Inhibition of VCAM-1 expression in the arterial wall is shared by structurally different antioxidants that reduce early atherosclerosis in NZW rabbits

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Abstract **We previously established that probucol decreases basal expression of VCAM-1 in the aorta of WHHL rabbits and inhibits the up-regulation of VCAM-1 expression that normally accompanies atherogenesis. To determine whether this effect is shared by other antioxidants in vivo, we now investigated whether a structurally unrelated antioxidant, vitamin E, also inhibits arterial VCAM-1 expression and whether the degree of VCAM-1 inhibition correlates with the reduction of atherosclerosis or the antioxidant protection of LDL. Atherogenesis and VCAM-1 mRNA and protein were determined in four groups of NZW rabbits (n = 6–8) fed 0.5% cholesterol alone or supplemented with 0.1% vitamin E, a low dose (0.04–0.075%) of probucol yielding the same degree of antioxidant protection of plasma LDL as vitamin E, or a high dose (0.5%) of probucol, and in normocholesterolemic rabbits. After 81 days, extensive atherosclerosis and a greater than 4-fold up-regulation of VCAM-1 mRNA was seen in rabbits on high cholesterol diet, mostly in the intima. Treatment with vitamin E, high-dose probucol, and low-dose probucol significantly decreased VCAM-1 mRNA by 49.0, 74.9, and 57.5%, respectively, and reduced atherosclerosis in adjacent segments of the thoracic aorta to a similar degree as reported by previous studies. Immunocytochemistry confirmed that lesions of antioxidant-treated animals also contained less VCAM-1 protein. Neither the degree of VCAM-1 inhibition nor the extent of atherosclerosis correlated with the degree of antioxidant protection of plasma LDL. In summary, treatment with structurally unrelated antioxidants conveyed different degrees of antioxidant protection to plasma LDL but significantly reduced VCAM-1 expression in vivo and inhibited atherogenesis. This is consistent with the assumption that antiatherogenic effects of antioxidants may in part be mediated by interference with oxidation-dependent intracellular signaling.**—Fruebis, J., M. Silvestre, D. Shelton, C. Napoli, and W. Palinski. **Inhibition of VCAM-1 expression in the arterial wall is shared by structurally different antioxidants that reduce early atherosclerosis in NZW rabbits.** ***J. Lipid Res.*** **1999.** 40: **1958–1966.**

Supplementary key words adhesion molecules • antioxidants • arteriosclerosis • immunocytochemistry • oxidation • oxidized LDL • PCR • probucol • vitamin E

The recruitment of circulating monocytes into the intima is a key event in early atherogenesis. The vascular adhesion molecule 1 (VCAM-1), which interacts with the monocyte integrin VLA-4 (1), is assumed to play an important role in the adhesion of monocytes to the endothelium. VCAM-1 is expressed on endothelial cells overlying early fatty streaks of rabbits (2–5) and at sites of inflammation in human arteries (6, 7). In addition, VCAM-1 is expressed by vascular smooth muscle cells (8), preferentially in the deeper subendothelial layers near the internal elastic lamina (5, 8). Under laminar flow and low shear stress, VCAM-1 supports monocyte adhesion even on unactivated cultured endothelial cells (9). However, little is known about the contribution of VCAM-1 to the recruitment of monocytes into the intima of large arteries in vivo.

A number of atherogenic factors have been reported to increase VCAM-1 expression by cultured endothelial cells or in vivo. These include hypercholesterolemia (3, 4), which presumably acts by increasing lipid peroxidation (10), lysophosphatidylcholine (a byproduct of LDL oxidation) (11, 12), and advanced glycation endproducts (4, 13). Oxidized LDL (OxLDL) itself also enhances VCAM-1 expression by endothelial cells, but only in endothelial cells stimulated with cytokines, such as interleukin 1 or tumor necrosis factor α (14–16). Increasing evidence suggests that oxygen radicals act as second messengers in the NFκB pathway that regulates expression of many factors potentially involved in monocyte recruitment, including VCAM-1 and monocyte chemotactic protein 1 (MCP-1) (17). It is therefore conceivable that increased lipid peroxidation shifts the intracellular redox status and thus en-

Abbreviations: GAPDH, glyceraldehyde phosphate dehydrogenase; LDL, low density lipoprotein; MCP-1, monocyte chemotactic protein 1; mmLDL, minimally modified LDL; NZW, New Zealand white; OxLDL, oxidized LDL; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen 4; WHHL, Watanabe heritable hyperlipidemic.

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hances VCAM-1 expression. Mechanisms by which lipid peroxidation products could activate NFκB-like transcription factors in vascular cells have been proposed by Liao and colleagues (18, 19). OxLDL or byproducts of lipid oxidation enhance VCAM-1 expression by endothelial cells (12, 14–16). Evidence for the role of oxidative processes also includes the observation that several antioxidants inhibit VCAM-1 expression in cultured human umbilical vein cells (16, 20, 21).

