Comparison of atherosclerotic lesions and HDL-lipid levels in male, female, and testosterone-treated female mice from strains C57BL/6, BALB/c, and C3H

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Summary

In order to determine whether male and female mice differed in HDL-lipid levels or in atherosclerotic response to a high fat diet, we examined 3 inbred strains which differed in susceptibility to atherosclerosis; C57BL/6, BALB/c, and C3H. Mice were fed normal chow or an atherogenic diet containing 1.25% cholesterol, 15% fat, and 0.5% cholic acid. Lesion number and size were determined after 14 weeks on the diet; plasma HDL-lipid levels were determined at 0 and 4 weeks on the diet.

For C3H, the most atherosclerosis-resistant strain, HDL-lipid levels were very high and not affected by sex or diet. For BALB/c, HDL-lipid levels were intermediate between the other two strains. Male levels were significantly higher than the females, and the atherogenic diet caused a drop in HDL-lipid levels of 14–27% depending on sex. For C57BL/6, the most atherosclerosis-susceptible strain, HDL-lipid levels were low compared to the other two strains. Males and females on normal chow did not differ in HDL-lipid, but females showed a 50% decrease in HDL when fed the atherogenic diet. For both BALB/c and C57BL/6, testosterone-treated females resembled the males.

The HDL-lipid levels in mice on atherogenic diet differed over a 3-fold range among the nine groups. When HDL-lipid levels were compared to the number of atherosclerotic lesions or the total lesion area, a high degree of correlation was observed ($r = -0.95$ for lesion number and $-0.93$ for total lesion area). This suggests that HDL-lipid levels are important in determining atherosclerosis susceptibility in mice.

Key words: Atherosclerosis; Female; HDL-lipids, levels; Male; Mice; Strains, C57BL/6. BALB/c, C3H; Testosterone
**Introduction**

In the human population, males have a much higher prevalence of coronary heart disease than females [1]. Epidemiological studies show an association of a higher prevalence of heart disease with reduced high density lipoprotein (HDL)-cholesterol levels [2–4] and adult men do have lower levels of HDL cholesterol (HDL-C) or lower ratios of HDL-C to total cholesterol [4,5]. Whether this reduced HDL-C is due to different lifestyle of males or due to endogenous hormones is not known. However, testosterone itself does not appear to be associated with the lowered HDL-cholesterol typical of men; indeed the opposite appears to be true. Within the male population, higher levels of testosterone are associated with higher HDL-C levels and reduced risk of heart disease [6–11].

Previously there were two indications that male mice might differ from female mice in atherosclerosis susceptibility and HDL-lipid levels. When the effect of 3-methylcholanthrene on aortic lesion formation in mice fed an atherogenic diet was examined, male mice had fewer lesions and lesions of smaller size than females across all treatment groups although only one such difference reached statistical significance [12]. Later, in a cross between atherosclerosis susceptible strain C57BL/6 and atherosclerosis-resistant strain BALB/c, we examined progeny of both sexes and found that males had higher HDL-lipid levels than female progeny fed an atherogenic diet (Mitchell, Holmes and Paigen, unpublished data).

In order to further explore the male and female difference in HDL-lipid levels and atherosclerosis susceptibility, we examined mice of three inbred strains. Strain C57BL/6 is atherosclerosis-susceptible and strains BALB/c and C3H are atherosclerosis-resistant [13]. The difference between the resistant strains and C57BL/6 is due to a single major gene, Ath-I (Paigen et al., unpublished data). Further work with the two resistant strains revealed a slight difference between them in that C3H is more resistant to lesion formation in aorta and coronary arteries than is BALB/c (unpublished data). We examined male and female mice of all three strains. In order to test the effect of testosterone more directly, we also examined female mice treated with testosterone. We asked the following questions. Do male mice have more HDL-lipid than females? If so, is this true for both atherosclerosis-susceptible and atherosclerosis-resistant strains? Are males less likely to form aortic lesions than females or to have lesions of smaller size? If males differ from females, are the differences decreased when females are treated with testosterone?

**Materials and methods**

**Chemicals and diet**

Chemicals were obtained as follows: electrophoretic grade agarose from BioRad Laboratories, Richmond, CA; sodium barbital buffer from Sigma Chemical Co., St. Louis, MO; Precilip normal lipid control from Boehringer Mannheim Diagnostics, Houston, TX; and GelBond plastic support medium from FMC Corp., Marine Colloids Div., Rockland, ME. Normal chow was Purina chow containing 4% fat and atherogenic diet with concentrations of 15% fat, 1.25% cholesterol and 0.5% cholic acid was described earlier [13]. Testosterone pellets were a gift of Kurt Pfister, University of Zurich, Zurich, Switzerland.

