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Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia

(deficient low density lipoprotein receptors/lipid peroxidation/modified lipoproteins/macrophage)

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ABSTRACT **In this study, we questioned whether *in vivo* probucol could prevent the progression of atherosclerosis in homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model for familial hypercholesterolemia. At 2 months of age, eight WHHL rabbits were divided into two groups. Group A (n = 4) was fed standard rabbit chow for 6 months. Group B (n = 4) was fed standard rabbit chow containing 1% probucol for 6 months. At the end of the experiments, average plasma concentrations of cholesterol were 704 ± 121 mg/dl in group A and 584 ± 61 mg/dl in group B, respectively. The percentage of surface area of total thoracic aorta with visible plaques in group A versus group B was 54.2% ± 18.8% versus 7.0% ± 6.3%, respectively. What was noteworthy was that the percentage of plaque in the descending thoracic aorta was almost negligible (0.2% ± 0.2%) in group B rabbits compared to that in group A rabbits (41.1% ± 20.2%). Low density lipoproteins (LDL) isolated from WHHL rabbits under treatment with probucol (group B) were shown to be highly resistant to oxidative modification by cupric ion and to be minimally recognized by macrophages. On the contrary, LDL from group A rabbits incubated with cupric ion showed a 7.4-fold increase in peroxides (thiobarbituric acid-reactive substances) and a 4.3-fold increase in the synthesis of cholesteryl ester in macrophages compared to those of LDL from group B rabbits. Thus, probucol could definitely prevent the progression of atherosclerosis in homozygous WHHL rabbits *in vivo* by limiting oxidative LDL modification and foam cell transformation of macrophages.**

Familial hypercholesterolemia (FH) is one of the most common human genetic diseases. Homozygous FH patients have inherited allelic mutations in the gene specifying the low density lipoprotein (LDL) receptor located on the cell surface (1). In these patients, few or no functional LDL receptors are synthesized in the body. As a result, not only impairment of catabolism but also overproduction of LDL occurs in FH homozygotes, subsequently leading to a 6-fold to 8-fold increase in plasma LDL levels before birth (1–3). Elevation of plasma levels of LDL leads to characteristic xanthoma formation in tendons and skin and accelerated atherosclerosis (4). Symptomatic coronary atherosclerosis typically develops before the age of 20 years in homozygous FH patients (5). To protect FH patients against atherosclerosis including coronary artery disease, it is necessary to reduce the plasma levels of LDL to as normal a level as possible. In FH homozygotes, liver transplantation is the only treatment so far (6), and plasmapheresis and the portal-caval shunt operation are partially successful (5, 7). None of the antilipidemic drugs is effective in homozygous FH patients.

The foam cell has been recognized as a characteristic feature of xanthomas in skin and tendons and also of the atheromas. Many foam cells in these lesions share properties characteristic of the macrophages. Therefore, the macrophage may be the progenitor of certain foam cells that are involved in atherogenesis (8–10). Several investigators demonstrated *in vitro* that macrophages can ingest large amounts of certain chemically modified lipoproteins, such as acetylated LDL and malondialdehyde-treated LDL through the process of receptor-mediated endocytosis (8, 11–13), and thereby they become foam cells. In addition, Morel *et al.* (14) and Steinbrecher *et al.* (15) suggested that oxidative modification of LDL plays an important role in atherogenesis by facilitating the accumulation of lipids in macrophages *in vitro*. Parthasarathy *et al.* showed that probucol, originally developed as an antioxidant, prevents autooxidation of native LDL by cupric ion (16). Moreover, there are some clinical reports that probucol causes a regression of cutaneous and tendon xanthomas in homozygous FH patients (17–19). It still remains unknown, however, what kind of alteration of LDL is necessary to transform macrophages to foam cells *in vivo*.

An animal model for human familial hypercholesterolemia, known as the Watanabe heritable hyperlipidemic (WHHL) rabbit (3, 20–26), has become available. Homozygous WHHL rabbits resemble their counterparts in having an accumulation of LDL on a low-fat diet, tendon xanthomas, severe atherosclerosis (25, 27), and a genetic defect in LDL receptor (2, 21, 24).

