Probucol Does Not Affect Lipoprotein Metabolism in Macrophages of Watanabe Heritable Hyperlipidemic Rabbits

Yutaka Nagano, Toru Kita, Masayuki Yokode, Kenji Ishii, Noriaki Kume, Hideo Otani, Hidenori Arai, and Chuichi Kawai

We recently reported that the antioxidant action of probucol inhibited the progression of atherosclerosis in Watanabe heritable hyperlipidemic (WHHL) rabbits. In this study, we investigated another possible action of probucol: Its action as an antiatherogenic agent on macrophages. When WHHL rabbit peritoneal macrophages were pre-incubated in vitro with probucol and then incubated with several atherogenic lipoproteins, the incorporation of the lipoproteins was not significantly prevented. In the case of mouse peritoneal macrophages, pre-incubation with probucol showed slight, although not statistically significant, changes in the amount of lipoprotein incorporations. We also used macrophages obtained from mice and WHHL rabbits fed with probucol, but the amount of uptake of lipoproteins by these cells was not less than that by control macrophages. Furthermore, to investigate the incorporation of atherogenic lipoproteins into these cells, we prepared probucol-containing macrophages; however, probucol in macrophages failed to prevent the uptake of such lipoproteins. In conclusion, probucol did not prevent foam cell transformation of macrophages of WHHL rabbit or mice directly, and the effect of probucol against atherogenesis in WHHL rabbits was due mainly to its inhibitory effect on the oxidative modification of low density lipoprotein, as previously reported. (Arteriosclerosis 9:453-461, July/August 1989)

One of the characteristic events in the early stages of atherogenesis is the accumulation of lipid-laden "foam cells" in the subendothelial space. Many foam cells in these lesions preserve the characteristics of macrophages. Therefore, macrophages are supposed to be the progenitor of certain foam cells that are involved in atherogenesis. Today it is known that, in vitro, macrophages ingest large amounts of certain chemically modified lipoproteins, such as acetylated low density lipoprotein (acetyl LDL) or malondialdehyde-treated LDL, through the process of receptor-mediated endocytosis, designated the acetyl LDL receptor or the scavenger receptor. Recently, the presence of malondialdehyde-altered protein in the atheroma of Watanabe heritable hyperlipidemic (WHHL) rabbits was reported, suggesting the involvement of lipid peroxides in atherogenesis.

Probucol is a drug widely used in the treatment of hypercholesterolemia, and Parthasarathy et al. proved by both in vitro and ex vivo experiments that probucol acted as an antioxidant on LDL. We discovered that probucol could prevent the progression of atherosclerosis in vivo in WHHL rabbits. This strongly suggested the in vivo action of probucol as an antioxidant because LDL from probucol-fed WHHL rabbits was resistant to the oxidative modification. Carew et al. also reported the antiatherogenic effect of probucol in WHHL rabbits.

However, there may exist a mechanism of probucol action (other than as an antioxidant) that can explain the dramatic antiatherogenic effect. Therefore, in this study, we examined one such possibility, paying special attention to the interaction of probucol and macrophages by in vitro and ex vivo experiments, because macrophages play an important role with lipoproteins in atherogenesis. We used peritoneal macrophages obtained from WHHL rabbits, the very animals in which we proved the antiatherogenic effect of probucol.

Methods

Animals

Homozygous WHHL rabbits were raised in Kyoto by mating heterozygous or homozygous female WHHL rabbits with homozygous male WHHL rabbits. Japanese White rabbits and female DDY mice (25 to 30 g) were purchased from Shimizu Laboratories (Kyoto, Japan). The mice were fed mouse laboratory chow MF (Oriental Yeast...
Company, Tokyo, Japan). The rabbits were fed rabbit laboratory chow RM-1 (Oriental Yeast Company).

**Materials**

1-14C-oleic acid (57.0 mCi/mmol) and cholesteryl-
1,2,6,7-3H-N-oleate (75.3 Ci/mmol) were obtained from
New England Nuclear (Boston, MA). Bovine serum albumin
(BSA) was from Armour Pharmaceutical Company
(Kankakee, IL). 1-14C-oleic acid-BSA complex was pre-
pared as described.18 Fetal calf serum (FCS), obtained from
Flow Laboratories (Mclean, VA), was inactivated at
56°C for 30 minutes before use. Lactalbumin hydrolysate
was purchased from GIBCO Laboratories (Chagrin Falls,
OH). L-Glutamine and streptomycin-penicillin solutions
were from Flow Laboratories (North Ryde, N.S.W., Aus-
tralia). Dulbecco's Modified Eagle's Medium (DMEM) and
Dulbecco's phosphate-buffered saline (PBS) were pur-
chased from Nissui Pharmaceutical Company, Limited
(Tokyo, Japan). Plastic Petri dishes were obtained from
Nunc (Roskilde, Denmark), and liquid paraffin oil was from
Nakarai Chemical Company (Kyoto, Japan). Other chem-
icals were reagent grade.

