Extent of antioxidant protection of plasma LDL is not a predictor of the antiatherogenic effect of antioxidants

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Abstract Oxidation of LDL plays an important role in atherogenesis. The lag-time in the formation of conjugated dienes provides a sensitive measure of the resistance of plasma LDL to oxidation and is widely assumed to be an indicator of atherogenic risk. To test this assumption, we investigated whether different antioxidants yielding similar lag-times result in similar reduction of atherosclerosis. A 6-months intervention study was carried out in three groups of 10 LDL receptor-deficient rabbits each. Because previous studies indicated that antioxidants that reduce atherosclerosis resulted in very long lag-times, the first group was treated with an antioxidant combination containing 1,000 IU vitamin E, 0.05% probucol analogue (BM15.0639), and 0.025% probucol. The lag-times achieved throughout the intervention period by this combination (952 ± 39 min) were matched in a second group (829 ± 46 min) treated with a variable dose of probucol (0.0575–0.11%, average 0.091%). A third, untreated group served as a control. Plasma cholesterol levels of all groups were matched. Even though both treatments yielded similar antioxidant protection of plasma LDL, 0.091% probucol reduced aortic atherosclerosis by 51.7% compared to the untreated group ($P < 0.005$), whereas the antioxidant combination failed to reduce lesion formation. Thus, the lag-time is clearly not correlated with the antiatherogenic efficacy of different antioxidants. However, a weak correlation was found within the group treated with probucol only. Our results suggest that the degree of antioxidant protection of plasma LDL may not be a good indicator of the atherogenic risk, in general, and that probucol reduces atherogenesis by mechanisms not shared by all antioxidants.—**Fruebis, J., D. A. Bird, J. Pattison, and W. Palinski.** Extent of antioxidant protection of plasma LDL is not a predictor of the antiatherogenic effect of antioxidants. *J. Lipid Res.* 1997. **38:** 2455–2464.

Supplementary key words arteriosclerosis • oxidation • lag-time • probucol • vitamin E

Lipoprotein oxidation, and oxidative processes in general, play an important role in the pathogenesis of arteriosclerosis (reviewed in 1–4). Although in vitro studies have identified numerous properties of minimally and extensively oxidized LDL that could contribute to lesion formation, the most important in vivo evidence for the atherogenic role of oxidation was provided by the demonstration that several lipophilic antioxidants, i.e., probucol, butylated hydroxytoluene (BHT), and diphenyl-phenylenediamine (DPPD) significantly reduced the progression of atherosclerosis in rabbits (5–8), primates (9) and mice (10). However, intervention studies with natural antioxidants such as vitamin E yielded conflicting results (11–15), and studies with lipophilic analogues of probucol also failed to demonstrate an antiatherogenic effect comparable to that of probucol (16, 17).

The resistance of plasma LDL to in vitro oxidation, measured as the lag-time in the formation of conjugated dienes after addition of a prooxidant, such as copper ions (18), is generally considered to be the best indicator of the degree of antioxidant protection achieved in vivo. The lag-time is also frequently used in human studies to assess the extent of LDL oxidation or the susceptibility of various LDL fractions to oxidation. For example, the lag-time has been determined in LDL from subjects with conditions that may enhance oxidation or increase atherogenesis, such as high dietary intake of unsaturated fatty acids, diabetes, smoking, and menopause (19–24). The implicit assumption in such studies is that the degree of antioxidant protection of plasma LDL correlates with the extent of lipid peroxidation in the arterial wall and thus provides a measure of the atherogenic risk.

Several studies have yielded data supporting an inverse correlation between the lag-time and the extent of atherosclerosis (9, 25, 26). Furthermore, such a correlation is suggested by the observation that antioxidants that reduce atherogenesis yield a much longer lag-time than antioxidants that fail to inhibit lesion for-

Abbreviations: apoB, apolipoprotein B; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; DPPD, diphenyl-phenylenediamine; IL-1, interleukin 1; MCP-1, monocyte chemotactic protein 1; OxLDL, oxidized low density lipoprotein; TBARS, thiobarbituric acid reactive substances; VCAM-1, vascular cell adhesion molecule 1.