In this paper, we investigate whether probucol is able to prevent the progression of the atheromatous formation in the aorta of homozygous WHHL rabbits *in vivo*.

METHODS

Animals. Female DDY mice (25–30 g) were purchased from Shimizu Laboratories (Kyoto, Japan). Homozygous WHHL rabbits were raised in Kyoto by mating heterozygous and/or homozygous female WHHL rabbits with homozygous male WHHL rabbits (9). At 2 months of age, eight rabbits were divided into two groups (group A and group B). Rabbits in group A (two males, two females) were fed standard rabbit chow for 6 months (9). Rabbits in group B (two males, two females) were raised with rabbit chow enriched with 1% (wt/wt) probucol for 6 months. The amount of daily diet for

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Abbreviations: FH, familial hypercholesterolemia; WHHL rabbit, Watanabe heritable hyperlipidemic rabbit; LDL, low density lipoprotein; VLDL, very low density lipoprotein; β-VLDL, β-migrating VLDL.

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each animal was restricted to 100 g during the study period. Water was supplied ad lib. Six months later (at the age of 8 months), the rabbits were sacrificed and their blood and aortas were taken for analysis.

Calculation for the Area with Atheromatous Plaque. Thoracic aortas were opened longitudinally and photographs of the inner surface were taken. Then the photographs were copied onto graph paper with magnification (×2) and atheromatous plaques were delineated. Numbers of small squares surrounded by the line were counted on the graph paper, and the percentages of the areas of atheromatous plaque were calculated. Thoracic aorta was divided into two parts (aortic arch and descending portion) at the level of the first intercostal artery, and the percentage of each part was also calculated (27).

Lipoproteins. Blood was obtained from each rabbit with the anticoagulant EDTA. LDL (density, 1.019–1.063 g/ml) was isolated by ultracentrifugation from plasma and dialyzed against two changes of at least a 500-fold vol of 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl. For the oxidation of LDL, 150 μg of dialyzed LDL was suspended in 1 ml of phosphate-buffered saline (PBS) containing 0.5 μM $CuSO_4$ and was incubated at 37°C for 24 hr in a CO_2 incubator (28). Lipid peroxide formation was estimated as thiobarbituric acid reactive substances using a lipoperoxide test kit (Wako, Osaka, Japan) according to the method of Heinecke *et al.* (29) with a slight modification (28).

Preparation of Mouse Macrophage Monolayers. Peritoneal cells were harvested from unstimulated mice in PBS as described by Edelson and Cohn (30). The fluid from 20–40 mice (3–6 × 10^6 cells per mouse) was pooled and the cells were collected by centrifugation (400 × *g*, 10 min, 4°C) and washed once with 30 ml of PBS. The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at a final concentration of 3 × 10^6 cells per ml. Aliquots (1 ml) of this cell suspension were dispersed onto plastic Petri dishes (35 × 10 mm) and then incubated in a humidified CO_2 incubator (5% CO_2/95% air) at 37°C for 2 hr. Then, each dish was washed twice with 2 ml of DMEM without serum to remove nonadherent cells. After the monolayers were incubated for 18 hr at 37°C in 1 ml of DMEM containing 10% fetal calf serum, the cells were washed twice with 2 ml of DMEM and then used for the experiment.

Assays of Cholesterol Reacylation. To initiate the experiment, 0.6 ml of DMEM and 45 μg of oxidized LDL were added to the prepared macrophage monolayers and incubated in a humidified CO_2 incubator (5% CO_2/95% air) at 37°C for 6 hr. The amount of incorporation of [^{14}C]oleate-albumin (7400 dpm/nmol) into cellular cholesteryl [^{14}C]oleate by macrophage monolayers was measured as described by Brown *et al.* (12), except that serum was omitted from the incubation medium. Each data point represents an average of duplicate incubations.

