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The effects of probucol on the progression of atherosclerosis in mature Watanabe heritable hyperlipidaemic rabbits

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1 Probucol was administered to mature Watanabe heritable hyperlipidaemic (WHHL) rabbits (≈9 months old). Groups of WHHL rabbits were randomly selected and treated as follows: Group 1 killed at 9 months ($n = 9$); Group II placed on sham-treated diet at 9 months and followed for 6 months ($n = 8$); Group III placed on probucol at 9 months and followed for 6 months ($n = 8$). Probucol was administered by mixing 1% wt/wt drug with standard laboratory diet.

2 Plasma concentrations of probucol increased to $93 \pm 11\,\mu g\,ml^{-1}$ in Group III during the initial 2 weeks and increased further to $149 \pm 24\,\mu g\,ml^{-1}$ at the end of the treatment period.

3 Plasma concentrations of total cholesterol, unesterified cholesterol and phospholipids were significantly reduced overall by probucol, while triglycerides were not affected.

4 No statistically significant differences were observed in the presence of oxidized products in low density lipoproteins (LDL) isolated from plasma of controls compared to probucol-treated rabbits. However, LDL from probucol-treated animals was resistant to oxidation in the presence of Cu^{2+} (3 μM).

5 Group I had aortic atherosclerosis covering $70 \pm 5\%$ of intimal area of thoracic aortae, that increased to $91 \pm 3\%$ in Group II. This was associated with cholesterol contents of aortae increasing from $1.4 \pm 0.2\,\mu g\,mg^{-1}$ in Group I to $2.7 \pm 0.3\,\mu g\,mg^{-1}$ in Group II. Probucol administration did not produce a statistically significant reduction of atherosclerotic lesion area ($78 \pm 7\%$). However, probucol treatment reduced cholesterol content to $1.9 \pm 0.3\,\mu g\,mg^{-1}$ ($P < 0.01$). Collagen content of aortae was not affected by probucol treatment.

6 Thus, while probucol did not promote regression, the drug did retard the continued deposition of cholesterol esters into atherosclerotic lesions of mature WHHL rabbits.

Keywords: Probucol; Watanabe heritable hyperlipidaemic rabbits; oxidation; atherosclerosis; cholesterol

Introduction

Probucol (4,4′-(isopropylidenedithio)bis(2,6-di-*t*-butylphenol) is a hypolipidaemic agent that is modestly effective at reducing plasma concentrations of low density lipoprotein (LDL)-cholesterol (Buckley *et al.*, 1989). However, this reduction in LDL-cholesterol concentrations is associated with a consistent reduction in plasma concentrations of high density lipoprotein (HDL)-cholesterol. This effect on HDL-cholesterol has led to speculation that the probucol-induced alteration in lipoprotein profiles may aggravate the atherogenic process.

In addition to its hypolipidaemic properties, probucol is a potent scavenger of hydroxyl radicals. In fact the chemical structure of probucol is related to the commonly used hydroxyl radical scavenger, butylated hydroxytoluene. A current concept of atherogenesis is that lipid peroxidation within vascular tissue contributes to the disease process (Steinberg *et al.*, 1989). Therefore, it has been proposed that probucol may possess specific anti-atherosclerotic properties that are independent of its hypolipidaemic effects (Steinberg, 1986). In support of this hypothesis, Kita *et al.* (1987) and Carew *et al.* (1987) have demonstrated that the oral administration of probucol to Watanabe heritable hyperlidaemic (WHHL) rabbits within the first 2 months of birth has resulted in marked reductions in the extent of aortic atherosclerosis after approximately 7 months of the regimen. The study of Carew *et al.* (1987) discerned that the modest hypolipidaemic effect of probucol was not responsible for the protective action by comparing the probucol-treated animals to a group administered lovastatin. In contrast to probucol, lovastatin afforded no protection against the development of aortic atherosclerosis despite an equivalent hypolipidaemic response.