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mation. In LDL receptor-deficient rabbits, 1% probucol prolonged the lag-time of plasma LDL 8-fold and inhibited lesion formation by almost 50% (16). In contrast, a probucol analogue (BM15.0639) and vitamin E prolonged the lag-time only 4-fold, and neither of these antioxidants reduced atherogenesis (14, 16). These and other similar intervention studies with antioxidants support the assumption that the lag-time is a predictor of the antiatherogenic efficacy of antioxidants. Furthermore, they led to the proposal of the "threshold hypothesis", postulating that an antioxidant has to convey a threshold degree of protection to LDL in order to inhibit atherosclerosis (16). However, the fact that compounds yielding greater lag-times had greater antiatherogenic potency does not constitute evidence for a causal relationship between the antioxidant protection of plasma LDL and the extent of atherogenesis, because the antiatherogenic mechanisms of different compounds at the level of the artery wall may also be different.

Conclusive evidence for or against the correlation between antioxidant protection of plasma LDL and atherogenesis could be obtained by testing a corollary of this hypothesis: If the antioxidant protection of plasma LDL indeed determines atherogenesis, then treatment with different antioxidants achieving the same degree of antioxidant protection should result in the same extent of atherosclerosis. This concept has never been tested experimentally. The present paper describes an intervention study designed to test *1*) the correlation between the degree of antioxidant protection of plasma LDL and the extent of atherogenesis and *2*) the threshold hypothesis, by comparing the antiatherogenic effect of an antioxidant combination to that of a dose of probucol yielding the same degree of antioxidant protection of plasma LDL.

METHODS

Animals and diets

Thirty LDL receptor-deficient (Watanabe Heritable Hyperlipidemic, WHHL) rabbits from the colony of the La Jolla Specialized Center of Research on Arteriosclerosis were divided into three groups. Animals were matched for gender, litter, and initial plasma cholesterol levels. Rabbits were fed regular rabbit chow (#7009, Teklad Test Diets, Madison, WI) with or without added antioxidants. Probucol was a generous gift from Hoechst Marion Roussel Research Institute, Cincinnati, OH. The probucol analogue BM15.0639 was a generous gift from Boehringer Mannheim, Mannheim, Germany. Vitamin E (D-α-tocopherol acetate) was purchased from Sigma Chemical Co., St. Louis, MO. All antioxidants were dissolved in diethyl ether and added to the chow which was then air dried for at least 24 h. The control chow was also treated with the solvent. Rabbits were fed 110 g per day for 24 weeks, starting at 6 weeks of age.

Analytical methods

Blood samples were collected from an ear vein in tubes containing EDTA. Total plasma cholesterol and triglyceride levels were determined using an automated enzymatic technique (Boehringer Mannheim Diagnostics). Plasma levels of vitamin E, probucol, and the probucol analogue were determined by HPLC as previously described (14, 16). The concentration of the antioxidants in aortic and carotid tissues was determined by the same HPLC method after enzymatic digestion, homogenization, and extraction of tissues as described (16). Because the procedure used to determine the extent of lesions in the aorta (see below) includes fixation and staining in organic solvents, which rules out a subsequent determination of the antioxidant concentrations, one unfixed aorta from each group was used for these measurements. Three 1-cm long segments of the arch, the thoracic aorta, and the upper abdominal aorta of each aorta were assayed separately. In addition, antioxidant concentrations in seven animals from each group were determined in the carotid arteries, which were dissected, tied off at both ends, and removed prior to perfusion-fixation of the systemic circulation.

Isolation of LDL and determination of lag-times

LDL (d 1.021–1.060 g/ml) was isolated from plasma by sequential ultracentrifugation, as previously described (16, 27). The LDL was then extensively dialyzed against PBS and sterile-filtered. Protein concentrations were determined with the BCA Protein Assay (Pierce, Rockford, IL) using BSA as the standard. The resistance of LDL to oxidation was determined immediately after isolation and dialysis. LDL was diluted to 100 μg/ml with PBS and Cu^{2+} was added at a final concentration of 5 μmol/l. The formation of conjugated dienes at 30°C was determined by measuring the absorption at 234 nm in a Uvikon 910 spectrophotometer for 24 h. Absorption at the beginning of the reaction was set to zero. Lag-times were determined graphically as the time point at which the tangent to the curve at the maximum slope of the propagation phase intercepted the time axis (18).