Others. Plasma levels of cholesterol were measured by the enzymatic method. Concentrations of probucol in plasma and LDL were measured by high-performance liquid chromatography (HPLC) (31). The protein content of lipoproteins and cells was determined by the method of Lowry *et al.* (32) with bovine serum albumin as a standard. The values shown represent mean ± SD, and statistical significance was determined by the Student's *t* test.

RESULTS

Rabbits ate their chow completely during the period of the study, and their body weights increased (Table 1). Statistically significant differences were not seen between the body weights of the two groups at the start and at the end of the study. The plasma levels of total cholesterol at the start and at the end of the study are shown in Table 1. The mean value in group B rabbits at the end of the study was slightly lower than in the control rabbits (group A), but the difference was not statistically significant.

Table 1. Body weight and plasma cholesterol at the start and end of the study in control (group A) and treated (group B) WHHL rabbits

	Group A	Group B
Treatment duration, mo	6.0	6.0
Weight, kg		
Start	1.23 ± 0.15	1.25 ± 0.18
End	2.80 ± 0.35	2.71 ± 0.13
Plasma cholesterol, mg/dl		
Start	545 ± 83	539 ± 76
End	704 ± 121	584 ± 61
Probucol in plasma, μg/ml		
End	ND	42.9 ± 8.0
Probucol in LDL, μg/ml		
End	ND	308 ± 66

The percentage of probucol in LDL was 69.2% ± 11.0% in plasma. Data are reported as mean values ± SD. ND, not detectable.

Fig. 1 shows photographs of the inner surface of the aorta of WHHL rabbits in group A and group B. Percentages of aortic lesions in all rabbits are summarized in Table 2. The percentage of surface area of total thoracic aorta with visible plaques was 54.2% ± 18.8% in group A, which is similar to that described previously (27, 33), and 7.0% ± 6.3% in group B ($P < 0.01$). In group A rabbits, the degree of atheromatous change was much more severe in the aortic arch than it was in the descending aorta. The percentage surface area of plaques in the aortic arch of group B rabbits was 14% ± 12.4%, which was considerably less severe than that in group A rabbits (67.8% ± 19.1%). Surprisingly, in group B, which had been treated with probucol, the percentage of plaque in the descending thoracic aorta was almost negligible (0.2% ± 0.2%) compared with that in group A (41.1% ± 20.2%). Moreover, only 0.3% of the entire thoracic aorta was affected with atheromatous plaque in one rabbit among group B rabbits (B-1) (Fig. 1), which clearly indicates that the B-1 rabbit had the least atherosclerosis.

As shown in Table 1, the probucol concentrations in plasma and LDL at the end of the study were 42.9 ± 8.0 μg/ml and 308 ± 66 μg/ml, respectively. The percentage of probucol in LDL was 69.2% ± 11.0% in plasma. The mean plasma concentration of probucol in group B rabbits is just near the level of that in human patients who are treated with probucol for a long time with a standard administration schedule (16, 34). The probucol level in LDL was significantly higher than in plasma, suggesting that high levels of this drug accumulate in lipoprotein particles.

As shown in Table 3, LDL from group A animals incubated with cupric ion showed a 7.4-fold increase in peroxide (thiobarbituric acid reactive substances) content and a 4.3-fold increase in the synthesis of cholesteryl ester in macrophages, compared with that of LDL from group B animals. In other words, LDL isolated from the plasma of probucol-treated animals (group B) was resistant to cupric ion-induced modification. These values are consistent with previous studies (15, 28, 29).