**Animals**

Mice 2-4 months of age from strains C57BL/6J, C3H/HeJ, and BALB/cJ were obtained from a colony at the University of California, Berkeley, CA. These mice are derived from Jackson Laboratory stock (Bar Harbor, ME) and mice are no more than two generations removed from Jackson stock. The mice were housed in a temperature-controlled facility with a 12 h light and dark cycle.

**Experimental design**

Blood samples were obtained before beginning the high-fat diet (Day 0) and after 4 weeks of diet. Changes in HDL-lipid levels are stable from 3 to 14 weeks on atherogenic diet (Fig. 1), so 4 weeks was chosen as the standard protocol for determining Ath-I phenotype by HDL levels in our laboratory. The mice were fed atherogenic diet for 14 weeks and killed after an overnight fast. Hearts and the first portion of the aorta were removed, washed in physiological saline, fixed in formaline-saline solution and evaluated for atherosclerotic lesions.
Testosterone treatment of female mice

Testosterone was administered in 3 mm diameter pellets containing 30 mg testosterone implanted subcutaneously in the area of loose skin behind the ears. One testosterone pellet was administered 4 weeks before Day 0 and a second at 6 weeks after Day 0.

Evaluation of aortas for lesions

The formalin-fixed hearts and aortas were embedded in gelatin, sectioned on a cryostat, and stained with oil-red-O as described earlier [12,13]. Aortic wall lesions were evaluated in 5 sections from each mouse taken at 80-μm intervals, beginning with the area identified by the presence of the valve cusps and an aorta that was round in shape. The number of lesions and the area occupied by each lesion was measured in each section. The area was evaluated using a grid inserted into the microscope eyepiece (20 × 20 micrometer disc #478, AO Scientific Instruments, Buffalo, NY). The length of a lesion and the average thickness were determined and multiplied to obtain a cross-sectional area in μm². Hearts were coded so that the scoring was done blindly as to identity of the heart, and one person performed all the scoring. Results are reported as the mean number of lesions per section (number of lesions/number of sections) and as the mean area occupied by lesions per section. This last value was obtained by summing the areas of each individual lesion in each section.

Determination of plasma HDL and cholesterol

Plasma was obtained from blood samples containing Na-EDTA as anticoagulant (final EDTA concentration approximately 1 mM). Plasma samples were stored at 4°C for no longer than 1 week before analysis. HDL was determined after the LDL and VLDL (low density- and very low density lipoproteins) had been precipitated out with an equal volume of polyethylene glycol 8000 solution (200 g/l, pH 10) [14]. Agarose gel electrophoresis of the HDL-containing supernatants was performed using the method of Noble et al. [15]. The following modifications to the method were employed. The supernatants were diluted with a 40% glycerol/10 mM EDTA solution (4 parts supernatant and 1 part solution). The resulting mixture was kept at 4°C until analyzed (up to 5 days). A 1.1% solution of agarose in 0.025 M sodium barbital, pH 8.6 was layered on GelBond plastic support medium and 4 × 1.5 mm wells made with a slab gel comb. Electrophoresis was carried out in a submarine gel apparatus in 0.025 M sodium barbital at 20 mA for 40–50 min. The gels were fixed in 5% glacial acetic acid/60% ethanol solution for at least 60 min. After fixation gels were stained in a 0.1% solution of Sudan black in 60% ethanol overnight, destained in 60% ethanol, and dried.

HDL peaks were measured by densitometer. The amount of HDL per sample was expressed as a percentage of a human plasma standard, Precilip, included in each agarose gel. Thus a HDL-lipid value of 100 is approximately equal to the HDL in average human plasma. Total cholesterol and HDL-cholesterol after precipitation of VLDL and LDL was determined by the method of Rudel and Morris [16].

![Graph](image-url)

Fig. 1. HDL-lipid levels in female C57BL/6 mice fed atherogenic diet for 14 weeks.