Quantification of atherosclerosis

At the end of the study period, rabbits were injected intravenously with 1,000 IU heparin and killed with an

overdose of sodium pentobarbital. The systemic circulation was perfused at a pressure of 80 mm Hg with 1 liter of isotonic PBS containing 2 mmol/l EDTA, pH 7.4, through a large-bore cannula inserted into the apex of the left ventricle, allowing free efflux from an incision in the right ventricle. Segments of both carotid arteries (approximately 3 cm long) were tied off at both ends and removed for determination of antioxidant concentrations (see above). The aorta was then fixed in situ with formal-sucrose (4% paraformaldehyde, 5% sucrose, 20 μmol/l BHT, 2 mmol/l EDTA, pH 7.4) for 20 min. The entire aorta was dissected and cleaned of loose adventitial tissue. The thoracic and abdominal aortas were divided 5 mm proximal to the celiac artery. Each segment was opened longitudinally and fixed in formal-sucrose for an additional 24 h. The aortas were stained with Sudan IV, pinned flat on black wax beds using 0.2-mm diameter stainless steel pins (Fine Science Tools, Foster City, CA), and covered with PBS. Digital images of the aorta were captured with a Sony DXC-960MD color video camera. The extent of atherosclerosis was determined by computer-assisted image analysis using Optimas 4.1 software (Bioscan, Seattle, WA), as previously described (14, 28). Results were expressed as percent of the arch, thoracic, abdominal, or entire aorta covered by atherosclerotic lesions.

Statistical analysis

Results are expressed as mean ± SEM. Differences between treatment groups were assessed by analysis of variance (ANOVA). The statistical analyses were performed using Excel 5.0 and Systat 5.01 statistical software (Systat, Evanston, IL).

RESULTS

To test whether the same degree of antioxidant protection would lead to the same antiatherogenic effect, we studied three groups of 10 LDL receptor-deficient rabbits matched for plasma cholesterol, triglycerides, litter, and gender (**Table 1**). The first (combination group) was treated with a combination of antioxidants (1,000 IU vitamin E, 0.05% probucol analogue BM15.0639, and 0.025% probucol), the second (probucol group) received varying doses of probucol ranging from 0.0575 to 0.11%, and the third (untreated group) was a true control. We had initially tried to maximize the level of antioxidant protection of LDL by combining the same doses of vitamin E (1,000 IU) and probucol analogue BM15.0639 (0.05%) used in our previous studies (14, 16). However, preliminary in vivo experiments indicated that this yielded lag-times considerably smaller than those typically obtained with 1% probucol. Because no further increase in antioxidant protection of LDL could be achieved by increasing the dose of vitamin E or the analogue (data not shown), we added a very low dose of probucol (0.025%) to the antioxidant combination. A previous study had indicated that 0.025% probucol by itself does not reduce atherogenesis in rabbits (29). Addition of 0.025% probucol to our antioxidant combination resulted in a substantial further increase in lag-times, as indicated below.

To avoid differences in the plasma cholesterol levels among the three groups resulting from lipid-lowering effects of probucol, the plasma cholesterol levels of the combination and the probucol group were matched to that of the control group by adding small amounts of cholesterol to their diets. This was aided by a preliminary experiment assessing the hypocholesterolemic effect of various doses of probucol, in which four matched groups of 3 LDL receptor-deficient rabbits each had been fed a diet containing 0.05%, 0.1%, 0.25%, and 0.5% probucol. After 16 days of treatment, plasma cholesterol levels were decreased by 18.1%, 27.9%, 23.1%, and 22.3%, respectively, compared to the levels prior to intervention. These results not only

TABLE 1. Body weight, total plasma cholesterol, and triglyceride levels

Variable	Treatment Group		
	Untreated (n = 10)	Combination (n = 10)	Probucol (n = 10)
Body weight (kg)			
Initial	0.95 ± 0.09	0.98 ± 0.09	0.84 ± 0.08
Final	2.68 ± 0.09	2.70 ± 0.06	2.60 ± 0.06
Plasma cholesterol level (mg/dl)			
Time-average	814 ± 19	906 ± 35[a]	891 ± 31[a]
Plasma triglyceride level (mg/dl)			
Time-average	636 ± 96	623 ± 124	672 ± 107

Data represent mean ± SEM.
[a] $P < 0.05$ compared to the untreated group.