DISCUSSION

Several investigators have reported that among the antilipidemic drugs, probucol is the only one that is partially effective in homozygous FH patients (17, 18). Probucol causes a rapid and remarkable regression of palpebral skin xanthoma and also achilles tendon xanthomas in both homo-

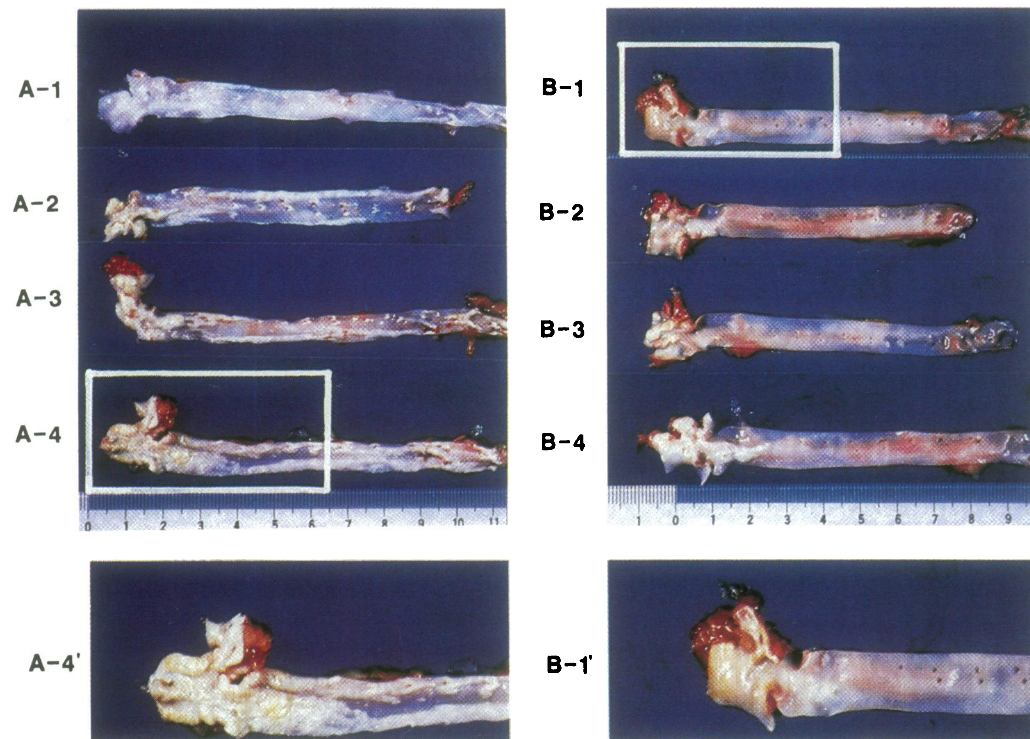

FIG. 1. Aortic specimens showing the extent of atherosclerotic plaques. The left four aortas (A-1–A-4) are from control group A. The right four aortas (B-1–B-4) are from probucol-treated group B. A-4′ and B-1′ are 2-fold magnifications of the white square area of A-4 and B-1, respectively.

zygous and heterozygous FH patients (17–19). This induction of regression of xanthomas by probucol is much more remarkable than would be expected from the reduction in LDL cholesterol (17–19, 35).

In this study, we have shown that probucol prevented the progression of atherosclerosis in homozygous WHHL rabbits (Fig. 1; Table 2) in the absence of a significant reduction in plasma cholesterol levels (Table 1). There was a significant reduction of atheromatous plaques in the thoracic aorta of the treated animals (7.0% ± 6.3%) compared to that in untreated controls (54.2% ± 18.8%). Moreover, in the treated rabbits, there were almost no atheromatous plaques in the descending thoracic aorta (0.2% ± 0.2%). What mechanism can explain the prevention of the progression of atherosclerosis in WHHL rabbits by probucol?

Harman (36) and Yagi (37) proposed that free radicals and lipid peroxidation may play an important role in the pathogenesis of atherosclerosis. In addition to the fact that peroxidized lipids are found in the atherosclerotic lesions, recently, cell-modified (oxidized) LDL, in which acyl chains are peroxidized, has been reported to transform macrophages into foam cells *in vivo* (14, 28, 38). These foam cells are recognized as a characteristic feature in the initial stages of atherosclerosis. These findings raise the possibility that oxidized LDL may be the source of cholesteryl esters in foam

Table 2. Percentages of intimal surface area of thoracic aorta involved with atheromatous plaque in control (group A) and treated (group B) WHHL rabbits