**Preparation of Probucol Diet**

Pure probucol powder kindly supplied by Daiichi Phar-
aceutical Company Limited (Tokyo, Japan) was simply
added to the raw animal chow at the rate of 1% (wt/wt).
No organic solvent was used. Then the chow was ground
to the appropriate size for the animals.

**Administration of Probucol**

Six WHHL rabbits (ages 2 to 4 months in the probucol
treatment group, Group P) were started on 100 g/day of
chow containing 1% (wt/wt) of probucol. Six control WHHL
rabbits (ages 2 to 4 months, Group C) were started on the
same amount of standard rabbit chow. One month later,
we checked the plasma concentrations of probucol in the
Group P rabbits. The value was 98.9±22.7 µg/ml
(mean±SD), which already exceeded the level that we
previously reported after 6 months of probucol feeding
(42.9±8.0 µg/ml).11 Because most probucol in plasma
exists in particles of lipoproteins,14 it was expected that
probucol had already exerted enough influence on the
LDL of these rabbits by this time. Based on these obser-
vations, LDL prepared from the blood of WHHL rabbits fed
probucol for more than 1 month was used as the probucol-
treated LDL. For the experiments, we used LDL prepared
from both groups of WHHL rabbits before they were 10
months old.

In some experiments, we used macrophages obtained
from probucol-fed animals. To obtain peritoneal macroph-
geces from probucol-fed WHHL rabbits, we fed the
rabbits the probucol diet more than 1 month, considering
the plasma concentration of probucol as stated above. We
also obtained peritoneal macrophages from probucol-fed
mice. For this purpose, mice ages 6 to 7 weeks were
started on mouse chow enriched with 1% probucol (wt/wt).
Before obtaining the macrophages, we measured the
concentrations of plasma cholesterol and probucol of
mice at 2, 4, and 8 weeks after beginning the diet, and we
determined the sufficient period of probucol feeding for
the mice. Because the amount of plasma obtained from
one mouse was too small for all the experiments, and
because macrophages obtained from mice were gathered
in one tube and then dispersed onto dishes, we pooled the
plasma of 10 mice and used this to measure the concen-
trations of probucol and plasma lipids. The mice con-
sumed an unrestricted amount of chow.

**Preparation of Macrophage Monolayers**

Peritoneal macrophages from a WHHL rabbit were
prepared by the method described by Ishii et al.16 First,
liquid paraffin oil (40 ml/rabbit) was injected into the
peritoneal cavity of a WHHL rabbit. Four days later, cells
were harvested by peritoneal lavage with 500 ml of saline
under anesthesia with pentobarbital. The cells were col-
rlected by centrifugation (1500 rpm for 10 minutes at 4°C),
were washed once with 30 ml of saline, and were resus-
pended in DMEM containing 0.2% (wt/vol) lactalbumin
hydrolysate,16 penicillin (100 U/ml), and streptomycin
(100 µg/ml) at a final concentration of 2×10^6 cells/ml.
Aliquots (1 ml) of this cell suspension were dispersed onto
plastic Petri dishes (35 × 10 mm) and were incubated in a
humidified CO2 (5%) incubator at 37°C for 2 hours. Then
each dish was washed twice with 2 ml of DMEM to
to remove nonadherent cells. After the monolayers were
incubated for 18 hours at 37°C with 1 ml of DMEM
containing 0.2% lactalbumin hydrolysate in the incubator,
the cells were washed twice with 2 ml of DMEM and
then used for the experiments.

In the mice, peritoneal cells harvested from unstimu-
lated mice were pooled in PBS as described by Kita et
al.17 The incubation of cells was performed with the same
procedure as described above for rabbit peritoneal macro-
phages except that the population of cells dispersed per
dish was 3×10^5, and 10% FCS (vol/vol) was used instead
of 0.2% lactalbumin hydrolysate.

In some experiments, macrophages were brought into
contact with probucol during the 18 hours of pre-
icubation time. In these cases, 5 µl of the stock solution
of probucol (1 or 10 mg/ml dissolved in ethanol) was
added to 1 ml of DMEM containing 10% FCS (final
concentration, 5 or 50 µg/ml, respectively) and this was
incubated for 18 hours, then washed twice with 2 ml of
DMEM, and used for the experiments. Pre-incubation time
was 18 hours because a previous study of the in vitro
effects of probucol18 had shown that probucol had a
significant effect on macrophages within 12 hours of
incubation. The viability of the cells was checked after
pre-incubation by using trypan blue dye. More than 99% of
the cells in each dish excluded the pigment regardless of
whether probucol or ethanol was present in the pre-
icubation medium.