Lipophilic antioxidants such as probucol, probucol analogues, butylated hydroxytoluene (BHT), and diphenylphenylenediamine (DPPD) are powerful inhibitors of atherogenesis in rabbits, primates, and mice (22–29). Vitamin E also inhibited atherosclerosis in some but not all studies performed in rabbit models (30–34). The antiatherogenic effect of these compounds is widely attributed to the antioxidant protection of LDL and other lipoproteins (35, 36), but to date it is not clear which of the atherogenic mechanisms induced by oxidation is most relevant in vivo. We recently demonstrated that probucol reduced the basal expression of VCAM-1 in vivo and completely prevented both atherogenesis and the up-regulation of VCAM-1 expression that normally accompanies lesion formation in WHHL rabbits (5). In contrast, probucol did not affect expression of MCP-1 (5). However, it could not be ruled out that inhibition of VCAM-1 expression resulted from effects specific to probucol, rather than from intra- or extracellular antioxidant effects.

To test the hypothesis that antioxidants may inhibit atherogenesis by reducing the expression of atherogenic genes, we now investigated whether a structurally different antioxidant, vitamin E, also reduces gene and protein expression of VCAM-1 and whether the antiatherogenic efficacy of probucol and vitamin E is linked to the degree of VCAM-1 inhibition or antioxidant protection of LDL. For this purpose, we determined VCAM-1 expression and early atherosclerosis in normo- and hypercholesterolemic NZW rabbits and in cholesterol-matched rabbits treated with vitamin E, a low dose of probucol that yields the same antioxidant protection of plasma LDL, or a high dose of probucol.

METHODS

Animals and experimental design

Four groups of male NZW rabbits matched for body weight and baseline plasma cholesterol level were fed a diet containing 0.5% cholesterol (high cholesterol control group) or high cholesterol diets supplemented with either 0.1% vitamin E (d-α-tocopherol; Sigma) (vitamin E group), a variable dose (0.04–0.075%) of probucol (low-dose probucol group), or 0.5% probucol (high-dose probucol group) for 81 days. The cholesterol content of the diet given to the high cholesterol control group (n = 8) was kept constant (0.5%) throughout the study. The plasma cholesterol level in the three antioxidant-treated groups (n = 6, each) was matched to that achieved in the high cholesterol control group by adjusting the cholesterol content of their diets. The antioxidant protection of plasma LDL in the vitamin E and low-dose probucol groups was matched. This was achieved by gradually reducing the dose of probucol in the low-probucol group from 0.075% to 0.04%. Both vitamin E and probucol slightly lowered plasma cholesterol levels. To achieve similar plasma cholesterol levels in all four groups, the amount of dietary cholesterol given to the three antioxidant groups (0.50–0.65%) was therefore slightly adjusted at several time points. The time-averaged dietary cholesterol concentration in all three antioxidant treatment groups was 0.57%. In addition to the four high cholesterol groups, a group of 8 animals was used to determine baseline levels of VCAM-1 expression in normocholesterolemic, non-atherosclerotic rabbits (low cholesterol control group).

Probucol and vitamin E were added to the chow dissolved in diethyl ether. The control chow was similarly treated with solvent only. Animals received a fixed amount of food (100 g/day) and water ad libitum. Total plasma cholesterol and triglycerides were measured by automated enzymatic assay with an Abbott VP bichromatic analyzer (Abbott Laboratories), high performance cholesterol reagent kit (No. 704036, Boehringer Mannheim Diagnostics), and triglyceride reagent (No. 6097, Abbott Laboratories). Plasma probucol and vitamin E levels were determined by HPLC, as described (27). Plasma LDL was isolated by ultracentrifugation using standard procedures (27, 37). The protection of plasma LDL against in vitro oxidation was determined at days 9, 33, and 71 of the study, using 5 μmol/l copper ions as prooxidant (27, 38, 39). A summary of the experimental design is provided in Table 1.

At the end of the intervention period, the animals were heparinized and given an overdose of sodium pentobarbital (150–200 mg). The systemic circulation was perfused with ice-cold RNAse-free PBS containing 2 mmol/l EDTA and the descending thoracic aorta was dissected. The aorta was thoroughly cleaned of adventitia, opened longitudinally and pinned out, all the while being kept submerged in ice-cold PBS. An electronic image of the artery was captured as described (40). The proximal part of the descending thoracic aorta comprising the first three pairs of intercostal artery orifices was then divided into three equal segments, each containing one pair of branch sites. The most proximal, segment 1, was further subdivided longitudinally between the orifices of the intercostal arteries in two equal segments (1a and 1b), each containing one branch site. Segment 3 was subdivided the same way into 3a and 3b, whereas segment 2 was left intact. A fourth segment of the thoracic aorta containing the 4th–6th pairs of intercostal artery orifices was also divided longitudinally into segments 4a and 4b.

Segments 1a and 3a were fixed with 4% paraformaldehyde, paraffin-embedded, and used for immunocytochemistry with antibodies against macrophages and smooth muscle cells. Segments 1b and 3b were snap frozen in OTC embedding media, cryo-sectioned, and used for immunocytochemistry with an antibody to VCAM-1. Segments 2 and 4a were frozen in liquid nitrogen and used for RNA isolation and RT-PCR analysis. To obtain separate quantitation of VCAM-1 mRNA expression in the intima and media, segment 4b was first dissected under a stereo microscope. As a clean separation of intima and media is difficult, dissection was performed so that the medial tissue was completely free of intima. The intimal sample could still contain a small amount of medial smooth muscle cells. Both tissues were then frozen and processed the same way as segments 2 and 4a. The above dissection scheme was chosen to ensure that tissues used for PCR and immunocytochemistry contain similar lesions. We have previously shown that this is true for the lesions formed distal to adjacent intercostal artery orifices (5).

