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 antitherogenic 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.

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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...
Determined the sufficient period of probucol feeding for the plasma concentration of probucol as stated above. We concentrations of plasma cholesterol and probucol of from probucol-fed animals. To obtain peritoneal macrophages from probucol-fed WHHL rabbits before they were also obtained peritoneal macrophages from probucol-fed WHHL rabbits, we fed the cells were harvested by peritoneal lavage with 500 ml of saline under anesthesia with pentobarbital. The cells were collected by centrifugation (1500 rpm for 10 minutes at 4°C), were washed once with 30 ml of saline, and were resuspended in DMEM containing 0.2% (wt/vol) lactalbumin hydrolysate, penicillin (100 U/ml), and streptomycin (100 µg/ml) at a final concentration of 2x10^6 cells/ml. Aliquots (1 ml) of this cell suspension were dispensed onto plastic Petri dishes (35x10 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 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 unstimulated mice were pooled in PBS as described by Kita et al. The incubation of cells was performed with the same procedure as described above for rabbit peritoneum macrophages except that the population of cells dispersed per dish was 3x10^6, 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 preincubation 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. Preincubation time was 18 hours because a previous study of the in vitro effects of probucol had shown that probucol had a significant effect on macrophages within 12 hours of incubation. The viability of the cells was checked after preincubation 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 preincubation medium.

**Probucol Concentration**

When we used macrophages from probucol-fed animals 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 collected 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. No-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 of LDL 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

| Table 1. Plasma Levels of Total Cholesterol, Its Subfractions, Triglyceride, and Probucol before and 4 Months after Start of Probucol Feeding |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                 | Control group                  |                           | Probucol group                  |                           |
|                                 | Before treatment | At 4 months | Before treatment | At 4 months |
| TChol                            | 594±87                     | 599±174         | 546±70                     | 557±74                     |
| HDL-C                            | 20±4                        | 11±2             | 20±6                       | 6±2                         |
| LDL-C                            | 317±82                      | 439±153          | 298±49                      | 439±93                      |
| VLDL-C                           | 256±37                      | 149±132          | 229±66                      | 112±85                      |
| TG                               | 860±144                     | 481±201          | 803±318                     | 483±274                     |
| Probucol                         | ND                          | ND               | ND                          | 74.1±8.3                    |

Values are mg/dl, means±SD, except for probucol 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 electromobility 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 β-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 rabbits was not less than that in 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.
Table 2. Effect of Probucol Feeding on Plasma Levels of Cholesterol and Probucol in Mice

<table>
<thead>
<tr>
<th>Duration of treatment</th>
<th>TChol</th>
<th>HDL-C</th>
<th>LDL-C</th>
<th>VLDL-C</th>
<th>Probucol</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.

However, Figure 4 shows that probucol feeding did not prevent the foam cell transformation of mouse macrophages. 5. Dose-response effect of acetylated low density lipoprotein (acytel LDL) modified from LDL of probucol-fed Watanabe heritable hyperlipidemic rabbits (acytel P-LDL) on cholesteryl ester formation in macrophages. 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 (acytel 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.

Figure 5 shows that acetyl LDL modified from P-LDL (acytel 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 (acytel 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 we and Carew et al. 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. In addition, cell-modified (oxidized) LDL which acyl chains were peroxidized has reportedly transformed macrophages into foam cells in vitro. As previously reported, LDL obtained from patients treated with probucol were resistant against oxidative modification in vitro, and we confirmed this in LDL obtained from WHHL rabbits.
Figure 6. Light microscopic appearances of mouse peritoneal macrophages incubated with acetylated low density lipoproteins (acetetyl 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. x970
whether the uptake of atherogenic lipoproteins, such as WHHL rabbits with probucol in vitro, then examined acetyl LDL, β-VLDL, or oxidized LDI could be inhibited. From WHHL rabbits to investigate the possibility that probucol could act on macrophages and prevent their prevention of atherogenesis with a mechanism other than as Watanabe, the plasma levels of triglyceride in WHHL rabbits drop without drugs as they get older. This may be due to a decrease in triglycerides. According to Watanabe, 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. 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 half level of control incubation. More, we could not change such a feature 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. 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 lipoprotein, 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.

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. 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, 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.

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>Acetyl LDL</td>
<td>43 ± 3</td>
<td>54 ± 10</td>
</tr>
<tr>
<td>After additional 30 hours of incubation with</td>
<td>95 ± 13</td>
<td>104 ± 24</td>
</tr>
<tr>
<td>Acetyl P-LDL</td>
<td>115 ± 13</td>
<td>120 ± 21</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>363 ± 24</td>
<td>333 ± 23</td>
</tr>
<tr>
<td>115 ± 13</td>
<td>333 ± 23</td>
<td></td>
</tr>
</tbody>
</table>

Monolayers of mouse peritoneal macrophages (3 x 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 x 10⁶ cells were dispersed onto plastic Petri dishes (100 x 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.

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.

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.
acetylated \( P \)-\text{LDL} was significantly incorporated into macrophages, although \( P \)-\text{LDL}, which contained probucol with the concentration of 9.1 \( \mu \text{g} / \text{mg} \) protein, was hardly incorporated. Figure 6A shows the macrophages transformed into foam cells by incubation with acetylated \( P \)-\text{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 acetylated \( C \)-\text{LDL}. In contrast, the cells in Figure 6C, which were incubated with \( C \)-\text{LDL}, were virtually devoid of lipid droplets. The probucol concentration in macrophages pre-incubated with acetylated \( P \)-\text{LDL} was 0.37 \( \mu \text{g} / \text{mg} \) cellular protein, but probucol was not detectable in macrophages pre-incubated with \( P \)-\text{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 acetylated \( P \)-\text{LDL} during the pre-incubation. Then we washed the medium completely and added acetylated \( \beta \)-\text{LDL} modified from human \( \beta \)-\text{LDL}, rabbit \( \beta \)-\text{VLDL}, or oxidized \( \text{LDL} \) 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 acetylated \( P \)-\text{LDL} and those pre-incubated with acetylated \( C \)-\text{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 acetylated \( \beta \)-\text{VLDL}, and oxidized \text{LDL} in WHHL rabbit macrophages pre-incubated with acetylated \( C \)-\text{LDL} were 127.9, 68.1, and 48.3 nmol/mg protein; those in macrophages pre-incubated with acetylated \( P \)-\text{LDL} were 121.4, 69.3, and 45.2 nmol/mg protein, respectively. These results indicate 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,11 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 \text{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 \( \text{LDL} \) receptor?

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Index Terms: probucol • oxidized LDL • macrophages • foam cell transformation • atherosclerosis • lipid peroxides • WHHL rabbit
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