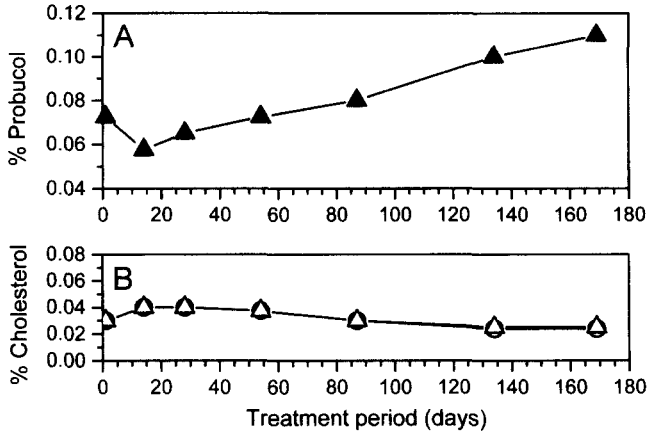

Fig. 1. **A:** Amount of probucol added to the diet of the probucol group. Variable amounts of probucol were administered to this group in order to match the extent of antioxidant protection of plasma LDL (lag-time) to that of the combination group receiving a constant dose of antioxidants (1000 IU vitamin E, 0.05% probucol analogue BM15.0639, and 0.025% probucol). **B:** Amount of cholesterol added to the diets of the probucol group and the combination group. Cholesterol was added to the chow of these groups to compensate for the cholesterol-lowering effect of probucol and the antioxidant combination, i.e., to maintain similar plasma cholesterol levels in all three experimental groups; △, probucol group; ○, combination group.

confirmed that in this animal model probucol has a significant lipid-lowering effect even at a very low dose, but that within the range of concentrations tested the hypocholesterolemic effect was similar. Thus, in the intervention study, minor variations in the dose of probucol in the probucol group would not significantly affect the plasma cholesterol levels.

The amount of each antioxidant in the combination group was kept constant throughout the study period (1000 IU vitamin E, 0.05% probucol analogue BM15.0639, and 0.025% probucol per kg diet). The amount of probucol added to the diet of the probucol-only group was adjusted throughout the intervention period to match the lag-time of the antioxidant combination (**Fig. 1A**). As shown, the dose ranged from 0.0575% to 0.11%, with a time-average of 0.091%. The amount of cholesterol added to the chow of the combination group and the probucol group varied between 0.024% and 0.040% (Fig. 1B) with a time-average of 0.031%. The two curves are almost identical, even though the dose of probucol administered to the probucol group was increased almost 2-fold towards the end of the intervention period. Note that during the intervention period, the weight of the animals in the probucol-only group increased from 0.84 to 2.60 kg. Therefore, increasing amounts of probucol were needed merely to maintain the plasma concentration of the drug.

Plasma antioxidant concentrations, cholesterol and triglyceride levels, and body weights

Plasma cholesterol and triglyceride levels were determined prior to the start of the study and subsequently at 2- to 5-week intervals. **Figure 2** shows that the average plasma cholesterol levels in the combination and probucol groups were very similar over the entire treatment period. Levels in the untreated group tended to be lower, but the difference between the untreated group and both antioxidant groups only reached significance at day 70. The difference between the untreated group and the combination group was also significant at day 105. The time-averaged cholesterol level in the untreated group was 814 ± 19 mg/dl, significantly lower than in the probucol group (891 ± 31 mg/dl, $P = 0.047$) and the combination group (906 ± 35 mg/dl, $P = 0.034$) (Table 1). No significant difference was found between the probucol and the combination group ($P = 0.762$). Plasma triglyceride levels in the three groups were similar. Likewise, the average body weights in the three groups increased in parallel throughout the 24 weeks of intervention (Table 1).

The concentrations of vitamin E, probucol analogue, and probucol in the diet, the plasma, and the carotid arteries are shown in **Table 2**. As expected, plasma concentrations generally reflected the amount of compound added to the diet. For example, the plasma concentration of probucol in the probucol-only group that received an average dose of 0.091% probucol was 4-fold higher than in the combination group that received a constant dose of 0.025%. The combination group that received 1000 IU of vitamin E showed a 4-fold higher vi-

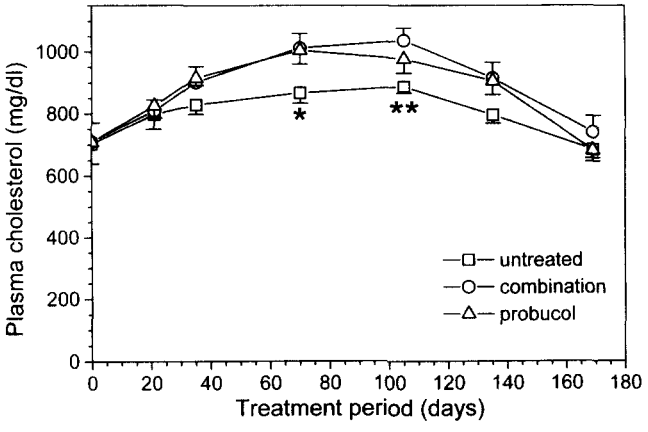

Fig. 2. Total plasma cholesterol levels. Blood was collected from each rabbit at the time points indicated and plasma cholesterol was determined as described in Methods. Data represent the mean ± SEM (n = 10). The plasma cholesterol levels of the untreated group were significantly lower ($P < 0.05$) than those of the combination and probucol groups on day 70 (*) and that of the combination group on day 105 (**).