Determination of VCAM-1 gene expression

The procedures used to isolate RNA and to perform the reverse transcription and PCR reaction were identical to those previously described in detail (5). Briefly, total RNA was isolated from frozen tissue within 24 h after preparation using an adaptation of

the method of Chomczynski and Sacchi (41). The tissue was homogenized in 1 ml of RNAzol B™ (Tel-Test) using a polytron PT1200 at setting 4 for 3 × 20 s. RNA was prepared according to the manufacturer's protocol and the amount of RNA isolated was determined by measuring absorption at 260 nm. Quantitative measurement of VCAM-1 mRNA by PCR was then performed as previously described, using GAPDH as internal standard (5).

Determination of atherosclerosis

The extent of atherosclerosis was determined on the electronic images of the unstained thoracic aortas (area of segments 1–4) captured prior to dissection, using Optimas image analysis software (Bioscan Optimas). Image analysis was performed in analogy to the method described in great detail in reference 40, except that black and white images were used. Results were expressed as the percentage of the arterial surface covered by atherosclerotic lesions.

Immunocytochemistry

After fixation in paraformaldehyde and embedding in paraffin, 7-μm thick sections were prepared from segments 1a and 3a and immunostained with monoclonal antibodies against macrophages (RAM 11; 1:1000 dilution (42)) and muscle cell actin (HHF-35; 1:500 dilution (43)), using an avidin–biotin–alkaline phosphatase method (5). Serial 8-μm thick frozen sections from segments 1b and 3b on coated slides were fixed for 5 min with acetone at −20°C and immediately immunostained with Rb1/9 (1:100 dilution), a monoclonal antibody against rabbit VCAM-1 (a generous gift from Dr. M. I. Cybulsky and Dr. M. A. Gimbrone) (3), using an avidin–biotin–peroxidase system (5). For both immunostaining methods, control sections in which the primary antibody was omitted were devoid of specific staining.

Statistical analysis

Results were expressed as mean ± SEM. Differences among groups were assessed by ANOVA followed by Student's *t*-test (consistent with variance and normal distribution of data), using Systat software (Systat Inc).

RESULTS

Probucol markedly lowers plasma cholesterol levels and vitamin E may also have a mild hypolipidemic effect in rabbits (22, 23, 27, 30, 33, 44). By slightly adjusting the amount of dietary cholesterol given to the antioxidant-treated groups, the plasma cholesterol levels in the two probucol groups and the vitamin E group were kept at very similar average levels throughout the 81 days of intervention (**Fig. 1**). The time-averaged plasma cholesterol level in the high cholesterol control group was 1157 ± 138 mg/dl, compared to 1178 ± 27, 1149 ± 70, and 1112 ± 112 mg/dl in the low and high dose probucol and vitamin E groups, respectively. Plasma cholesterol in the additional control group fed regular rabbit chow was 39 ± 4.4 mg/dl.

Plasma levels of probucol and vitamin E were determined twice during the intervention period and at the end of the study and showed no significant changes over time. The final antioxidant concentrations are shown in **Table 1**. As expected, the high cholesterol control group showed almost 3-fold higher plasma vitamin E levels, compared to the low cholesterol control group. A further 5-fold increase was observed in the vitamin E group. The plasma probucol levels achieved in the low and high dose probucol groups were similar to those previously reported (22, 27, 39).

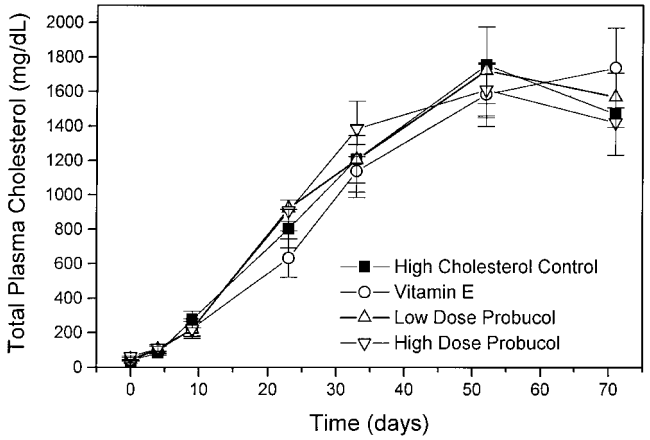

Fig. 1. Plasma cholesterol levels during the intervention period in the four groups of NZW rabbits fed high cholesterol diets. Data are mean ± SEM; n = 6–8 for each group.

The average body weight in the four groups fed high cholesterol diets at baseline ranged from 2.22 to 2.34 kg. Over the course of the study, all animals gained weight equally and no significant differences among the groups were observed at any time point. The final body weight ranged from 3.49 to 3.69 kg.

The antioxidant protection in plasma was determined as the resistance of plasma LDL to copper-oxidation and is reported as the lag time between the addition of copper ions and the onset of the rapid phase of oxidation (38). Even though we have previously demonstrated that the lag time does not necessarily correlate with the antiatherosclerotic efficacy of different antioxidants (39), this parameter provides a good cumulative indicator of the antioxidant content of the LDL particles in vivo (38). Lag times were determined in individual LDL samples isolated from 3 animals per group and in one LDL sample isolated from a plasma pool containing equal volumes from all animals in the same group. The average lag times of the three individual samples and that of the pooled samples were very similar in all groups (data not shown). The lag time of the pooled LDL of the high cholesterol group at the end of the study was 190 min. Treatment with antioxidants markedly increased the lag time to 835 min in the vitamin E group, 838 min in the low dose probucol group, and >1440 min in the high dose probucol group (Table 1). Thus, as intended, a very similar degree of antioxidant protection was achieved in the groups treated with vitamin E and low dose probucol.