The number of mice at each time point was 5 except for the group at 0 weeks which had 16 animals. Mean and standard error bars are depicted. HDL-lipid levels are expressed relative to Precilip, a human plasma standard. HDL-lipid levels in strains C3H and BALB/c were stable over the 14 weeks (data not shown).
Results

**Total cholesterol and HDL cholesterol levels**

Female mice from each of the 3 strains were evaluated for total cholesterol and HDL-cholesterol after precipitation of VLDL and LDL. The total cholesterol levels did not differ greatly among the 3 strains either for chow-fed (range 66–93 mg/dl) or atherogenic diet-fed (range 181–207 mg/dl) animals (Table 1). Likewise HDL-cholesterol did not differ among the 3 strains in chow-fed mice (range 62–69 mg/dl). However, HDL-cholesterol did differ considerably in animals fed an atherogenic diet for 4 weeks. The atherosclerosis-susceptible strains C57BL/6 showed a drop in HDL-cholesterol levels to 39 mg/dl, but the atherosclerosis-resistant strains showed little change. The combined LDL and VLDL cholesterol, as determined by subtraction of HDL from total cholesterol, did not show much difference among the strains.

In subsequent experiments, HDL-lipid levels were determined by agarose gel electrophoresis. Chemical determination of cholesterol levels requires killing of the animal to obtain sufficient plasma, but agarose gels can be done on small quantities of plasma that can be obtained without killing animals.

**TABLE 1**

<table>
<thead>
<tr>
<th>Cholesterol pool</th>
<th>Diet</th>
<th>C57BL/6 (mg/dl ± SD)</th>
<th>BALB/c (mg/dl ± SD)</th>
<th>C3H (mg/dl ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>Chow</td>
<td>66 ± 14</td>
<td>93 ± 10</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>Total</td>
<td>Atherogenic</td>
<td>192 ± 26</td>
<td>181 ± 10</td>
<td>207 ± 32</td>
</tr>
<tr>
<td>HDL</td>
<td>Chow</td>
<td>62 ± 9</td>
<td>69 ± 14</td>
<td>69 ± 13</td>
</tr>
<tr>
<td>HDL</td>
<td>Atherogenic</td>
<td>39 ± 7</td>
<td>64 ± 8</td>
<td>69 ± 9</td>
</tr>
<tr>
<td>LDL, VLDL</td>
<td>Chow</td>
<td>4</td>
<td>15</td>
<td>24</td>
</tr>
<tr>
<td>LDL, VLDL</td>
<td>Atherogenic</td>
<td>153</td>
<td>117</td>
<td>138</td>
</tr>
</tbody>
</table>

*Each value is the mean of 3 female mice fed chow or atherogenic diet for 4 weeks as described in Methods.*

**HDL-lipid levels**

The HDL-lipid levels of male, female, and testosterone-treated females for all three strains of mice are shown in Fig. 2. The first question is whether males differ from females, and if they do, whether testosterone-treatment of females caused the HDL-lipid levels to resemble the male more closely. The answer to this question is different for each strain. For C57BL/6 mice on normal chow, males, females, and testosterone-treated females are quite similar. However, on atherogenic diet, the pattern is different. Males and testosterone-treated females have levels of HDL-lipid that are higher than females (P < 0.0001) primarily because female HDL-lipid levels have dropped by 50%. For BALB/c, males have significantly higher levels of HDL-lipid than females regardless of diet. Testosterone-treated females are significantly higher than females for both diets, but not quite as high as males. For strain C3H, no difference between males and females was observed regardless of diet.

The three strains also show a pattern of increasing HDL-lipid levels consistent with previous work on susceptibility to lesion formation [13]. For, females on normal chow, the levels of HDL-lipid are 202, 228, and 310 for C57BL/6, BALB/c, and C3H, respectively. For females on an atherogenic diet, the HDL-lipid levels are 101, 174, and 290, respectively. This raises the question as to whether any correlation exists between the HDL-lipid levels and the susceptibility to aortic lesions. In order to

![Fig. 2. Comparison of HDL-lipid levels in male, females, and testosterone-treated females fed normal chow or an atherogenic diet.](image-url)

HDL-lipid levels are expressed relative to Precilip, a human plasma standard. Mean and standard error bars are depicted. The number of animals in each group was between 9 and 12 except for 16 animals in the C57BL/6 females fed an atherogenic diet.
TABLE 2
RELATIVE HDL-LIPID LEVELS AND ATHEROSCLEROTIC LESIONS IN MICE FED AN ATHEROGENIC DIET