	Aortic arch, %	Descending aorta, %	Total thoracic aorta, %
	Group A		
A-1	47.4	12.7	31.1
A-2	51.0	39.6	44.5
A-3	80.4	42.3	59.7
A-4	92.4	69.7	81.6
Mean ± SD	67.8 ± 19.1	41.1 ± 20.2	54.2 ± 18.8
	Group B		
B-1	0.6	0	0.3
B-2	7.0	0	3.3
B-3	14.8	0.6	7.4
B-4	33.6	0.1	17.1
Mean ± SD	14.0 ± 12.4	0.2 ± 0.2	7.0 ± 6.3*

*$P < 0.01$.

Table 3. Effect of Cu^{2+}-induced modification of LDL on TBARS contents and stimulation of cholesteryl ester formation in mouse peritoneal macrophages

Source	TBARS content, nmol of malondialdehyde per mg of protein	Incorporation of [^{14}C]oleate into cholesteryl ester, nmol per mg of cellular protein
LDL from group A		
Unincubated	4.2 ± 0.8	0.93 ± 0.07
Incubated 24 hr without Cu^{2+}	4.3 ± 0.52	1.34 ± 0.33
Incubated 24 hr with Cu^{2+}	39.8 ± 4.6*	9.0 ± 2.4†
LDL from group B		
Unincubated	0.34 ± 0.34	0.74 ± 0.09
Incubated 24 hr without Cu^{2+}	0.54 ± 0.27	1.82 ± 0.35
Incubated 24 hr with Cu^{2+}	5.4 ± 1.1*	2.1 ± 0.5†

LDL samples were prepared from the plasma of four untreated controls (group A) and from that of four probucol-treated animals. Incubation and analyses were carried out as described in duplicate for each sample and the results were averaged. The content of cholesteryl [^{14}C]oleate in cells incubated without lipoproteins was 0.66 nmol per mg of protein. TBARS, thiobarbituric acid reactive substances. Data are reported as mean values ± SD.

*$P < 0.001$.

†$P < 0.01$.

cells *in vivo*. Up to this point, however, there has been no definite evidence for such a role *in vivo*.

Probucol was originally developed as an antioxidant (39, 40). Recently Parthasarathy *et al.* (16) showed that probucol inhibits oxidative modification of LDL by cupric ions or endothelial cells *in vivo*. Our present data might be explained by the mechanism that probucol inhibits atherogenesis by limiting oxidative modification of LDL and subsequently by limiting foam cell transformation of macrophages *in vivo* (Table 3).

There is also a possibility that probucol could inhibit the uptake of certain lipoproteins [including acetyl LDL, oxidized LDL, and β-migrating very low density lipoprotein (β-VLDL)] by macrophages, as suggested by Yamamoto *et al.* (40). However, we could not successfully demonstrate that probucol inhibits the uptake of modified LDL and β-VLDL by macrophages (data not shown).

Kritchevsky *et al.* (41) reported that probucol in cholesterol-fed rabbits reduced the severity of aortic atherosclerosis, but to a degree compatible with the degree of plasma cholesterol lowering. In these rabbits, however, atherosclerosis is likely to be caused by an increase in β-VLDL, which can be recognized without any modifications by macrophages via a β-VLDL receptor. Recently, we reported that WHHL VLDL can be recognized by macrophages via a β-VLDL receptor, and it thereby transforms macrophages into foam cells (9). WHHL VLDL may be playing some role in the formation of the small number of atheromatous plaques that occur in the aortic arch of probucol-treated animals.

The dose of probucol for the WHHL rabbits, however, is 20–30 times the human dose on a mg/kg basis. Nevertheless, the plasma concentration of probucol in rabbits is near the level of that in human patients. We do not know whether a lower dose of probucol, which is the same amount of the human dose on a mg/kg basis, would be effective.

Finally, our study suggests the possibility that oxidized LDL is the true form of modified LDL that produces foam cells and atherosclerosis *in vivo*.

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