**Probucol Concentration**

When we used macrophages from probucol-fed ani-
mals or from control animals incubated with probucol-
containing lipoproteins, the concentrations of probucol in
macrophages were also measured. For this purpose,
macrophages incubated under each condition were col-
llected by a rubber policeman (Costar, Cambridge, MA,
Catalog No. 3010), were centrifuged at 1500 rpm for
10 minutes, and were dissolved with 0.2 N NaOH. An aliquot of this solution was used to determine the probucol concentrations in macrophages. The amount of probucol in saline used for peritoneal lavage and that in the media used to cultivate macrophages of probucol-fed animals were also measured.

To measure the concentration of probucol, the lipid fraction was extracted from samples with ethanol and diethyl ether, then dissolved with methanol, and analyzed with high-performance liquid chromatography.

**Lipoproteins**

To take LDL from the WHHL rabbits, blood was obtained from ear arteries of WHHL rabbits in both groups with ethylenediamine tetraacetic acid (EDTA) disodium as an anticoagulant. LDL (d=1.019 to 1.063) was isolated from plasma by ultracentrifugation and was dialyzed against two changes of at least a 500-fold volume of 150 mM NaCl containing 0.24 mM EDTA (pH 7.4). LDL was also isolated from the plasma of normal Japanese White rabbits as described earlier. Acetyl LDL was prepared from the LDL of healthy human subjects or from both groups of WHHL rabbits by the method described by Basu et al. For the oxidation of LDL, 150 μg of LDL dialyzed with PBS was suspended in 1 ml of PBS containing 0.5 μM CuSO₄ and was incubated at 37°C for 24 hours in a CO₂ incubator. Beta-very low density lipoprotein (β-VLDL) was isolated as the d<1.006 fraction of a Japanese White rabbit fasted overnight after being fed a 2% cholesterol and 10% corn-oil diet for more than 14 days.

**Assays of Cholesterol Reacylation**

To initiate the experiment, 0.6 ml of DMEM and 27 μg of lipoprotein were added to the prepared macrophage monolayers (final concentration, 45 μg/ml). This was incubated in a humidified CO₂ incubator (5%) at 37°C. We followed the method described by Brown et al. except that the serum was omitted from the incubation medium. Lactalbumin hydrolysate was not added to the medium of WHHL rabbit macrophages during the incubation time. Cellular cholesteryl esters were isolated by thin-layer chromatography, and the cholesteryl ¹⁴C-oleate content was determined by liquid scintillation counting with ³H-cholesteryl oleate as an internal standard. The data were obtained by averaging duplicate incubations.

**Oil Red O Staining**

Monolayers of peritoneal macrophages were prepared on a Slide Flask (Nunc, Roskilde, Denmark, Catalogue No. 170920) and were incubated with lipoproteins in DMEM containing 10% FCS for 24 hours. Then the cells were dried, fixed with 6% formalin for 5 minutes, stained with oil red O for 60 minutes, and counterstained with Meyer’s hematoxylin for 10 minutes.

**Other Measurements**

Serum levels of cholesterol were measured with an enzymatic method with a Choles Color Ace kit from the Ono Pharmaceutical Company, Limited (Osaka, Japan). The cholesterol levels in the subfractions of lipoprotein were measured after they had been isolated by ultracentrifugation.

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**Table 1. Plasma Levels of Total Cholesterol, Its Subfractions, Triglyceride, and Producol before and 4 Months after Start of Probucol Feeding**

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Before treatment</th>
<th>At 4 months</th>
<th>Producol group</th>
<th>Before treatment</th>
<th>At 4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>TChol</td>
<td>594±87</td>
<td>599±174</td>
<td>546±70</td>
<td>557±74</td>
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<tr>
<td>HDL-C</td>
<td>20±4</td>
<td>11±2</td>
<td>20±6</td>
<td>6±2</td>
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<td></td>
</tr>
<tr>
<td>LDL-C</td>
<td>317±82</td>
<td>439±153</td>
<td>298±49</td>
<td>439±93</td>
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<td></td>
</tr>
<tr>
<td>VLDL-C</td>
<td>256±37</td>
<td>149±132</td>
<td>229±69</td>
<td>112±85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>860±144</td>
<td>461±201</td>
<td>803±318</td>
<td>483±274</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Producol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>74.1±8.3</td>
</tr>
</tbody>
</table>

Values are mg/dl, means±SD, except for Producol values, which are μg/ml.

