Oxidized LDL bind to nonproteoglycan components of smooth muscle extracellular matrices

Mary Y. Chang,* Susan Potter-Perigo,* Thomas N. Wight,* and Alan Chait[†,1]

Departments of Pathology* and Medicine,[†] Division of Metabolism, Endocrinology, and Nutrition, School of Medicine, 1959 NE Pacific, BB-928 Health Sciences, Box 356426, University of Washington, Seattle, WA 98195-6426

Abstract **Arterial wall lipid retention is believed to be due primarily to ionic interactions between lipoproteins and proteoglycans. Thus, oxidized low density lipoproteins (LDL), with decreased positive charge relative to native LDL, should have decreased interaction with negatively charged proteoglycans. However, oxidized LDL does accumulate within arterial lesions. Therefore, this study investigated the binding of native and oxidized LDL to a complex smooth muscle extracellular matrix and the role of ionic charge interactions in their binding. LDL was modified with 2,2-azo-bis(2-amidinopropane)-2HCl, hypochlorite, soybean lipoxygenase, and phospholipase or copper sulfate. The extracellular matrix had 15- to 45-fold greater binding capacity for the different forms of oxidized LDL than for native LDL. However, the affinity of binding for all forms of oxidized LDL was high (K_a = ~10−9 M) and was similar to that for native LDL. Preincubation of the lipoproteins with chondroitin sulfate decreased the binding of native LDL, but had no effect on the binding of oxidized LDL. Digestion of matrices with chondroitin ABC lyase and heparinase decreased the binding of native LDL, but increased the binding of oxidized LDL; matrix digestion with pronase or trypsin markedly reduced the binding of both native and oxidized LDL. Thus, the binding of native LDL involves matrix proteoglycans, whereas the binding of oxidized LDL involves a nonproteoglycan component(s) of the matrix. The markedly enhanced retention of oxidized LDL compared with native LDL may play an important role in the progression of atherosclerosis.**—Chang, M. Y., S. Potter-Perigo, T. N. Wight, and A. Chait. **Oxidized LDL bind to nonproteoglycan components of smooth muscle extracellular matrices.** ***J. Lipid Res.*** **2001.** 42: **824–833.**

Supplementary key words soybean lipoxygenase • hypochlorite • copper • glycosaminoglycan • chondroitin sulfate • chondroitin ABC lyase • heparinase • collagen • matrix

The vascular extracellular matrix consists of a variety of molecules that are capable of interacting with lipoproteins and may be responsible for lipoprotein deposition in atherosclerosis. However, proteoglycans of the extracellular matrix are hypothesized to be the major lipoprotein binding site within atherosclerotic lesions (1–4). In vitro studies suggest that the interaction between lipoproteins and proteoglycans occurs predominantly as a result of electrostatic interactions between clusters of positively charged amino acids of the apolipoprotein (apo)B protein moiety of low density lipoproteins (LDL) and negatively charged residues on the glycosaminoglycan chains of proteoglycans (5, 6). These studies suggest that oxidatively modified LDL, which has decreased positive charge relative to native LDL, should bind less well to proteoglycans (7). However, oxidized LDL is atherogenic and is likely to play an important role in atherogenesis (8–13). In addition, oxidized LDL or oxidation-specific epitopes have been identified in vivo in atherosclerotic lesions both in humans (14) and in animal models of atherosclerosis (15–17). Therefore, this study examined the interaction of oxidized LDL with the extracellular matrix of cultured smooth muscle cells (SMC) to understand the nature of the molecular interaction by which lipoprotein oxidation could contribute to lipid retention in the arterial wall.

For this purpose, the binding of native and oxidatively modified LDL to a complex extracellular matrix produced by arterial SMC in culture was evaluated. Use of such a complex matrix rather than isolated proteoglycans, collagen, elastin, or other matrix components enables examination of LDL binding to matrix components in a more physiological presentation.

To compare the binding of native LDL to that of oxidatively modified LDL, several methods of oxidation were used. These different methods of oxidation generated oxidized LDL with differing degrees of protein and/or lipid modification. Importantly, this allowed for a correlation to be made between the extent of modification and the

Abbreviations: AAPH, 2,2-azo-bis(2-amidinopropane)-2HCl; DTPA, diethylenetriaminepentaacetic acid; EBSS, Earle's balanced salt solution; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HOCl, hypochlorite; LDL, low density lipoproteins; SLO, soybean lipoxygenase; SMC, smooth muscle cell; TBARS, thiobarbituric acid-reactive substances; TNBS, trinitrobenzenesulfonic acid.

[1] To whom correspondence should be addressed.
e-mail: achait@u.washington.edu.

amount of binding. The role of electrostatic interactions in the binding of native and oxidized LDL to proteoglycans within this complex extracellular matrix also was examined by competition-binding studies and selective enzymatic digestions of the matrix.

In summary, both native and oxidized LDL bound with high affinity to SMC extracellular matrices. However, the binding capacity of the matrix for each of the different forms of oxidized LDL was much greater than for native LDL. The binding of native LDL could be diminished by removal of matrix glycosaminoglycan chains with enzymatic digestion or by competition with free glycosaminoglycans. In contrast, the binding of oxidized LDL could not be diminished by removal of matrix glycosaminoglycan chains or by competition with free glycosaminoglycans. Thus, retention of native LDL is mediated by matrix proteoglycans, whereas the retention of oxidized LDL is not mediated by direct ionic interactions with matrix proteoglycans. The enhanced retention of oxidized LDL compared with native LDL could have important consequences in the development and progression of atherosclerosis.