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Relative a HDL levels</th>
<th>n b</th>
<th>Number of c lesions</th>
<th>Area involved d in lesions (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>female</td>
<td>0.50</td>
<td>80</td>
<td>0.61 ± 0.09</td>
<td>0.66 ± 0.14</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>female + test.</td>
<td>0.85</td>
<td>35</td>
<td>0.34 ± 0.14</td>
<td>0.23 ± 0.10</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>male</td>
<td>0.89</td>
<td>50</td>
<td>0.27 ± 0.03</td>
<td>0.29 ± 0.06</td>
</tr>
<tr>
<td>BALB/c</td>
<td>female</td>
<td>0.86</td>
<td>45</td>
<td>0.44 ± 0.07</td>
<td>0.47 ± 0.12</td>
</tr>
<tr>
<td>BALB/c</td>
<td>female + test.</td>
<td>1.13</td>
<td>40</td>
<td>0.18 ± 0.06</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>BALB/c</td>
<td>male</td>
<td>1.21</td>
<td>30</td>
<td>0.23 ± 0.08</td>
<td>0.16 ± 0.06</td>
</tr>
<tr>
<td>C3H</td>
<td>female</td>
<td>1.29</td>
<td>25</td>
<td>0.07 ± 0.04</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>C3H</td>
<td>female + test.</td>
<td>1.30</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C3H</td>
<td>male</td>
<td>1.52</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Relative to 202, the HDL lipid level of C57BL/6 females fed normal chow.
b n = number of aortic cross-sections examined: 5 sections examined for each mouse.
c Expressed as number of lesions/aortic cross-section.
d Cross-sectional area of each lesion added to give total.

For BALB/c, the pattern was somewhat different. Males had higher HDL-lipid levels regardless of diet, and testosterone-treated females had HDL-lipid levels that were about 1.8-fold higher than the females. These higher levels were associated with a reduction in atherosclerotic lesions. This observation is consistent with an earlier report [12] showing that males had fewer lesions and reduced lesion size in another atherosclerosis-susceptible strain, AKXL-38a. In contrast, the atherosclerosis-resistant strain C3H had high levels of HDL-lipid that were not affected by sex or testosterone treatment.
atherosclerosis-resistant [13] and both differ in atherosclerosis-susceptibility from C57BL/6 due to a single major gene which maps on chromosome 1 in the mouse (unpublished data). In spite of the fact that BALB/c and C3H apparently share the same major gene for atherosclerosis-resistance, our data are consistent with the hypothesis that BALB/c has some other modifying genes which make it less resistant to lesion formation than C3H. In earlier work, we showed that neither strain had aortic lesions after 14 weeks on the atherogenic diet [13], but by 8 months, BALB/c did have aortic lesions as well as lesions in the coronary arteries (unpublished data). In this report, BALB/c has HDL-lipid levels and lesion formation that are intermediate between C57BL/6 and C3H.

Thus the answers to the questions we started with are not simple. Male mice do have more HDL-lipid than females but only in some strains and on some diets. When males differ from females, then testosterone-treatment does cause female mice to have HDL-lipid levels that resemble the male more closely. We also questioned whether male mice were less likely to get lesions than female mice. What we found was that HDL-lipid level is highly correlated with lesion formation and that HDL-lipid level can be affected by genetics or sex.

The finding that HDL-lipid levels are higher for males than females of some strains appears at first to be contrary to the human data. However, the discrepancy is more apparent than real. For mice, males had higher HDL-lipid levels than females for 3 of 6 conditions (3 strains on 2 diets). In the human population, male HDL-C levels average higher than females but considerable variation occurs. Furthermore, lifestyles of males differ on the average from lifestyles of females and, in the past, included a higher prevalence of HDL-cholesterol-lowering behavior such as cigarette smoking. Among children there is either no sex difference in HDL-cholesterol [1,17] or males have higher levels [1,2,18]. What is more relevant is that among adult males, a higher level of testosterone is associated with higher HDL-cholesterol [6-11]. In one report showing that higher testosterone levels are associated with higher HDL-C levels in adult men, the authors present 5 models for the relationship [6]. If the mouse is an appropriate model, the data in this study rule out 3 of the 5 models leaving only the models that testosterone increases HDL-C or that testosterone affects factor x which then increases HDL-C.

Probably the most interesting observation is the close correlation between HDL-lipid levels and lesion formation. Whether the HDL-lipid levels were altered by diet, sex, or hormone treatment, the HDL-lipid level correlated closely with lesion formation. This suggests that more than an association may exist and lends support to the hypothesis that the relationship is causal.

Acknowledgements

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