While there is good evidence that probucol can prevent the initiation of atherosclerosis in WHHL rabbits, the effects of the drug on established disease have not been demonstrated. It has been speculated that probucol may promote regression of atherosclerosis, based on the observations of reductions in xanthoma during treatment of familial hypercholesterolaemic subjects (Yamamoto *et al.*, 1983). Indeed, a preliminary report has provided some evidence of probucol-induced regression of atherosclerotic lesions in rhesus monkeys fed a high-fat, high-cholesterol diet (Wissler & Vesselinovitch, 1983). The purpose of the present study was to determine whether probucol exerted any effects on established atherosclerosis lesions in WHHL rabbits (Havel *et al.*, 1982; Buja *et al.*, 1983).

Methods

Animals

A colony of WHHL rabbits at Washington University School of Medicine was initiated from animals kindly provided by Dr Joseph L. Goldstein (University of Texas). Animals were maintained in isolation from other colonies under aseptic conditions, and given water and a standard laboratory rabbit diet *ad libitum*. A total of 25 rabbits was used. All procedures performed on the animals were approved by the Washington University Animal Studies Committee.

Probucol (a gift from Merrell Dow Research Institute, Cincinnati, OH, U.S.A.) was dissolved in chloroform and sprayed evenly over the diet in the proportion of 1% wt/wt. Diet for the control group was sprayed with chloroform alone. Treated diets were kept under a fume hood until the odour of the solvent had dissipated. Food intake was monitored daily for

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both control and drug-treated groups. Body weights were determined weekly.

Characterization of plasma lipids

Concentrations of triglycerides, cholesterol esters, unesterified cholesterol and phospholipids in plasma and in isolated lipoprotein fractions were determined with commercially available enzyme kits (Wako Chemical Company, Dallas, TX, U.S.A.) Concentrations of phospholipids and triglycerides were calculated based on mean molecular weights of 722 and 866 respectively.

Probucol determinations in plasma

Plasma concentrations of probucol were determined by reverse phase high performance liquid chromatography (h.p.l.c.) using the method of Satonin & Coutant (1986). Concentrations of probucol in plasma were determined relative to an internal standard supplied by the Merrell Dow Research Institute (MDL 27,272).

Oxidation of isolated low density lipoproteins

LDL was isolated by sequential ultracentrifugation in a Beckman L8-55 instrument equipped with a 50.3 Ti rotor between densities of 1.019 to 1.063 g ml^{-1} using sodium bromide (Havel *et al.*, 1955). Isolated lipoprotein fractions were dialyzed against 100 volumes of EDTA (1 mM)/sodium chloride (0.15 M), with three changes of dialysis fluid over 24 h. Volumes of fractions were reduced by placing LDL in dialysis bags that were coated with Aquacide (Calbiochem, La Jolla, CA, U.S.A.). Mass of protein in lipoprotein fractions was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Pierce Chemical Company, Rockford, IL, U.S.A.) as standard.

Lipid peroxidation was initiated by incubation of LDL fractions with copper sulphate, 3 μM (McLean & Hagaman, 1989). The extent of lipid peroxidation was quantified as thiobarbituric acid-reacting substances (TBARS) as described by Satoh (1978). Standards were prepared by dissolution of malondialydehyde-*bis*-dimethyl-acetal (Aldrich Chemical Company, Milwaukee, WI, U.S.A.) in distilled water immediately before the start of the assay. Samples (100 μg protein in 0.5 ml EDTA/saline) were incubated with sulphuric acid (0.05 M) and thiobarbituric acid (0.2% wt/vol, dissolved in sodium sulphate, 2 M) for 30 min at 100°C. After cooling, n-butyl alcohol (4 ml) was added and mixtures vortexed vigorously. The two phases were separated by centrifugation at 500 *g* for 5 min. The absorbance of the chromogen was determined at 530 nm with a Beckman DU-30 spectrophotometer. Results are expressed as nmol equivalents of malondialdehyde and the assay was linear between 0.5 to 20 nmol.

Characterization of atherosclerosis

Animals were killed by overdosing with pentobarbitone (120 mg kg^{-1}, administered intravenously). Aortae were rapidly dissected free from the ascending arch to the ileal bifurcation. Extraneous tissue was removed and full length incisions exposed intimal areas. Intimal areas were photographed with a Polaroid camera. The areas of grossly discernible normal and atherosclerotic intima were digitized from these photographs by use of a Numonics model 2210 tablet (Numonics Corporation, Lansdale, PA, U.S.A.) and Sigma-Scan (Jandel Scientific, Corte Madera, CA, U.S.A.) run on a DOS-based computer.