TABLE 2. Concentration of antioxidants

	Treatment Group		
	Untreated	Combination	Probucol
Vitamin E			
In the diet (IU/kg)	40	1000	40
In plasma (time average, μmol/l, n = 10)	91.4 ± 6.9	422.2 ± 51.7[a]	97.0 ± 7.0
In carotid tissue (ng/mg, n = 7)	3.6 ± 0.4	15.0 ± 0.75[a]	4.0 ± 0.14
Analogue			
In the diet (%)	none	0.05	none
In plasma (time average, μmol/l, n = 10)	N.D.	124.8 ± 26.2	N.D.
In carotid tissue (ng/mg, n = 7)	N.D.	14.1 ± 2.96	N.D.
Probucol			
In the diet (%)	none	0.025	0.091*
In plasma (time average, μmol/l, n = 10)	N.D.	33.5 ± 3.7	128.6 ± 11.6[b]
In carotid tissue (ng/mg, n = 7)	N.D.	4.2 ± 0.8	11.5 ± 3.2[c]

Plasma from each rabbit was obtained and the antioxidant concentration was determined by HPLC analysis. Tissue concentrations were determined as described in Methods. Data represent mean ± SEM; N.D., not detectable; *, time average.

[a] $P < 0.001$ compared to both the untreated and the probucol groups.

[b] $P < 0.001$ compared to the combination group.

[c] $P = 0.145$ compared to the combination group.

tamin E concentration in the plasma than the control group. The baseline plasma concentration of vitamin E determined in the untreated group at day 21 was 77.1 ± 3.9 μmol/l. The time-averaged plasma vitamin E concentrations were 91.4 ± 6.9 μmol/l in the untreated group, 97.0 ± 7.0 μmol/l in the probucol group, and 422.2 ± 51.7 μmol/l in the combination group. The plasma concentration of vitamin E in the combination group was similar to that previously achieved by treating rabbits with the same dose of vitamin E only (14). The plasma concentration of the probucol analogue in the combination group was 124.8 ± 26.2 μmol/l, very similar to the concentrations reported in our earlier study (16). The plasma concentration of probucol in the probucol only group was significantly higher than that in the combination group (128.6 ± 11.6 vs. 33.5 ± 3.7 μmol/l, $P < 0.001$).

The antioxidant concentration in the combined tissue of the left and right carotid arteries was determined in a subset of seven animals and generally reflected the plasma levels (Table 2). None of the compounds showed selective accumulation in the carotid artery. In addition, the levels of all three antioxidants were measured in three aortic segments (arch, thoracic and abdominal aorta) of one animal from each treatment group. Whereas the above measurements in the carotid arteries represent antioxidant concentrations in macroscopically normal arterial tissues, the aortic segments varied in their extent of atherosclerosis. We therefore separately determined the concentration of the antioxidants in lesioned and non-lesioned aortic tissue. Concentrations in normal aortic segments were about 4-fold greater than in the normal carotids and were dramatically higher in lesion-containing segments (data not shown).

Antioxidant protection of LDL

The antioxidant protection of plasma LDL was determined by following conjugated diene formation in a standardized assay using LDL isolated from each individual animal. All determinations were performed within 3 days after LDL isolation and every effort was made to ensure identical assay conditions. **Figure 3** shows the average lag-times in each group throughout the intervention period. Compared to the untreated group, a highly significant increase in lag-times was observed in both the combination and the probucol group ($P < 0.001$). Animals treated with the antioxidant combination showed consistently greater lag-times than animals treated with probucol only (except at the final time point). However, the difference only reached statistical significance ($P < 0.05$) at two time points (days 21 and 135). The time-averaged lag-times were 952 ± 39 min in the combination group, 829 ± 46 min in the probucol group, and 214 ± 14 min in the untreated group. The difference in the time-averaged lag-time between the combination and the probucol group was marginally significant ($P = 0.058$).