Extent of atherosclerosis

After 81 days of intervention, all animals receiving cholesterol-enriched diets showed macroscopic lesions in the aorta, whereas control animals fed regular chow were free of lesions. As expected, the high cholesterol control group had the most extensive atherosclerosis (38.6 ± 12.2%), compared to 12.8 ± 8.0% in the high dose probu-

TABLE 1. Experimental design, plasma cholesterol and antioxidant levels, and antioxidant protection of LDL

Variable	Low Cholesterol Control	High Cholesterol Control	Vitamin E	Low Dose Probucol	High Dose Probucol
Number of animals	8	8	6	6	6
Cholesterol added to diet (%)[a]	none	0.5	0.568	0.565	0.565
Dietary antioxidant supplementation (%)[a]	none	none	0.1	0.047	0.5
Plasma cholesterol level (mg/dl)[a]	39 ± 4.4	1157 ± 138	1112 ± 112	1178 ± 27	1149 ± 70
Plasma vitamin E concentration (μmol/L)[b]	20.6 ± 4.2	55.9 ± 13.9	288.9 ± 32.9	44.8 ± 4.1	51.5 ± 8.4
Plasma probucol concentration (μmol/L)[b]	0	0	0	44.5 ± 7.1	116.7 ± 14.6
Lag time (min)[b]	N.D.	190	835	838	>1440

The degree of antioxidant protection was expressed as the lag time in the formation of conjugated dienes during in vitro oxidation of LDL by copper ions. Data shown represent the lag time of LDL isolated from a pooled sample containing equal amounts of plasma from all animals in the group; N.D., not determined.

[a] Time averaged.

[b] At the end of the intervention period.

col, 19.5 ± 9.2% in the low dose probucol, and 11.5 ± 5.0% in the vitamin E group (**Fig. 2**). These results are comparable to those reported in several other studies (22, 23, 27, 31). However, the reduction of atherosclerosis in the present study did not reach statistical significance ($P = 0.092$ to 0.26), probably because NZW rabbits obtained from a commercial source could not be litter-matched and showed considerable variability in lesion sizes within each group.

The extent of lesions determined separately in segments 2, 4a, and 4b closely reflected the extent of lesions in the entire thoracic aorta (**Table 2**). Lesions near the left and right orifices of the same pair of intercostal arteries were also very similar (segments 4a and 4b). This validated our assumption that in NZW rabbits with early atherosclerosis, adjacent segments of the thoracic artery consistently contain similar size lesions and can therefore be used to analyze parameters that cannot be determined in the same tissue sample.

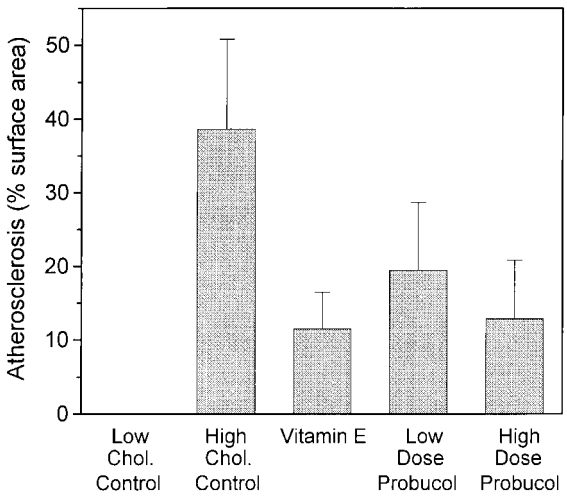

Fig. 2. Effect of antioxidant treatment on the extent of atherosclerosis in the entire thoracic aorta. At the end of the 81-day intervention period, rabbits were killed and the aortas were dissected as described in Methods. Lesions were quantified by computer-assisted image analysis and results are expressed as percent of the aortic surface covered by atherosclerotic lesions. Data are mean ± SEM; n = 6–8 for each group.

Determination of gene expression in aortic tissues

Gene expression of VCAM-1 in arterial tissue was determined by quantitative PCR using GAPDH as internal standard. Results are expressed as the ratio between VCAM-1 and GAPDH. We have previously demonstrated the reliability of this method and established that GAPDH represents a suitable internal standard (5). This was verified under the experimental conditions used in the present study. The expression of GAPDH was examined quantitatively by competitive PCR using as competitor a shortened cDNA fragment of the PCR product (5). No statistically significant difference was found when GAPDH expression in the combined medial and intimal tissue was compared to that in the corresponding intimal tissue alone. GAPDH expression was not affected by treatment with probucol or vitamin E (data not shown).

In agreement with our previous results, we found a basal level of VCAM-1 expression even in control animals that were fed regular chow and that had no atherosclerosis (**Fig. 3**). In these animals, the expression (VCAM-1/GAPDH ratio) was lowest in medial tissue (0.41 ± 0.07) and highest in intimal tissue (1.26 ± 0.24). Expression of VCAM-1 in the combined medial and intimal tissue of high cholesterol control animals determined in segments 2 and 4a was 0.652 ± 0.125 and 0.781 ± 0.148, respectively.