Total cholesterol content of vascular tissue was determined by gas liquid chromatography (g.l.c.) as described by Ishikawa *et al.* (1974) with 5-α-cholestane as a standard. For the determination of collagen, weighted segments of tissue were hydrolyzed by incubation in the presence of concentrated hydrochloric acid incubated overnight at 80°C. Hydroxyproline content of the tissue was determined with Erlich's solution as described by Prockop & Undenfreind (1960).

Immediately following the dissection of aortic tissue from the body, segments of tissue were cut in a region that included a branch of an intercostal artery, since this region is a site of predilection for the development of atherosclerosis. Tissue was immersion fixed in formaldehyde (2% wt/vol) dissolved in phosphate-buffered saline at room temperature for 4 h. Further fixation was achieved by overnight incubation in this solution at 4°C. Sections of tissue (5 μm thick) were cut, mounted and stained with haematoxylin and eosin.

Experimental protocol

Twenty five rabbits were randomized into 3 groups when animals were approximately 9 months of age. Group I was killed at this interval and served as the control group for determining the extent of atherosclerosis at the initiation of drug administration. Group II was given a diet that had been soaked in chloroform in a manner identical to that used to coat probucol on the diet, and was followed for 6 months. Group III was fed a diet for 6 months that was coated with probucol.

Statistical analyses

Statistical comparisons between groups were made with Student's *t* test (two-tailed) performed with Stats-+ (Statsoft, Tulsa, OK, U.S.A.). A repeated-measures analysis of variance (ANOVA) was used to assess the statistical significance of overall plasma lipid concentrations between Groups II and III. Duncan's multiple-range test was used to assess the statistical significance between each interval that plasma lipid concentrations were determined for Groups II and III. A probability value of less than 5% was considered statistically significant. Values are presented as means with s.e.means.

Results

General characteristics

The WHHL rabbits used in the present study weighed approximately 3.75 kg at the initiation of the study i.e., when the rabbits were ≈ 9 months of age. During the 6 months of observation, Groups II and III did not increase in body weight, and no significant differences in body weight were apparent between them (Figure 1). In accord with this finding, the food consumption was both constant and comparable between the groups. All animals maintained good health during the entire course of this study and all rabbits that entered the study survived to completion of the protocol.

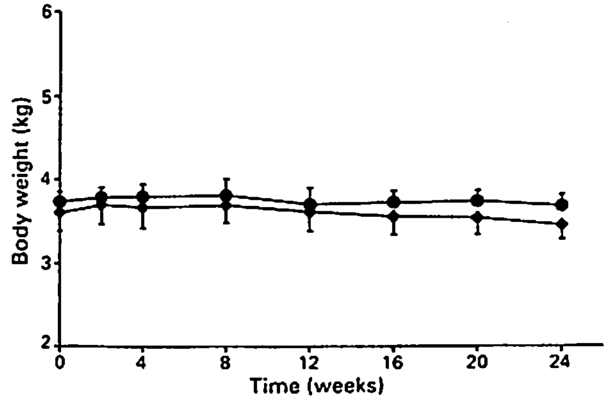

Figure 1 Body weights of control rabbits (Group II, ●) and probucol-treated (Group III), WHHL rabbit (◆). Points represent the means of 8 observations and bars the s.e.mean.

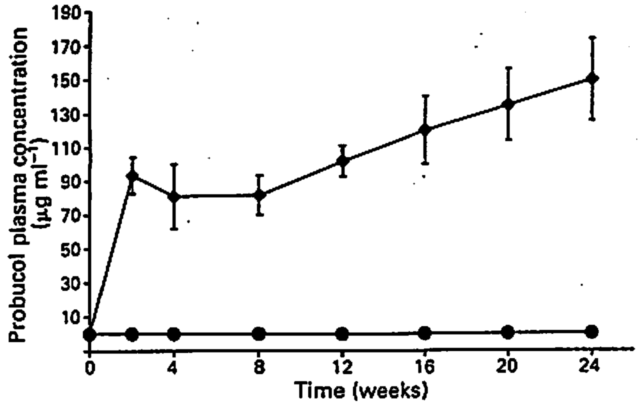

Figure 2 Plasma concentrations of probucol. Plasma concentrations of probucol were determined at selected intervals by h.p.l.c. in control (Group II, ●) and probucol-treated (Group III), WHHL rabbit (◆). Points represent the means of 8 determinations and bars represent the s.e.mean.