TChol = total cholesterol, HDL-C = high density lipoprotein cholesterol, LDL-C = low density lipoprotein cholesterol, VLDL-C = very low density lipoprotein cholesterol, TG = triglyceride, ND = not detectable.

The protein content of lipoproteins and macrophages was determined by the method described by Lowry et al. with BSA as a standard. In the case of macrophages, the cells in the 35 × 10 mm dish were dissolved with 0.2 N NaOH in advance, and an aliquot of this solution was used to determine the protein content.

The values shown represent the means±SD, and statistical significance was determined using Student’s t-test. When the p value was less than 0.01, the difference was considered significant.

**Results**

Table 1 shows the mean levels of cholesterol and probucol in the plasma of WHHL rabbits before and 4 months after probucol treatment. There was no significant reduction of plasma cholesterol levels even in Group P WHHL rabbits although the subfractions of cholesterol levels changed in both groups.

Before examining the interaction of probucol and macrophages, we ascertained that the LDL obtained from Group P rabbits (P-LDL) were resistant to oxidative modification compared with the LDL obtained from Group C rabbits (C-LDL); that is, copper-treated C-LDL showed increased electrornobility on agarose gel electrophoresis and fragmentation of apo B-100 on NaDodSO₄ gel electrophoresis; however, copper-treated P-LDL did not show these changes. Consequently and in contrast to C-LDL, P-LDL did not cause foam cell transformation of WHHL rabbit macrophages even after it was incubated with 0.5 μM cupric ion (data not shown).

To evaluate the direct effect of probucol on peritoneal macrophages, we pre-incubated WHHL rabbit macrophages with probucol in vitro for 18 hours, then incubated them with ¹⁴C-oleate-BSA complex in DMEM containing β-VLDL, acetyl LDL, or oxidized LDL, which was modified from LDL of normal rabbits by incubation with 0.5 μM cupric ion for 24 hours. Regardless of the pre-incubation with probucol, these lipoproteins stimulated cholesteryl ester accumulation in WHHL rabbit macrophages as shown in Figure 1. Statistically significant differences were not observed. When the same experiment was
performed with mouse peritoneal macrophages, the uptake of \( \beta \)-VLDL by macrophages was slightly reduced, and that of acetylated LDL and oxidized LDL showed a tendency to increase although none of these were statistically significant (Figure 2). We performed two preliminary experiments whereby macrophages were co-incubated with probucol during both pre-incubation and incubation time or during incubation time. The results of the two preliminary experiments showed the same tendency as the experiments in which co-incubation was performed during the pre-incubation time (data not shown).

We also used peritoneal macrophages taken from WHHL rabbits fed with probucol for more than 1 month to evaluate the in vivo effect of probucol on macrophages. We found that the amount of cholesteryl ester synthesis stimulated by several lipoproteins in the macrophages of probucol-fed WHHL rabbits was not less than that of macrophages of control WHHL rabbits. Neither change was statistically significant. In other words, probucol in vivo did not prevent the incorporation of several lipoproteins in the macrophages (Figure 3).

The same experiment was performed with macrophages obtained from mice fed with probucol. Beforehand, we determined the adequate period of probucol feeding for mice. In Table 2, the mean plasma concentrations of cholesterol and probucol in probucol-fed mice are shown. The plasma levels of cholesterol dropped significantly only 2 weeks after the start of the feeding, and no further decrease was observed during the next 6 weeks. The plasma concentration of probucol showed similar levels at 2, 4, and 8 weeks after feeding was begun. Therefore, in this experiment we used the macrophages of mice fed probucol for more than 2 weeks.

![Figure 1](http://atvb.ahajournals.org/content/9/4/456F1)