Cholesterol feeding led to a 4.0- to 4.2-fold increase in VCAM-1 gene expression. This increase was very similar in all four tissue samples analyzed. The highest level of VCAM-1 expression was again seen in intimal tissue (5.09 ± 0.70), the lowest in the media (1.71 ± 0.47). VCAM-1 expression in the intact segments 2 and 4a of high cholesterol control animals was 2.71 ± 0.43 and 3.10 ± 0.36, respectively.

Treatment with probucol and vitamin E was consistently associated with a decrease of VCAM-1 gene expression in the vessel wall, compared to the high cholesterol control group (Fig. 3). Probucol treatment reduced VCAM-1 expression in all four thoracic segments analyzed to levels close to the basal level measured in control rabbits, even though these groups clearly had developed substantial atherosclerosis. Treatment with vitamin E also resulted in a significant reduction of VCAM-1 expression in the intima and in the intact segment 4a, compared to high cholesterol controls. Vitamin E also reduced VCAM-1 expression in segment 2 and in the media, but this did not reach statistical significance.

TABLE 2. Extent of atherosclerosis in the entire thoracic aorta and in individual segments

	Low Cholesterol Control	High Cholesterol Control	Vitamin E	Low Dose Probucol	High Dose Probucol
Entire thoracic	0	38.62	11.49	19.48	12.84
Segment 2	0	38.49	11.25	18.14	14.14
Segment 4a	0	37.27	12.60	20.82	10.00
Segment 4b	0	40.20	10.23	17.06	10.06

The descending thoracic aorta was prepared, imaged, and further subdivided into segments 2, 4a, and 4b, as described in Methods. Atherosclerosis was quantitated by computer-assisted image analysis and results are expressed as percent of the luminal surface covered by atherosclerotic lesions.

No significant correlation between VCAM-1 gene expression and the extent of atherosclerosis was found in individual groups or when data from all groups were analyzed together (e.g., $r = 0.12$; $P = 0.64$ for pooled data of segment 4b).

Presence of VCAM-1 protein in atherosclerotic lesions

To verify that regulation of VCAM-1 mRNA was reflected by protein expression, comparative immunocytochemistry was performed on frozen sections from 3–4 rabbits each from the high cholesterol control and the an-