Plasma concentrations of probucol were monitored at selected intervals after the initiating treatment as indicated in Figure 2. The mean plasma concentration in the drug treated group was $93 \pm 11\,\mu g\,ml^{-1}$ after 2 weeks and this concentration was maintained to 8 weeks. Thereafter there was a trend toward increasing concentrations, reaching $149 \pm 24\,\mu g\,ml^{-1}$ at the end of the study. As expected, probucol was never detected in the plasma of the control group (Figure 2). A green colouration was present in the plasma of probucol-treated animals that was attributed to the accumulation of oxidized probucol (Barnhart *et al.*, 1990).

Probucol on plasma concentrations of lipoproteins

Plasma concentrations of total cholesterol at the initiation of the study were $674 \pm 80\,mg\,dl^{-1}$ in Group II and $683 \pm 123\,mg\,dl^{-1}$ in Group III (NS). In Group II, plasma concentrations of cholesterol were maintained until the animals were approximately one year old, after which there was a gradual increase. Probucol treatment produced an overall significant decrease in plasma concentrations of total cholesterol ($P < 0.001$) as analyzed by ANOVA. Despite the significance of the overall effect, comparison of each interval by Duncan's multiple-range test showed that only the 8 and 12 week intervals were significantly different between the groups ($P < 0.0375$ and $P < 0.003$, respectively; Figure 3a). Probucol treatment also produced overall reductions in unesterified cholesterol and phospholipids that were significantly different when analyzed by ANOVA ($P < 0.0001$ and $P < 0.0008$, respectively; Figures 3b and 3c). Plasma concentrations of triglycerides were not significantly affected by probucol administration (Figure 3d).

Oxidation of lipoproteins in vitro

LDL was isolated from the plasma of Groups II and III, and the extent of lipid peroxidation was determined by the extent of TBARS present. The concentrations of TBARS in native plasma-derived LDL were low and were not significantly changed compared to LDL isolated from probucol-treated rabbits (Figure 4). LDL from Groups II and III was incubated with copper (3 µM) to simulate a microenvironment in which LDL may be subjected to increased oxidative stress. Under these conditions, there was a 110% increase ($P < 0.001$) in the concentrations of TBARS in LDL from untreated animals. In contrast, there was only a modest increase in the concentration of TBARS in LDL derived from probucol-treated animals which was not significantly higher than that in native LDL. Consequently, TBARS were present in significantly ($P < 0.004$) increased concentrations in copper-treated native LDL compared to that isolated from probucol-treated animals.

Extent of atherosclerosis

The extent and nature of atherosclerosis was assessed in all 3 groups of animals. At ≈ 9 months of age (Group I), WHHL rabbits had extensive involvement of the aorta of grossly discernible atherosclerotic lesions of the aorta with 71 ± 5% and