**Figure 1.** Effect of in vitro addition of probucol on cholesteryl ester formation in peritoneal macrophages of Watanabe heritable hyperlipidemic (WHHL) rabbits by several lipoproteins. Macrophages were brought into contact with ethanol or probucol dissolved in ethanol during an 18-hour pre-incubation. The cells were washed twice with 2 ml of Dulbecco’s Modified Eagle’s Medium (DMEM). To each monolayer (2 \( \times \) 10^6 cells), 0.6 ml of DMEM containing 0.2 mM \( ^{14} \text{C}-\text{oleate-albumin} \), and several lipoproteins (45 \( \mu \)g/ml) were added, and the cells were incubated for 6 hours at 37°C. The amount of cholesteryl \( ^{14} \text{C}-\text{oleate} \) formed in cells pre-inoculated under each condition was reported as a percentage of that formed in cells that underwent control pre-incubation. The mean values of cholesteryl ester formation in control WHHL rabbit macrophages by acetylated low density lipoprotein (acetylated LDL), \( \beta \)-very low density lipoprotein (\( \beta \)-VLDL), and oxidized LDL were 22.9, 12.1, and 8.4 nmol/mg protein, respectively; these data are shown as 100% in the figures. The letters at the bottom represent the conditions of pre-incubation: C=control pre-incubation, E=5 \( \mu \)l ethanol added to 1 ml of medium, S=5 \( \mu \)l of ethanol solution of probucol (1 mg/ml) added to 1 ml of medium (final concentration of probucol: 5 \( \mu \)g/ml), 50=5 \( \mu \)l of ethanol solution of probucol (10 mg/ml) added to 1 ml of medium (final concentration of probucol: 50 \( \mu \)g/ml).

![Figure 2](http://atvb.ahajournals.org/content/9/4/456F2)

**Figure 2.** Effect of in vitro addition of probucol on cholesteryl ester formation in peritoneal macrophages of mice by several lipoproteins. Macrophages were brought into contact with ethanol or probucol dissolved in ethanol during an 18-hour pre-incubation. The cells were washed twice with 2 ml of Dulbecco’s Modified Eagle’s Medium (DMEM). To each monolayer (3 \( \times \) 10^6 cells), 0.6 ml of DMEM containing 0.2 mM \( ^{14} \text{C}-\text{oleate-albumin} \), and several lipoproteins (45 \( \mu \)g/ml) were added, and the cells were incubated for 6 hours at 37°C. The amount of cholesteryl \( ^{14} \text{C}-\text{oleate} \) formed in cells pre-inoculated under each condition was reported as a percentage of that formed in cells that underwent control pre-incubation. The mean values of cholesteryl ester formation in control mouse peritoneal macrophages by acetylated low density lipoprotein (acetylated LDL), beta-very low density lipoprotein (\( \beta \)-VLDL), and oxidized LDL were 50.6, 39.9, and 4.9 nmol/mg protein, respectively; these data are shown as 100% in the figure. See the legend for Figure 1 for an explanation of the abbreviations.

![Figure 3](http://atvb.ahajournals.org/content/9/4/456F3)

**Figure 3.** Effect of several lipoproteins on cholesteryl ester formation in macrophages obtained from probucol-treated Watanabe heritable hyperlipidemic (WHHL) rabbits. Rabbits were fed a probucol diet (1%, wt/wt) for more than 1 month. To each monolayer (2 \( \times \) 10^6 cells), 0.6 ml of Dulbecco’s Modified Eagle’s Medium (DMEM) containing 0.2 mM \( ^{14} \text{C}-\text{oleate-albumin} \), and several lipoproteins (45 \( \mu \)g/ml) were added; this was incubated at 37°C for 6 hours. The values of cholesteryl \( ^{14} \text{C}-\text{oleate} \) formation in probucol-fed WHHL rabbit macrophages are given as a percentage of those in control WHHL rabbit macrophages. The mean values of cholesteryl ester formation in these experiments with acetylated low density lipoprotein (acetylated LDL), beta-very low density lipoprotein (\( \beta \)-VLDL), and oxidized LDL were 19.5, 13.4, and 7.7 nmol/mg protein, respectively, in control macrophages. These data are reported as 100% in the figure. C=control WHHL rabbit, P=probucol-fed WHHL rabbit.
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Table 2. Effect of Probucol Feeding on Plasma Levels of Cholesterol and Probucol in Mice

<table>
<thead>
<tr>
<th>Duration of treatment</th>
<th>TC (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
<th>LDL-C (mg/dl)</th>
<th>VLDL-C (mg/dl)</th>
<th>Probucol (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>123</td>
<td>82</td>
<td>29</td>
<td>12</td>
<td>ND</td>
</tr>
<tr>
<td>2 weeks</td>
<td>39</td>
<td>21</td>
<td>10</td>
<td>8</td>
<td>11.1</td>
</tr>
<tr>
<td>4 weeks</td>
<td>40</td>
<td>19</td>
<td>9</td>
<td>12</td>
<td>9.5</td>
</tr>
<tr>
<td>8 weeks</td>
<td>46</td>
<td>20</td>
<td>11</td>
<td>15</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Values are mg/dl except probucol, which is µg/ml. The data were obtained from plasma pooled from 10 mice. Consequently, the mean values of the 10 mice in each group are shown. Abbreviations are given in the footnote to Table 1.