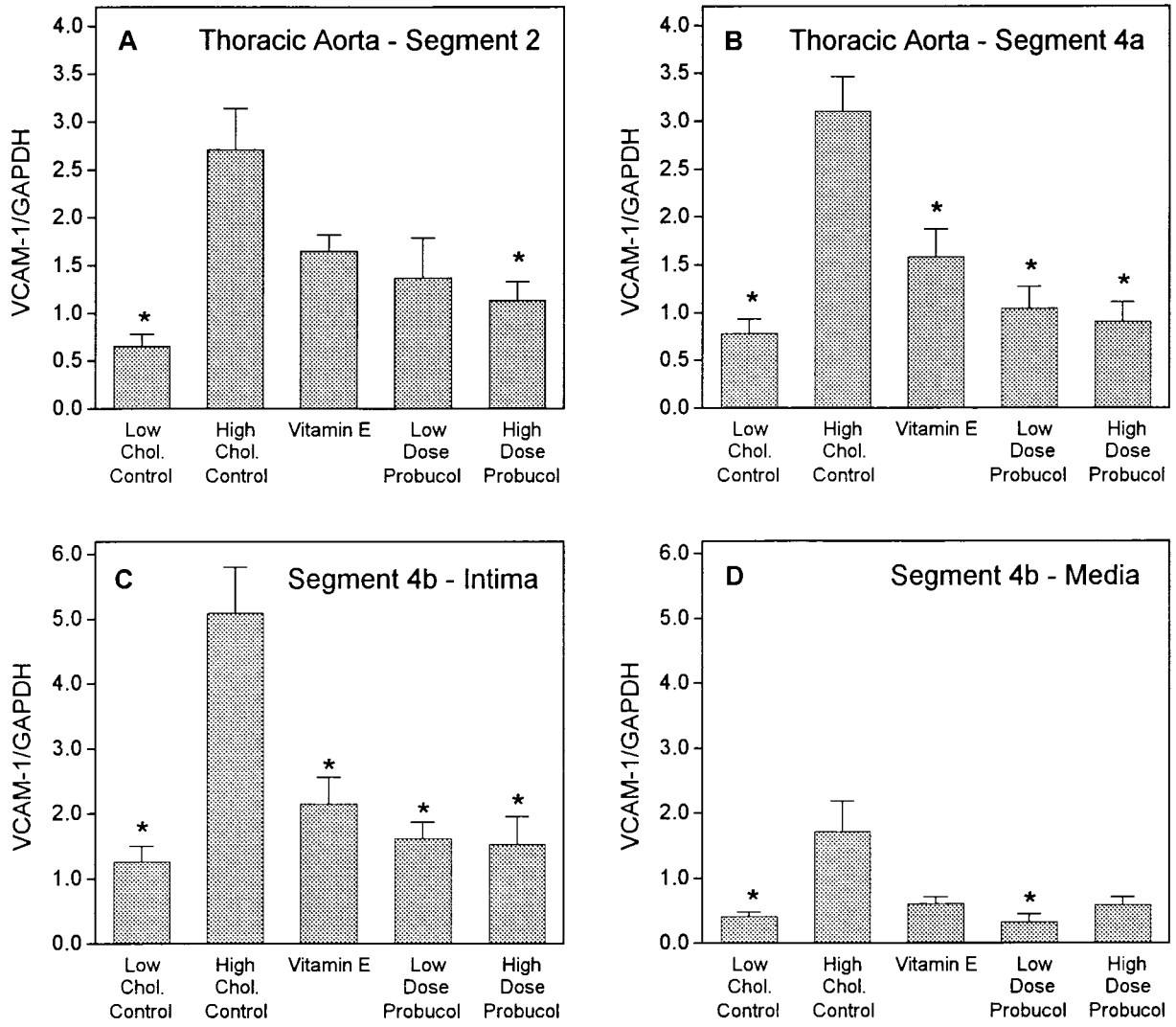

Fig. 3. Effect of antioxidant treatment on VCAM-1 mRNA expression in the thoracic aorta. Aortic segments were prepared and VCAM-1 gene expression was determined by quantitative PCR using GAPDH as internal standard, as described in Methods. Gene expression was determined separately in segments 2 (panel A) and 4a (panel B) of the thoracic aorta. The adjacent thoracic segment 4b was dissected into intima (panel C) and media (panel D) before mRNA isolation and quantification. Data are mean ± SEM; *, $P < 0.05$, compared to the high cholesterol control group.

tioxidant-treated groups. Because immunocytochemistry is far less sensitive than PCR, VCAM-1 protein was detected only in the atherosclerotic intima (**Fig. 4**). In general, animals with the highest mRNA levels in adjacent lesions showed the most intense staining. In the high cholesterol control group, even the earliest lesions consisting of only a few layers of macrophage/foam cells (Fig. 4A) stained intensely for VCAM-1 (Fig. 4B). Some larger lesions showed VCAM-1 expression mainly on the endothelium (Fig. 4C), but in most, intense staining was seen