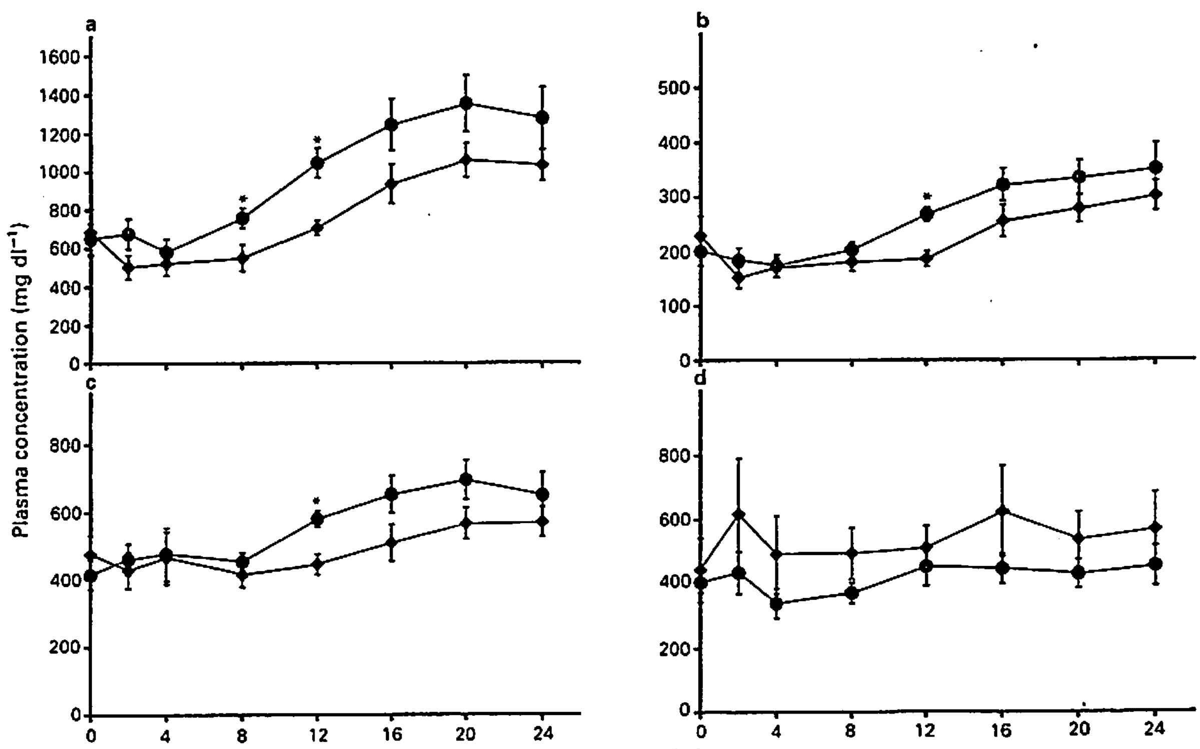

Figure 3 Plasma concentrations (mg dl⁻¹) of total cholesterol (a), unesterified cholesterol (b), phospholipids (c), and triglycerides (d) of control rabbits (Group II, ●) and probucol-treated (Group III), WHHL rabbit (◆). Points represent the means of 8 determinations and bars represent the s.e.mean.

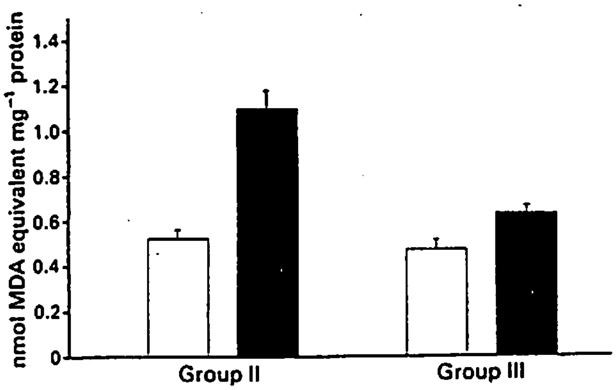

Figure 4 Presence of thiobarbituric acid-reacting substances (TBARS) in low density lipoproteins (LDL) from control (Group II, solid columns) and probucol-treated WHHL rabbits (Group III, open columns). TBARs were determined in the native material and following a 3 h incubation with Cu^{2+} for 18 h. Columns are the means of 4 determinations and bars are s.e.mean.

55 ± 7% of the intimal surface of the thoracic and abdominal segments, respectively, being covered (Figure 5a). The distributions of these atherosclerotic lesions were predominately at the arch and at arterial branch points. After a further 6 months, coverage in Group II had increased to 91 ± 3% and 71 ± 8% for the thoracic and abdominal segments, respectively. The extent of aortic atherosclerosis in Group III was 77 ± 7% and 59 ± 7% for abdominal and thoracic segments respectively. These reductions did not attain statistical significance.

As may be expected, there was a high content of cholesterol in the aortae from WHHL rabbits in Group I (1.4 ± 0.2 μg cholesterol mg^{-1} wet wt; Figure 5b). Both abdominal and thoracic segments in Group II had marked increases in cholesterol content. However, this increased cholesterol content was attenuated in both segments from the probucol-treated Group III ($P < 0.01$).

Although there was a decrease in the cholesterol content of the aorta from the probucol-treated animals compared to age-matched controls, no changes in collagen content were detectable as judged by the concentration of hydroxyproline (Figure 5c).