Figure 4. Effect of several lipoproteins on cholesteryl ester formation in macrophages obtained from probucol-treated mice. Mice were fed a probucol diet (1%, wt/wt) for more than 2 weeks. To each monolayer (3x10⁶ cells), 0.6 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing 0.2 mM [14C]-oleate-albumin, and several lipoproteins (45 µg/ml) were added and incubated at 37°C for 6 hours. The values of cholesteryl [14C]-oleate formation in probucol-fed mouse macrophages are given as a percentage of those in control mouse macrophages. The mean values of cholesteryl ester formation in these experiments by acetylated low density lipoprotein (acetyl LDL), beta-very low density lipoprotein (β-VLDL), and oxidized LDL were 47.0, 32.1, and 3.9 nmol/mg protein, respectively, in control macrophages. These data are reported as 100% in the figure. C=control mouse, P=probucol-fed mouse.

However, Figure 4 shows that probucol feeding did not prevent the foam cell transformation of mouse macrophages.

Figure 5 shows that acetyl LDL modified from P-LDL (acetyl P-LDL) was significantly incorporated by mouse peritoneal macrophages, showing saturation kinetics. The amount of cholesteryl ester synthesis in macrophages stimulated by acetyl P-LDL was almost the same as acetyl LDL modified from C-LDL (acetyl C-LDL). Figures 6A and 6B show oil red O-stained macrophages transformed into foam cells by acetyl P-LDL and acetyl C-LDL. The amounts of intracellular lipid droplets in these macrophages were similar. In contrast, no lipid droplets were stained in macrophages incubated with C-LDL (Figure 6C).

Based on these results, we performed the experiment shown in Table 3. Mouse peritoneal macrophages were pre-incubated with acetyl P-LDL or acetyl C-LDL for 18 hours. Then the cells were washed completely and incubated for 30 hours with either acetyl LDL modified from human LDL, rabbit β-VLDL, or oxidized LDL. We measured the intracellular concentration of probucol after 18 hours of pre-incubation and after another 30 hours of incubation. We found that probucol existed in macrophages that were pre-incubated with acetyl P-LDL throughout the incubation time. The amounts of cholesteryl ester formation stimulated by both acetyl C-LDL and acetyl P-LDL during the pre-incubation time (18 hours) were similar. In addition, further incorporation of human acetyl LDL, rabbit β-VLDL, and oxidized LDL occurred during the 30 hours of incubation time regardless of the lipoprotein used for the pre-incubation, and the amount of the uptake of several lipoproteins during the incubation time by macrophages pre-incubated with acetyl P-LDL did not differ from that by macrophages pre-incubated with acetyl C-LDL. In other words, probucol did not inhibit the incorporation of several lipoproteins even when it had been incorporated into macrophages in advance.

Discussion

Recently we11 and Carew et al.12 have ascertained that probucol prevented the progression of atherosclerosis in WHHL rabbits without marked reduction of plasma cholesterol levels in vivo. It has been suggested that peroxidized lipids are closely related to the initiation and progression of atheromatous plaque formation.25,26,27 In addition, cell-modified (oxidized) LDL in which acyl chains were peroxidized has reportedly transformed macrophages into foam cells in vitro.28,29,30 Foam cells have been recognized as a characteristic feature in the initial stage of atherosclerosis.1 As previously reported, LDL obtained from patients treated with probucol were resistant against oxidative modification in vitro,10 and we confirmed this in LDL obtained from WHHL rabbits.11
Figure 6. Light microscopic appearances of mouse peritoneal macrophages incubated with acetylated low density lipoproteins (acetyl LDL) modified from LDL of probucol-treated Watanabe heritable hyperlipidemic (WHHL) rabbit (A), acetyl LDL modified from LDL of control WHHL rabbit (B), and LDL obtained from control WHHL rabbit (C). Aliquots of lipoprotein (45 μg/ml) were added to monolayers of macrophages and incubated for 24 hours in a CO₂ incubator at 37°C. Staining was performed with oil red O and Meyer's hematoxylin as described in the text. ×970
whether the uptake of atherogenic lipoproteins, such as WHHL rabbits with probucol in vitro, then examined used probucol in several ways in our experiments. To investigate this, we transformation into foam cells. To investigate this, we from WHHL rabbits to investigate the possibility that probucol could act on macrophages and prevent their

Many investigators are interested in whether probucol prevents atherogenesis with a mechanism other than as an antioxidant. In the present study, we used materials from WHHL rabbits to investigate the possibility that probucol could act on macrophages and prevent their transformation into foam cells. To investigate this, we used probucol in several ways in our experiments.