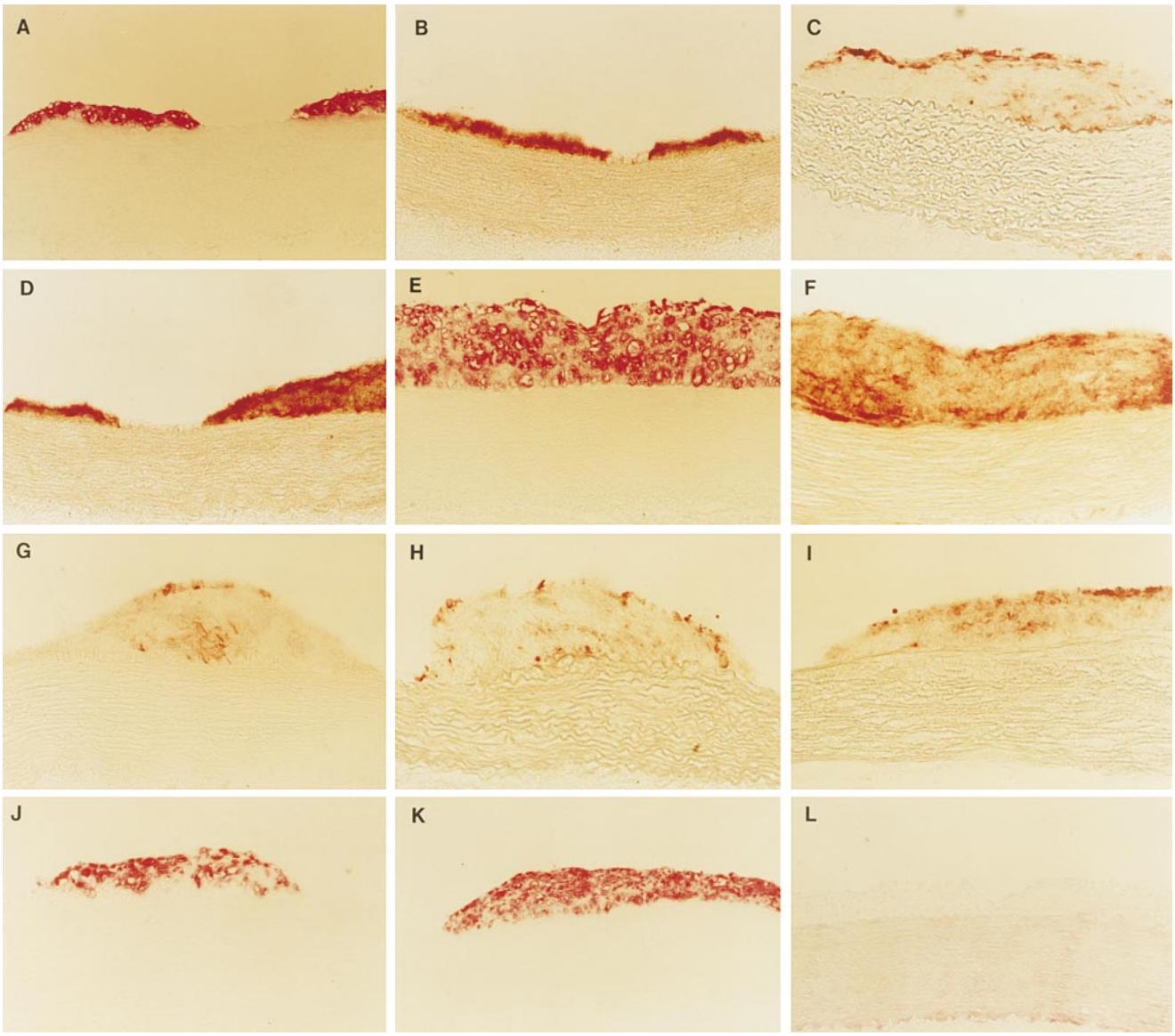

Fig. 4. Presence of VCAM-1 protein, macrophages, and smooth muscle cells in early atherosclerotic lesions of NZW rabbits fed high cholesterol diets with or without antioxidants. Frozen sections from segments 1b and 3b of the thoracic aorta were fixed for 5 min with acetone at −20°C and immunostained with Rb1/9, a monoclonal antibody against rabbit VCAM-1, as described in Methods. Antibodies bound were detected with an avidin–biotin–peroxidase system. For comparison, paraformaldehyde-fixed and paraffin-embedded sections from the adjacent thoracic segments 1a and 3a were immunostained for macrophages or smooth muscle cells using monoclonal antibodies RAM 11 and HHF-35, respectively, and an avidin–biotin–alkaline phosphatase detection system, as described in Methods. Representative lesions from the high cholesterol control group (A–F), the vitamin E group (G, H, J), and high dose probucol group (I, K, L) are shown. A: Very early lesions consisting of only a few layers of macrophage/foam cells staining with RAM 11. B: Comparable early lesions showing intense staining for VCAM-1. C: Larger lesion showing mostly endothelial expression of VCAM-1. D: Early lesion and shoulder area of a transitional lesion displaying intense staining for VCAM-1. E: Macrophage/foam cells in a large lesion. F: Comparable lesion from an adjacent frozen segment displaying intense VCAM-1 staining in the deeper layers of the intima. In contrast, lesions from rabbits treated with vitamin E (G, H) or high dose probucol (I) showed relatively little VCAM-1 staining. Note that all immunostaining with the same antibody was performed in the same assay and that the amplification step in the procedure was determined by the sections with the weakest staining. RAM-11 staining of lesions from the vitamin E (J) and probucol groups (K) showed that lesions in the antioxidant-treated groups consisted mainly of macrophages and did not differ in this respect from those of the high cholesterol control group shown above. Smooth muscle cells were absent from the intima of most lesions of all groups (L), and isolated smooth muscle cells were limited to the vicinity of the internal elastic lamina of larger lesions. Magnification × 50 (A, B, D, K); × 62.5 (E–L); × 80 (C).

throughout the lesion (Fig. 4D). In addition, in some larger macrophage-rich lesions of this group (Fig. 4E) staining also occurred near the internal elastic lamina (Fig. 4F), which may reflect smooth muscle cell-derived VCAM-1 (5, 7, 8). In contrast, in all three antioxidant-treated groups, only patchy and much less intense VCAM-1 staining was observed (Fig. 4G–I). This was also true when only lesions of comparable size were compared. Thus, it appears that the protein expression generally reflects mRNA expression. No qualitative differences between the vitamin E (Fig. 4G, H), the high dose probucol (Fig. 4I), or the low dose probucol groups was discernible by immunostaining. Given the fact that the overall extent of atherosclerotic surface area tended to be smaller in the antioxidant treatment groups than in the high cholesterol control, it was conceivable that lesion composition, in particular macrophage presence, might be different. Such a difference could reflect a more advanced stage of lesions in the high cholesterol control group or specific effects of antioxidants on lesion cells (45). To rule out that the difference in VCAM-1 expression merely reflects differences in lesion composition, immunocytochemistry with monoclonal antibodies against macrophages and smooth muscle cells (actin) was performed. This yielded no indication of differences in lesion composition. Lesions from the treated groups showed macrophage staining patterns (Fig. 4J, K) similar to those from the high cholesterol control group (Fig. 4E). In all four groups, most lesions were completely devoid of smooth muscle cells (Fig. 4L). Only the largest lesions from all groups contained some smooth muscle cells in the vicinity of the internal elastic lamina (data not shown).

DISCUSSION

The present study demonstrates that vitamin E and probucol significantly reduce the up-regulation of VCAM-1 mRNA and protein expression in the vascular wall of NZW rabbits during cholesterol-induced atherogenesis. We have previously shown that probucol reduces baseline expression of VCAM-1 and prevents the up-regulation of VCAM-1 expression that normally accompanies lesion formation in WHHL rabbits (5). However, this could not be unequivocally attributed to the antioxidant effect of probucol. The present observation that vitamin E, a structurally and metabolically very different compound, causes a down-regulation of VCAM-1 expression in vivo similar to that of probucol strongly supports the assumption that this effect is due to the antioxidant effects of both agents. Lesion formation in the present experiment was decreased by 57–75%, in contrast to the previous study, where atherogenesis was completely prevented by probucol. This provides clear evidence that the effect on VCAM-1 expression is not limited to a reduction of the baseline level expressed in the normal intima prior to the onset of atherogenesis, but that it persists throughout the early stages of lesion formation.