In agreement with these quantitative data, histological sections of aorta demonstrated that there was an increase in the lipid deposition in the core region of lesions comparing Groups I to II. Generally, there was decreased lipid deposition in the core region of Group III. However, there were no major differences in the morphology of the atherosclerotic lesions between the groups studied. Thus, the atherosclerotic lesions differed in extent of lipid deposition rather than in the cellular composition.

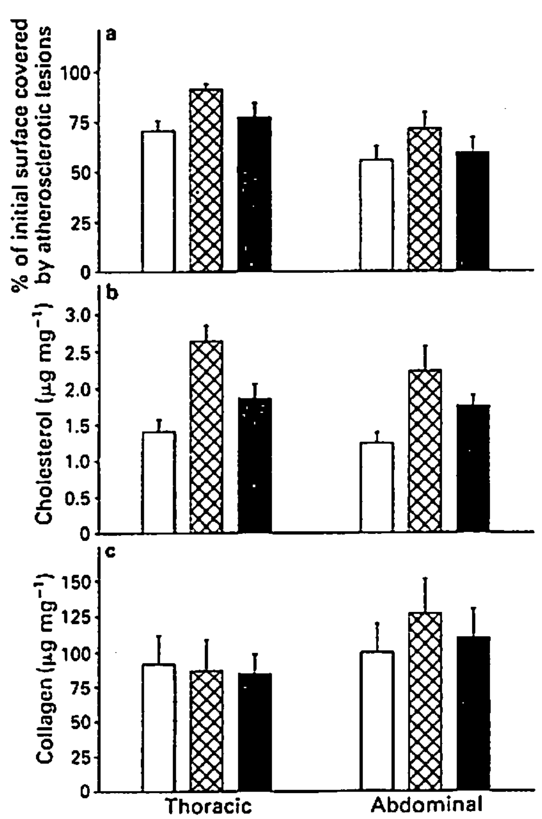

Figure 5 Characterization of aortic atherosclerosis in WHHL rabbits. Parameters determined were the percentage of intimal area that was covered by grossly discernible atherosclerotic lesions (a), total cholesterol content (b), and collagen content (c). Data are represented for Group I (open columns, $n = 9$), Group II (cross-hatched columns, $n = 8$), and Group III (solid columns, $n = 8$). Columns are the means of a minimum of 4 determinations and bars are s.e.mean.

Discussion

Probucol has been found to attenuate the initiation and development of atherosclerosis in cholesterol-fed rabbits (Kritchevsky *et al.*, 1971; Tawara *et al.*, 1986; Daugherty *et al.*, 1989) and WHHL rabbits (Kita *et al.*, 1987; Carew *et al.*, 1987). Although several reports have demonstrated anti-atherosclerotic properties of probucol in experimental studies, a negative study has also been published (Stein *et al.*, 1989). Those reports that have described an anti-atherosclerotic effect have generally not ascribed this protection to an alteration in lipid metabolism, but rather to the hydroxyl radical scavenging properties of the drug (Steinberg, 1986). Indeed there is increasing evidence that the changes in metabolic properties that have been described for oxidized LDL *in vitro* (Fogelman *et al.*, 1980; Henriksen *et al.*, 1981; Steinbrecher *et al.*, 1984; Parthasarathy *et al.*, 1986a) may be relevant to the lipoprotein metabolism *in vivo* (Daugherty *et al.*, 1988; Haberland *et al.*, 1988; Boyd *et al.*, 1989; Palinski *et al.*, 1989).

The temporal cellular events that are involved in the progression of atherosclerosis in WHHL rabbits have been described in detail by Rosenfeld *et al.* (1987a,b). Macrophages are deposited in the subintimal space of aortic tissue of WHHL rabbits within the first month following parturition. These initial lesions develop into fatty streaks by the continued deposition of macrophages that are transformed to foam cells, and the persistent deposition of extracellular matrix. Thus, the atherosclerotic lesions investigated in the present study were mature fatty streaks predominantly containing macrophages and numerous smooth muscle cells.

