolase (EC 3.1.1.13) from Schizophyllum commune (2 U/mg) was purchased from Toyobo Co.; cholesterol oxidase (EC 1.1.3.6) from Nocardia sp. (0.9 U/mg) from Oriental Yeast Co.; horseradish peroxidase (EC 1.11.1.7) (100 U/mg) from Seikagaku Kogyo Co.; homovanillic acid, Triton X-100 and EDTA from Nakarai Chemicals; and the standard cholesterol solution (Precimat) from Boehr-

<table>
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<tr>
<th>Table 1. Composition of the enzymatic cholesterol reagent</th>
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<tbody>
<tr>
<td>Cholesteryl ester hydrolase</td>
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<tr>
<td>Cholesterol oxidase</td>
</tr>
<tr>
<td>Peroxidase</td>
</tr>
<tr>
<td>Homovanillic acid</td>
</tr>
<tr>
<td>Triton X-100</td>
</tr>
<tr>
<td>0.1 M Na-Na Phosphate buffer (pH 7.0)</td>
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</table>
inger Mannheim Co. Other chemicals were of analytical grade.

Statistics: Experimental data were compared statistically by Student's t-test. Correlations were calculated according to the least-squares method.

Results

Body weight: Changes in the body weight are shown in Fig. 1. The growth rate of mice was significantly suppressed by feeding a high-cholesterol diet, but all of them survived. This suppression of the growth rate may be due to the anorectic effect of cholic acid, which was added to the diet to facilitate the absorption of cholesterol from the intestine and to obtain higher hypercholesterolemia. In preliminary studies, the mice refused to eat the high-cholesterol diet when the content of cholic acid in the diet was increased to 1–2%.

Serum cholesterol level: The results given in Table 2 show that the serum TC level increased about 80% in mice fed the high-cholesterol diet in comparison with the basal diet control. FC level was also increased about 110%. As a consequence, the ester ratio (the ratio of esterified cholesterol (EC) to TC) was slightly, but significantly decreased in the high-cholesterol diet group.

HDL-cholesterol level: The high-cholesterol diet group showed about 50% decrease in both HDL-TC and HDL-FC levels in comparison with the control (Table 2). Consequently, there was no major difference in the ester ratio of HDL-cholesterol. The ratio of HDL-TC to serum TC (HDL-TC/serum-TC) was remarkably decreased, while the atherogenic index ((serum-TC-HDL-TC)/HDL-TC) was remarkably increased in the high-cholesterol diet mice.

LCAT activity: LCAT activities were markedly decreased in mice fed the high-cholesterol diet compared to the control animals. The fractional LCAT activity (% esterified/hr) and molar LCAT activity (μmole esterified/l/hr) decreased 61% and 44%, respectively (Fig. 2). The fractional LCAT activity was positively correlated to the HDL-FC level (r=0.916, P<0.01), but negatively

Table 2. Serum cholesterol and high density lipoprotein (HDL)-cholesterol levels of inbred C57BL/6Cr mice fed the high-cholesterol diet

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Basal diet (mg/100 ml)</th>
<th>High-cholesterol diet (mg/100 ml)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum TC</td>
<td>175±4</td>
<td>317±16</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Serum FC</td>
<td>36.1±0.9</td>
<td>76.4±4.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ester ratio (%)</td>
<td>79.4±0.3</td>
<td>75.9±0.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL-TC (mg/100 ml)</td>
<td>101.8±2.4</td>
<td>48.3±3.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL-FC (mg/100 ml)</td>
<td>25.2±0.9</td>
<td>11.8±1.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ester ratio (%)</td>
<td>75.2±0.3</td>
<td>75.8±0.7</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-TC/serum-TC</td>
<td>0.581±0.011</td>
<td>0.154±0.016</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Atherogenic index</td>
<td>0.731±0.033</td>
<td>5.78±1.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

TC: Total cholesterol, FC: Free cholesterol, EC: Esterified cholesterol. Ester ratio: EC/TC×100. Atherogenic index: (Serum TC−HDL-TC)/HDL-TC. Each value represents the mean±S.E. of 10 animals. NS means no statistical significance.
correlated to the serum FC level \((r = -0.950, P < 0.01)\). The correlation between the fractional LCAT activity and HDL-FC level is shown in Fig. 3. The molar LCAT activity was also positively correlated to the HDL-FC level \((r = 0.843, P < 0.01)\).