First, we pre-incubated the peritoneal macrophages of WHHL rabbits with probucol in vitro, then examined whether the uptake of atherogenic lipoproteins, such as acetyl LDL, β-VLDL, or oxidized LDL, could be inhibited. However, as shown in Figure 1, there was no statistically significant difference between the control and probucol-treated macrophages in the mean amount of cholesteryl ester synthesis stimulated by acetyl LDL, β-VLDL, and oxidized LDL.

Table 3. Effect of Cell-associated Probucol on Incorporation of Lipoprotein

<table>
<thead>
<tr>
<th>Lipoprotein used for 18-hour pre-incubation</th>
<th>Acetyl C-LDL</th>
<th>Acetyl P-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 18 hours of pre-incubation</td>
<td>43±3</td>
<td>54±10</td>
</tr>
<tr>
<td>After additional 30 hours of incubation with</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl LDL</td>
<td>363±24</td>
<td>333±23</td>
</tr>
<tr>
<td>β-VLDL</td>
<td>95±13</td>
<td>104±24</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>115±13</td>
<td>120±21</td>
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</tbody>
</table>

Monolayers of mouse peritoneal macrophages (3×10⁶ cells) were incubated in 0.6 ml of Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal calf serum (FCS), 0.2 mM ¹⁴C-oleate-albumin, and 100 μg/ml of acetylated low density lipoprotein (acetyl LDL) modified from LDL obtained from control Watanabe heritable hyperlipidemic (WHHL) rabbits (C-LDL) (acetyl C-LDL) or from LDL obtained from probucol-treated WHHL rabbits (P-LDL) (acetyl P-LDL) for 16 hours. Cells in some dishes were harvested at this time to measure the amount of cholesteryl ¹⁴C-oleate formed. Other cells were washed twice with 2 ml of DMEM and received 0.6 ml of fresh DMEM containing 10% FCS and 0.2 mM ¹⁴C-oleate albumin. Then 100 μg/ml of acetyl LDL modified from LDL of healthy human subjects, beta-very low density lipoprotein (β-VLDL) obtained from rabbits fed with a 2% cholesterol/10% com oil-enriched diet, or oxidized LDL modified from rabbit LDL was added, and this was incubated for 30 hours. Probucol concentrations in macrophages were measured as follows: 3×10⁶ cells were dispersed onto plastic Petri dishes (100×20 mm) and were incubated with 6 ml of DMEM containing 10% FCS for the same period as for cells incubated to measure the cholesteryl ester formation. At the end of the incubation time, the cells were harvested, centrifuged, and dissolved with 0.2 N NaOH to measure protein contents and concentrations of probucol in macrophages. Probucol concentrations in macrophages pretreated with acetyl P-LDL were 0.37 μg/mg protein at the end of 18 hours pre-incubation and 0.4, 0.36, and 0.38 μg/mg protein after an additional 30 hours of incubation with acetyl LDL, β-VLDL, or oxidized LDL, respectively.

Table 1 shows that the plasma levels of total cholesterol did not decrease as expected after 4 months of probucol. We do not know the reason for the lack of cholesterol lowering in WHHL rabbits, but recent reports showed the same tendency.²¹²² This strongly suggests that the antiatherogenic action of probucol is caused by a mechanism different from its cholesterol-lowering effect. The changes in the values of cholesterol substractions might be due to a decrease in triglycerides. According to Watanabe,²³ the plasma levels of triglyceride in WHHL rabbits drop without drugs as they get older. This may have caused the decrease in triglyceride-rich VLDL and the relative increase of LDL in our study.

Many investigators are interested in whether probucol prevents atherogenesis with a mechanism other than as an antioxidant. In the present study, we used materials from WHHL rabbits to investigate the possibility that probucol could act on macrophages and prevent their transformation into foam cells. To investigate this, we used probucol in several ways in our experiments.

First, we pre-incubated the peritoneal macrophages of WHHL rabbits with probucol in vitro, then examined whether the uptake of atherogenic lipoproteins, such as acetyl LDL, β-VLDL, or oxidized LDL, could be inhibited. However, as shown in Figure 1, there was no statistically significant difference between the control and probucol-treated macrophages in the mean amount of cholesteryl ester synthesis stimulated by acetyl LDL, β-VLDL, and oxidized LDL.

Yamamoto et al.¹⁸ have reported that in vitro addition of probucol into culture medium prevented the foam cell transformation of human macrophage-like cells (UE-12) by acetyl LDL to at least a half level of control incubation. However, we could not confirm such a drastic change in our experimental system. It is possible that the different results were caused by the different species of cells used. We used cells from WHHL rabbits because it was in this animal that probucol has been definitely effective on the prevention of atherogenesis. So we regard this study as a counterpart of our previous report.¹¹ As shown in Figure 2, the addition of probucol to mouse peritoneal macrophages in vitro caused a response that was slightly different from the case of WHHL rabbit macrophages, although the changes were not significant.