The fact that VCAM-1 protein was observed only in the intima and that the up-regulation of VCAM-1 gene expression was most dramatic (but not limited to) the intima is consistent with the assumption that up-regulation of VCAM-1 expression is a constitutive element of lesion formation (2–7, 9, 46). It is also consistent with the hypothesis that powerful lipophilic antioxidants may reduce atherogenesis, at least in part, by inhibiting endothelial VCAM-1 expression and subsequently reducing the recruitment of circulating monocytes. However, our study does not provide evidence for a causal link between the inhibition of VCAM-1 expression and the reduction of atherosclerosis. The observation that VCAM-1 mRNA in the high cholesterol control group was markedly up-regulated, whereas the increase in the antioxidant treated groups was much smaller, could simply reflect the fact that lesions in the latter groups were less extensive. Lesions from the high cholesterol control group often showed more VCAM-1 immunostaining than those of the treatment groups, but they also were much larger. To establish a causal relationship, it would be necessary to show that lesions from the antioxidant-treated groups contain less VCAM-1 than control lesions of the same size and stage. The scarcity of transitional and advanced lesions in the treatment groups did not permit us to evaluate this.

The second argument suggesting that inhibition of VCAM-1 is not the sole mechanism reducing atherogenesis is the apparent lack of correlation between the extent of lesions and VCAM-1 mRNA expression. Vitamin E reduced atherosclerosis as effectively as the high dose of probucol (−74.6 vs. −74.9%; segment 4), but decreased VCAM-1 expression by only 49%, compared to 70.8% for high dose probucol. In contrast, low dose probucol reduced VCAM-1 expression by 66.5%, but appeared to be much less effective in reducing atherosclerosis than either vitamin E or high dose probucol. However, one has to keep in mind that VCAM-1 mRNA data may not reflect VCAM-1 protein or its biological activity.

The degree of antioxidant protection of plasma LDL (lag times) also failed to correlate with VCAM-1 expression. This is in agreement with a previous study (39), but may merely indicate that the antioxidant protection of plasma LDL is a poor indicator of the intracellular concentration or efficacy of different antioxidants.

There can be little doubt that LDL oxidation contributes to the recruitment of circulating monocytes into the arterial intima, as recently demonstrated in human fetal aortas (47), and that oxidative processes are involved in the regulation of VCAM-1 (36). Cell culture experiments have shown that some byproducts of LDL oxidation, such as lysophosphatidylcholine, increase the expression of VCAM-1 (12, 15), and Cominacini et al. (16) have recently provided direct evidence for an up-regulation of VCAM-1 by LDL oxidized with 1 μmol/l Cu^{2+}, endothelial cells, or phospholipase A_2/soybean lipoxygenase. Further support is provided by the fact that antioxidants inhibit VCAM-1 expression in cultured cells (16, 20, 21) and in vivo (present study and ref. 5). On the other hand, oxidation enhances the expression of other molecules that influence the adhesion and endothelial transmigration of monocytes, such as ICAM-1

(16), P-selectin (48), and E-selectin (21). For example, activation of NFκB and monocyte adhesion induced by minimally oxidized LDL (mmLDL) (18, 19, 36) does not appear to be VCAM-1 dependent (49). It is also increasingly recognized that OxLDL may influence intracellular signaling by pathways other than NFκB. Ricote et al. (50) recently showed that OxLDL stimulated expression of the peroxisome proliferator-activated receptor γ (PPARγ) in macrophages, and immunocytochemical colocalization of PPAR and OxLDL in macrophages (and probably also in endothelial cells) of human atherosclerotic lesions suggested that OxLDL also promotes expression of this nuclear transcription factor.

In analogy, antioxidants seem to inhibit monocyte adhesion by a variety of mechanisms. Probucol may decrease endothelial monocyte adhesion (51) by down-regulating expression not only of VCAM-1, but also of E-selectin (52). Erl et al. (21) showed that α-tocopheryl succinate (but not α-tocopherol) suppressed expression of VCAM-1 and E-selectin. Faruqi, de la Motte, and DiCorleto (20) reported that α-tocopherol and probucol inhibit agonist-induced monocyte adhesion to HUVEC in a dose-dependent manner, but not via NFκB. Work by Devaraj, Li, and Jialal (53, 54) also demonstrated that α-tocopherol has multiple effects on monocytes that may affect its binding to the endothelium. Finally, increasing evidence suggests that VCAM-1 is but one of the ligands for VLA-4 on the monocyte surface and that monocyte adhesion may also involve other endothelial binding sites (1, 55). Unfortunately, a VCAM-1 knockout model that might have been helpful in addressing the role of VCAM-1 in atherogenesis and the contribution of its inhibition by antioxidants to lesion reduction was not viable (56). Site- or time-specific knockout models or blocking antibodies may provide more insights.

Although the present results do not constitute proof for a causal role of VCAM-1 in atherogenesis, they provide convincing evidence that antioxidants interfere with oxidation-sensitive intracellular regulatory mechanisms in vivo. Together with the mounting evidence linking the expression of several adhesion molecules and other atherogenic factors to such oxidation-dependent signaling, our findings support the notion that antioxidants may reduce atherosclerosis in part by modulating the expression of these factors in the arterial wall.

We thank Jennifer Pattison, Joe Juliano, and Norma Pradis for excellent technical assistance. These studies were supported by National Heart, Lung, and Blood Institute grant HL56989 (La Jolla Specialized Center of Research in Molecular Medicine and Atherosclerosis). Probucol was a generous gift from Hoechst Marion Roussel Research Institute, HMR Inc.

Manuscript received 12 March 1999, in revised form 20 May 1999, and in re-revised form 20 July 1999.

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