While the determination of a drug to inhibit the initiation of atherosclerosis may have important implications for the prevention of the disease, the ability of a drug to induce regression could have profound implications for therapy of established disease. It has been hypothesized that probucol may induce regression of atherosclerotic lesions since the drug reduces the extent of xanthomas in familial hypercholesterolaemic patients (Yamamoto *et al.*, 1988). Since xanthomas consist of lipid-laden foam cells analogous to atherosclerotic lesions (Fowler *et al.*, 1979; Schaffner *et al.*, 1980), it is thought that these may represent a model of atherosclerotic lesions. The preliminary report of Wissler & Vesselinovitch (1983) demonstrated that there was a modest regression on aortic atherosclerosis as judged by the percentage of intimal area that was covered by grossly discernible lesions. The present study was designed to provide quantitative data to demonstrate whether probucol (A) promoted regression, (B) attenuated the progression, or (C) did not influence the atherogenic process of established atherosclerotic lesions.

Previous reports that have described the temporal cholesterol concentrations in plasma of WHHL rabbits have generally described a gradual reduction with age (Rosenfeld *et al.*, 1987a). In contrast, the present study has described a gradual increase in plasma concentrations. However, as distinct from other work, the present study was initiated with mature WHHL rabbits that were ≈9 months of age. Furthermore, plasma cholesterol concentrations began to increase only after the rabbits were a year of age. A systematic error in the measurement of plasma cholesterol concentrations is considered unlikely since rabbits were initiated into the study over a 8 month time span. Thus, there was considerable randomization of the plasma cholesterol determinations, which would have negated the effects of a drift in the assay calibration. The mechanism of this increase in plasma cholesterol concentrations in rabbit over one year of age is unknown.

Administration of probucol to WHHL rabbits produced high plasma concentrations of the drug but relatively modest reductions in concentrations of cholesterol for the initial 3 months; although the difference between the groups increased after 1 year of age. Variable responses have been demonstrated for probucol on plasma cholesterol concentrations in WHHL rabbits. Naruszewicz *et al.* (1984) observed a 36% decrease in plasma concentrations of LDL-cholesterol and attributed this reduction to a change in the inherent property of the LDL. However, a later study by the same group failed to demonstrate a hypolipidaemic effect despite comparable plasma concentrations of probucol (Carew *et al.*, 1987).

The moderate reduction in plasma concentrations of cholesterol observed in the present study may have contributed to the observed reduction in cholesterol in the aortae of probucol-treated rabbits. In addition to the hypolipidaemic effect contributing to the anti-atherosclerotic effects, the ability of probucol to scavenge hydroxyl radicals may also have contributed to the reduced cholesterol deposition. Oxidation of LDL in the plasma is likely to be limited because of the combination of the relatively low concentrations of oxidant and the extensive antioxidant defence mechanisms. Despite these considerations, modified LDL has been detected in the plasma of human subjects (Avogaro *et al.*, 1988). However, it is more likely that oxidative modifications of LDL occur in non-intravascular compartments such as the subendothelial space. To simulate this environment of increased oxidative stress, LDL was incubated with low concentrations of copper. Since probucol is highly lipophilic, it is transported by lipoproteins. Indeed, LDL from probucol-treated rabbits was highly resistant to oxidation in the presence of copper. Thus a significant contribution to the decrease of the continued lipid deposition may have been due to prevention of LDL oxidation at the loci of the arterial tissue. The primary protective effects attributable to the antioxidant properties of probucol are likely to be related to an effect on the inhibition of LDL modification rather than influencing the metabolism of the macrophages directly. Hence, although probucol prevents oxidation of LDL and metabolism by macrophages (Parthasarathy *et al.*, 1986b; Yamamoto *et al.*, 1986), the drug has no effect on the metabolism of acetylated LDL by this cell type (Ku *et al.*, 1990). In the present work, it was not feasible to study whether the macrophage metabolism was itself modified after the chronic administration of probucol. Despite this reduction in the extent of lipid deposition, no effect of probucol was demonstrable on the collagen content of aortae following probucol treatment. Thus, the effect on atherosclerotic lesions in established disease appeared to be related only to effects on lipid deposition.

In summary, the present study has demonstrated that the chronic administration of probucol to mature WHHL rabbits with established aortic atherosclerosis does not promote regression but does lead to a reduction in the continued deposition of cholesterol into arterial tissue. This beneficial effect may have been partially due to a slight hypolipidaemic effect that was observed during probucol administration. However, the marked potency in scavenging hydroxyl radicals may have contributed more significantly to the beneficial effect on the atherogenic process.

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