Extent of atherosclerosis

Atherosclerosis was determined morphometrically and expressed as the percentage of aortic surface area covered by Sudan-positive lesions (**Fig. 4**). The probucol group showed a 51.7% reduction in atherosclerosis in the entire aorta compared to the untreated control group ($P = 0.005$). The treatment effect was most striking in the thoracic region (minus 64.8%, $P = 0.029$). Atherosclerosis in the arch and abdominal aorta was reduced by 39.8% ($P = 0.0007$) and 52.1% ($P = 0.02$),

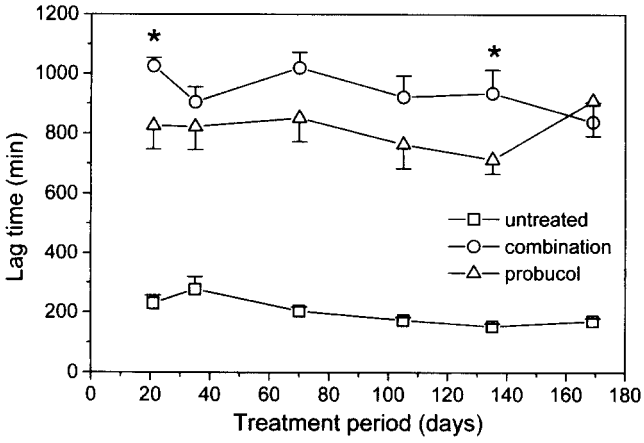

Fig. 3. Resistance of plasma LDL to oxidation. LDL was isolated by sequential ultracentrifugation from the individual plasma samples obtained at the time points shown in Fig. 1. After extensive dialysis against PBS and sterile filtration, 5 μmol/l $CuSO_4$ was added to each individual LDL (100 μg of protein/ml) and the formation of conjugated dienes was measured at 234 nm. The initial absorption was set to zero and the lag-times were determined as the intersection of the tangent to the curve at its maximum slope with the x-axis. Data represent mean ± SEM (n = 10). The difference between the untreated group and both the combination group and the probucol group was highly significant ($P < 0.001$). Differences between the probucol and the combination group reached significance ($P < 0.05$) only at days 21 and 135 (*).

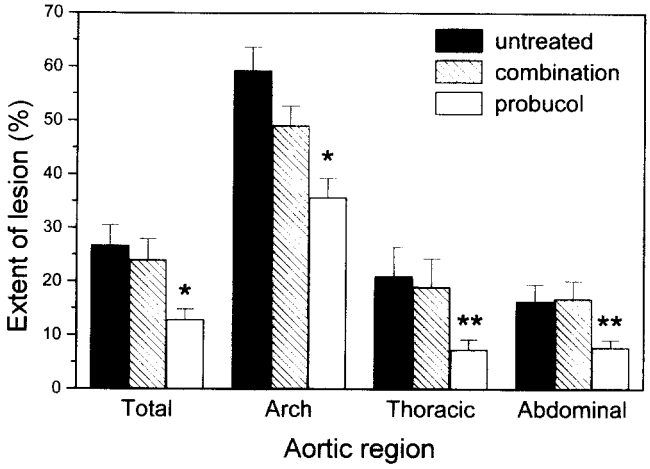

Fig. 4. Extent of atherosclerosis. At the end of the 24-week intervention period, rabbits were killed and the aortas were dissected and Sudan-stained as described in Methods. Lesions were quantitated by computer-assisted image analysis and results were expressed as percent of the aortic surface covered by atherosclerotic lesions. Data are mean ± SEM, n = 9 for each group. (The aorta from the 10th animal in each group was used for analysis of tissue drug levels.) *, $P < 0.005$ compared to the untreated group; **, $P < 0.05$ compared to the untreated group. The differences between the probucol group and the combination group were also significant ($P < 0.05$) in the entire aorta and in all segments except in the thoracic aorta ($P = 0.055$).

respectively. In stark contrast, the antioxidant combination failed to reduce lesion formation in the entire aorta (24.0% vs. 26.7% in the untreated group), as well as in the arch, thoracic and abdominal segments. The difference between the probucol and the combination group was significant (12.9% vs. 24.0%, $P = 0.021$).

Although clearly no correlation existed between lag-times and atherosclerosis when data from the different treatment groups were pooled, a weak inverse correlation between the extent of atherosclerosis in the entire aorta and the lag-times was found within the probucol group ($r = 0.65$, $P = 0.043$) (**Fig. 5**). No such correlation was found when data of the combination and untreated groups were analyzed separately. The plasma cholesterol levels did not correlate with lesion area in the entire aorta in any of the three groups (data not shown). The same was true when animals of the same litter or gender were compared.