We also tried the pre-incubation with a higher concentration level (200 μg/ml) of probucol, the level at which Yamamoto et al. proved the effect of probucol on macrophages. However, this nonphysiological concentration of probucol caused no significant inhibition or stimulation of lipoprotein uptake by macrophages (data not shown). This can be explained by the fact that probucol is very hydrophobic, so an excessive amount of probucol is not soluble in the culture medium and could not exert any further influence.

Because most probucol in plasma does not exist in free form but mainly in the particle of lipoproteins,¹⁴ it cannot be expected that probucol in vivo acts on macrophages in the same manner as in the in vitro experiments. For this reason, we investigated the possibility that macrophages obtained from probucol-fed animals might have been affected by probucol in vivo by mechanisms different from those in the in vitro experiments described above.

To make this point clear, we performed experiments with macrophages obtained from probucol-fed WHHL rabbits and mice. The concentration of probucol was detectable in the saline used for peritoneal lavage of probucol-fed WHHL rabbit (0.2 μg/ml). This suggested that these macrophages had contacts with probucol in vivo, although the concentration of probucol in macrophages themselves was not detectable by our system. As stated previously, oxidized LDL is the most likely atherogenic lipoprotein that exists in vivo and is thought to play the most important role in the atherogenesis of WHHL rabbits. However, foam cell transformation of macrophages by oxidized LDL was not affected by probucol feeding, although the behavior of cholesteryl ester synthesis in WHHL macrophages by other lipoproteins was slightly different, as shown in Figure 3. Figure 4 also revealed no in vivo effect of probucol on mouse peritoneal macrophages.

Furthermore, we carried out experiments using macrophages into which probucol was definitely incorporated. First, we pre-incubated mouse peritoneal macrophages with acetyl P-LDL, which contained probucol with the concentration of 10.3 μg/mg protein. Figure 5 shows that...
acetyl P-LDL was significantly incorporated into macrophages, although P-LDL, which contained probucol with the concentration of 9.1 µg/mg protein, was hardly incorporated. Figure 6A shows the macrophages transformed into foam cells by incubation with acetyl P-LDL. Foam cell transformation was observed in the cells of Figure 6A to the same degree as those of Figure 6B, which were incubated with acetyl C-LDL. In contrast, the cells in Figure 6C, which were incubated with C-LDL, were virtually devoid of lipid droplets. The probucol concentration in macrophages incubated with acetyl P-LDL was 0.37 µg/mg cellular protein, but probucol was not detectable in macrophages incubated with P-LDL, in spite of the similar levels of probucol concentrations in the medium. Thus, we confirmed that probucol was incorporated into macrophages as a component of acetyl P-LDL during the pre-incubation. Then we washed the medium completely and added acetyl LDL modified from human LDL, rabbit β-VLDL, or oxidized LDL in renewed medium and continued the incubation. The results in Table 3 show that, even when macrophages contain probucol, they show additional incorporation of atherogenic lipoproteins. There was no significant difference in the amount of this additional incorporation between macrophages pre-incubated with acetyl P-LDL and those pre-incubated with acetyl C-LDL, which was devoid of probucol. We also performed the same experiments with WHHL rabbit macrophages. The mean values of additional cholesteryl ester synthesis stimulated by acetyl LDL, β-VLDL, and oxidized LDL in WHHL rabbit macrophages pre-incubated with acetyl C-LDL were 127.9, 68.1, and 48.3 nmol/mg protein; those in macrophages pre-incubated with acetyl P-LDL were 121.4, 69.3, and 45.2 nmol/mg protein, respectively. These data indicated that probucol did not have an effect on macrophages for the prevention of foam cell transformation even when it was definitely incorporated into macrophages.

Our data in the studies above prove that probucol did not significantly prevent macrophages from transforming into foam cells, which is an important event in the early stage of atherogenesis. Considering these results and our previous study,2 we now think that the action of probucol as an antioxidant and as an antiatherogenic agent is tightly connected. Thus, the strong possibility of the existence of oxidized LDL in vivo could be one answer to the paradoxical question, How can lipoprotein-bound cholesteryl esters accumulate in the macrophages of WHHL rabbits and homozygous familial hypercholesterolemia patients who have no LDL receptor?

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Index Terms: probucol • oxidized LDL • macrophages • foam cell transformation • atherosclerosis • lipid peroxides • WHHL rabbit
Probucol does not affect lipoprotein metabolism in macrophages of Watanabe heritable hyperlipidemic rabbits.

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