Aorta cholesterol level: As shown in Table 3, the TC level of the aorta of mice was significantly increased by the high-cholesterol diet. The FC level was also significantly increased, while there was no difference in the EC level.

**Table 3. Aortic cholesterol levels of inbred C57BL/6Cr mice fed the high-cholesterol diet**

<table>
<thead>
<tr>
<th>Aorta</th>
<th>Dietary group</th>
<th>(\text{mg/g dry weight})</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal diet</td>
<td>High-cholesterol diet</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>7.39±0.16</td>
<td>8.18±0.13</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FC</td>
<td>6.54±0.15</td>
<td>7.12±0.13</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>EC</td>
<td>0.85±0.12</td>
<td>1.06±0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

TC: Total cholesterol, FC: Free cholesterol, EC: Esterified cholesterol. Each value represents the mean ±S.E. of 10 animals. NS means no statistical significance.

**Fig. 2.** Serum lecithin:cholesterol acyltransferase (LCAT) activities of inbred C57BL/6Cr mice fed the high-cholesterol diet. B: Basal diet group, HC: High-cholesterol diet group. Each column represents the mean±S.E. of 10 animals.

**Fig. 3.** Correlation between fractional LCAT activity and HDL-free cholesterol level. The solid line represents the linear regression equation obtained by least-squares analysis. \(r = \text{correlation coefficient}.\)

Discussion

Atherosclerosis has been studied in mice (4–6, 13), but little information is available on drug evaluation of anti-atherosclerotic compounds. Male inbred C57BL/6J mice are very good animals with which to study the production of dietary atheromata because they all develop atheromata relatively quickly and at approx. the same time (6). Thus, we used the same strain C57BL/6Cr mice to try to biochemically characterize the development of lipid deposits in the aorta of mice fed a high-cholesterol diet. We were successful using the fluoroenzymatic method (9) for the microdetermination of tissue cholesterol. This technique enables easy, sensitive and specific measurements of cholesterol in limited amounts of tissue such as in the mouse aorta (9). This is the first report of TC and FC concentration determi-
nations with mouse aorta.

In the present study, hypercholesterolemia and consistent but relatively gradual cholesterol accumulation in the aorta were observed in mice fed the high-cholesterol diet (Tables 2 and 3). C57BL/6J inbred mice show atheromatous lesions in the limited region of the aorta (valve sinus) (6). Therefore, cholesterol concentration in the total aorta might not increase much. Elevated concentration of serum cholesterol seems to facilitate lipid deposition in the arterial wall of the inbred mouse (4, 5). To produce more severe hypercholesterolemia than in this study, additional treatment such as prolongation of the feeding period, supplementation of an antithyroid drug, or alteration of the diet composition should be further examined.

The mechanism of dietary cholesterol accumulation in the aorta is not sufficiently understood. Recent studies of lipoproteins have shown that most of the cholesterol in the arterial wall is derived from the low density lipoprotein (LDL), while removal of cholesterol from the arterial wall occurs by transfer of FC to the HDL (14-16). Most of the FC entering the HDL is esterified by the serum LCAT. LCAT functions in connection with the HDL and seems to participate in the transfer of cholesterol between tissues and lipoproteins (16, 17). An elevated serum level of LDL and very low density lipoprotein (VLDL) and/or low concentration of HDL have been considered to increase the accumulation of the cholesterol in the arterial wall (18, 19). In the present study, LDL-TC plus VLDL-TC levels (considered as the difference between serum TC and HDL-TC) were increased, while the HDL-TC level was decreased in the mice fed the high-cholesterol diet. Consequently, the atherogenic index was markedly increased, and the HDL-TC/serum-TC ratio was markedly decreased (Table 2). These changes in the lipoprotein metabolism of mice appear to facilitate the cholesterol accumulation in the aorta.

The LCAT activity was remarkably reduced by the cholesterol feeding (Fig. 2). The LCAT activity was also positively correlated with HDL-cholesterol level (Fig. 3), and there was no major difference in the ester ratio of HDL-TC, suggesting that low activity of LCAT may be accompanied by a low HDL level. However, the possibility that dietary cholic acid may inhibit the LCAT activity in serum cannot be excluded, and further studies should be conducted to obtain more extensive data.

Our findings indicate that this biochemical approach using mice may be useful for evaluating anti-atherosclerotic agents, although further study is required of the diet composition and feeding time prolongation as a means of increasing cholesterol accumulation in the aorta.

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