DISCUSSION

It is generally accepted that LDL in the plasma is well protected from oxidation and that the atherogenic byproducts of OxLDL are formed primarily in extravascular domains in which prooxidant conditions prevail, including the arterial wall (1). Nevertheless, it would appear that the resistance of plasma LDL to oxidation should to some degree determine the extent of LDL oxidation in the vascular wall. The measurement of the resistance of plasma LDL to oxidation not only reflects the composition of LDL (e.g., its content in antioxidants, polyunsaturated fatty acids, etc.) but is also influenced by the extent of oxidation taking place in other tissues, in particular under conditions of hypercholesterolemia which increase the oxidative stress (30, 31). The resistance of plasma LDL to oxidation is therefore widely assumed to be a good indicator of the atherogenic risk (14, 16, 18–26, 32–34). However, the present study does not support this assumption.

In our intervention study, a relatively low dose of probucol (0.091%) resulted in a remarkable reduction of atherosclerosis (minus 51.7% in the entire aorta compared to the untreated control group; Fig. 4), whereas the antioxidant combination failed to reduce atherogenesis, even though it yielded equal or even greater antioxidant protection of plasma LDL than probucol (Fig. 3). These results demonstrate that the lag-time in the formation of conjugated dienes is not correlated with the antiatherogenic efficacy of different antioxidants, at least in the WHHL model characterized by high levels of plasma cholesterol. In contrast, we saw a weak but significant correlation between these

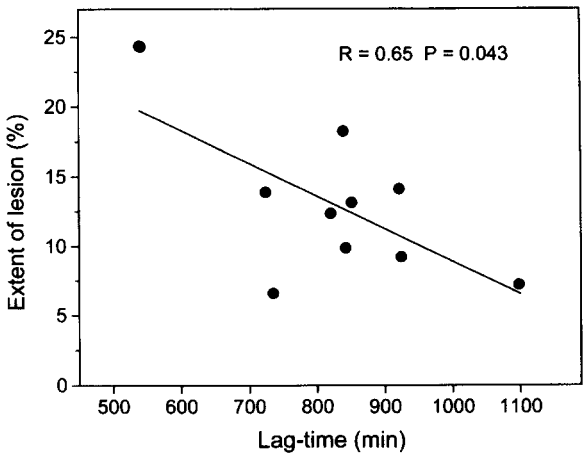

Fig. 5. Correlation between lag-time and extent of atherosclerosis in the group treated with probucol only. For each animal, the lag-time indicated in the figure represents the average value (area under the curve divided by time) of the lag times determined at days 21, 70, 105, 135, and 169 of the intervention period. Atherosclerosis was measured as the percent surface area of the entire aorta staining with Sudan IV.

two parameters within the group treated with probucol only, in agreement with a similar observation by Sasahara et al. (9) in probucol-treated primates. The lag-time provides a measure of the amount of active antioxidant present in LDL, and increasing lag-times may therefore be correlated with increasing antiatherogenic effect of an individual compound (or compounds sharing the same pharmacokinetic properties in the vascular wall). However, the degree of antioxidant protection of plasma LDL by itself is clearly not a comprehensive predictor of the extent of atherogenesis, as generally assumed.

Two different explanations may account for the fact that the same degree of protection of plasma LDL obtained by different lipophilic antioxidants resulted in vastly different antiatherogenic effects. The first is that the concentrations of individual antioxidants in plasma may not reflect the concentrations or activity of these compounds in the arterial wall. The second explanation is that probucol, as well as other antioxidants that reduce atherogenesis, such as BHT and DPPD, may act by mechanisms not shared by all antioxidants.

In theory, a greater selective accumulation of probucol might account for the reduction in lesion formation. Our measurements of plasma and tissue concentrations provided no indication for such differences in bioavailability (Table 2). None of the compounds showed selective accumulation in the carotid arteries or in the aorta. Tissue levels of probucol in the probucol group and probucol analogue in the combination group were similar, and if one also considers vitamin E and probucol in the combination group, the overall antioxidant concentration in arterial tissues of rabbits treated with the antioxidant combination was greater than that of animals receiving only probucol.

However, two important caveats have to be considered when comparing antioxidant levels. First, at least one of the probucol metabolites is itself a powerful antioxidant, whereas the same is not true for the analogue. This metabolite would not show in the measurement of the arterial probucol concentration. However, its protective effect would be reflected by the lag-time, assuming the metabolite was present in plasma LDL at similar concentrations as in the artery wall. The second caveat is more important. The concentrations of antioxidants determined in arterial tissues do not necessarily reflect the concentration of the compounds in smaller compartments, in particular the intracellular concentration. Substantial evidence points to the involvement of oxidative processes at the intracellular level in atherogenesis. Liao et al. (30, 35) have recently proposed mechanisms by which lipid peroxidation products, such as those generated during the oxidative modification of LDL, may activate NFκB-like transcription factors in vascular cells and induce expression of genes that in turn may enhance atherogenesis, such as VCAM-1, MCP-1, and other cytokines (for review see 4). Khan et al. (36) specifically showed that OxLDL increases cytokine-induced expression of VCAM-1 by an oxidation-sensitive regulatory mechanism. Antioxidants may affect the intracellular redox potential, either by reducing extracellular formation of lipid oxidation products that may be taken up into the cell or by exerting an antioxidant effect intracellularly. Indeed, several studies have reported that various antioxidants, including vitamin E, inhibit gene up-regulation induced by oxidation products in vitro (36–39). We have recently shown that probucol also lowers the basal expression of the monocytic adhesion molecule VCAM-1 in the artery wall of LDL receptor-deficient rabbits and prevents the up-regulation of VCAM-1 gene and protein expression that occurs during lesion formation in vivo (40). Some of the modulation of cellular gene expression may represent a direct cellular effect of the antioxidants independent of the inhibition of LDL oxidation.

Alternatively, the fact that the addition of high doses of vitamin E and probucol analogue to 0.025% probucol did not reduce lesion formation, whereas 0.091% probucol did, may be interpreted as evidence for a distinct antiatherogenic mechanism of probucol. In addition to exerting a more powerful antiatherogenic effect as a result of different pharmacokinetics in the artery wall, probucol may have inhibited atherogenesis by specific cellular effects. For example, probucol may have inhibited IL-1-induced monocyte adhesion to endo-

thelial cells (41, 42). Some of these cellular effects of probucol may even be unrelated to its antioxidant properties. A recent report by Shaish et al. (43) may support this notion. In this study, all-*trans* β carotene reduced atherogenesis in cholesterol-fed NZW rabbits in the absence of any antioxidant effect on plasma LDL. β-Carotene may be converted into derivatives of retinoic acid which could modulate gene expression of vascular cells. However, it is unknown whether modulation of gene expression is indeed responsible for the apparent antiatherogenic effect of β carotene and whether probucol affects the same regulatory pathways. The fact that the antiatherogenic potency of probucol is shared by structurally unrelated antioxidants, such as DPPD, and that a similar inhibition of IL-1 induced monocyte adherence in vitro is also caused by vitamin E (41) indicates that such effects are probably due to the antioxidant properties of probucol. Nevertheless, our results clearly suggest that differences in antiatherogenic mechanisms exist between different antioxidants at the level of the arterial wall that are not due to or reflected by the antioxidant protection of LDL. The difference in antiatherogenic efficacy of various antioxidants is likely to result from their effects on vascular cells.

Although the atherogenicity of OxLDL, or lipid oxidation products in general, is not in question, our results do not support the widespread assumption that the susceptibility of plasma LDL to oxidation is an indicator of "atherogenic risk." The lag-time is clearly not a valid parameter to compare the antiatherogenic potency of different antioxidants. Even less certain is the value of comparisons of the ex vivo resistance of LDL to oxidation for predicting CHD risk in patients. Our results also disprove the idea that a threshold degree of protection of plasma LDL (i.e., a threshold lag-time) has to be achieved by an antioxidant in order to obtain an antiatherogenic effect. Although the concept of a certain level of antioxidant protection being required to affect atherogenesis remains a valid one, the parameter that accurately reflects lipid oxidation in plasma, in the artery wall, in particular intracellular oxidation in vascular cells, will have to be redefined.

The lag-time is generally preferred to parameters reflecting only the extent of oxidation that has already occurred (measurements of lipoperoxides or TBARS, or functional assays measuring chemotactic effects on monocytes or uptake by macrophages) because it provides a cumulative measure of various factors that influence LDL oxidation (i.e., its content of natural and synthetic antioxidants, its fatty acid composition, the presence of oxidized lipids, etc.). The standardized lag-time assay uses a fixed concentration of Cu^{2+} to trigger oxidation of polyunsaturated fatty acids in LDL, presumably by abstraction of a proton from a preexisting hydroperoxy group and generation of a peroxy radical. However, it is unknown whether this reflects other oxidative processes hypothesized to contribute to the oxidation of LDL in the artery wall, such as free radicals generated by vascular cells or extracellular lipid oxidation, free metal ions, cellular enzymes such as lipoxygenases (44, 45), or the myeloperoxidase hydrogen peroxide system (46, 47). Furthermore, the relative contribution of these mechanisms to the oxidation of LDL in vivo remains uncertain.

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