MATERIALS AND METHODS

Materials

All chemical reagents were purchased from Sigma (St. Louis, MO), unless otherwise specified. All radiochemical reagents were purchased from ICN (Irvine, CA).

Lipoprotein preparation and characterization

LDL were isolated from plasma of normal human volunteers as described previously (18). LDL (d = 1.019–1.063 g/ml) were separated from normal human plasma by preparative ultracentrifugation in a Beckman VTi 65 vertical rotor (Beckman Instruments, Palo Alto, CA) (19) and purified by sequential density ultracentrifugation (18).

Oxidative modification of native LDL was performed by several methods. LDL was extensively oxidized in the presence of copper by incubation of LDL (300 μg/ml) with 5 μM copper sulfate for 18 h at 37°C in air (Cu^{2+}-LDL) (20). Oxidation by the thermally dependent free radical generator 2,2-azo-bis (2-amidinopropane)-2HC1 (AAPH) was achieved by incubation of LDL (500 μg/ml) with 2 mM AAPH for 18 h at 37°C in the presence of Chelex-treated phosphate-buffered saline (PBS; AAPH-LDL) (21). Oxidative modification of LDL by hypochlorite (HOCl) was achieved by incubation of 400 μl LDL (1.5 mg/ml) with 5 μl reagent-grade HOCl (HOCl-LDL) for 20 min on ice, followed by overnight dialysis against PBS (22). LDL also was oxidized by incubation with soybean lipoxygenase (SLO) and phospholipase A2 (PLA2) coupled to Sepharose 4B beads (23). In brief, 15 mg of enzyme-coupled beads (100 U/μl SLO and 0.25 U/μl PLA2) were incubated with 1 mg LDL in 1.5 ml PBS containing 2 μl linoleic acid for 48 h at room temperature with gentle agitation on a mechanical rocker (SLO-LDL). All incubations were terminated by the addition of butylated hydroxytoluene (25 μM final concentration). The extent of modification was assessed by measurement of thiobarbituric acid-reactive substances (TBARS) (24), lipid peroxides (25), conjugated dienes (20), electrophoretic mobility on agarose gels in barbital buffer at pH 8.6 (26), measurement of free amino groups by trinitrobenzenesulfonic acid (TNBS) reactivity (27), protein fragmentation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (18), and particle size by negative staining electron microscopy (28).

LDL was iodinated with [^{125}I]NaI by the method of McFarlane (29), as modified by Bilheimer, Eisenberg, and Levy (30).

SMC isolation and culture

Arterial SMC cultures were isolated from monkey (*Macaca nemestrina*) thoracic aorta by the explant method (gift from Dr. Russell Ross) and maintained as described previously (31). Cells between passage 4–13 were plated onto 2% gelatin-coated 35-mm dishes (5×10^6 cells/dish) or 48-well trays (5×10^5 cells/well). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) with high glucose, pyruvate, and nonessential amino acids supplemented with penicillin (10^5 U/l), streptomycin (10^5 μg/l), and 5% calf serum. Media were changed every 2–3 days.

At confluence, SMC cultures were metabolically labeled with 50 μCi/ml [^{35}S]SO_4,10 μCi/ml [^{3}H]leucine, 5 μCi/ml [^{3}H]thymidine, or 20 μCi/ml [^{3}H]proline for 48 h for characterization and selective enzymatic degradation studies.

Preparation of extracellular matrix

SMC were washed three times with PBS, incubated with 2.5 mM NH_4OH and 0.1% Triton X-100 for 30 min at room temperature, and then washed three times with distilled water to remove cellular debris (32).

Enzyme-linked immunosorbent assay (ELISA) for assessing lipoprotein binding to matrix

Dilutions of native or oxidized LDL in the physiological Earle's balanced salt solution (EBSS; Gibco) with 1% BSA (EBSS/BSA) were incubated with extracellular matrix preparations for 2 h at 37°C, after which the lipoproteins were removed and the matrix was washed three times with EBSS/BSA. The matrix was then incubated for 1 h at 37°C with the monoclonal antibody MB47 (1:4,000 in EBSS/BSA; gift from Dr. Linda Curtiss, Scripps Clinic, La Jolla, CA) (33), and washed, as above. Horseradish peroxidase-conjugated goat anti-mouse IgG (1:4,000 in EBSS/BSA) was added for 1 h at room temperature, then removed, and the matrix washed, as above. Colorimetric development was allowed to proceed for up to 10 min using o-phenylenediamine as substrate. The reaction was stopped by the addition of 8 N H_2SO_4 and the reaction product was measured at $A_{490\text{-}405}$.

This ELISA was characterized by high affinity saturable binding to the extracellular matrix with very little nonspecific binding compared with the findings with a radioisotope assay using radioiodinated LDL, which indicated nonsaturable binding to the matrix (data not shown). Absorbance units were converted into nanograms of lipoprotein bound to the matrix using a standard curve of ^{125}I-LDL of known specific activity. The B_t and K_a binding parameters were determined with SAAM II software. Further, the relationship between absorbance units and nanograms bound was similar for radioiodinated native LDL, AAPH-LDL, SLO-LDL, and Cu^{2+}-LDL (data not shown), indicating that the MB47 antibody detects apoB in native and oxidized LDL equally well, as previously reported (34). MB47 was not as accurate for determining binding of HOCl-LDL, in that the absorbance units overestimated the amount bound by 20–50% (data not shown). No nonspecific binding of the primary antibody (MB47) was observed, as evidenced by the lack of measurable $A_{490\text{-}405}$ in the absence of lipoproteins.

Free glycosaminoglycans and divalent metal ion chelators were used for competition binding studies. Chondroitin sulfate type C (chondroitin-6-sulfate, 0.1–1.0 mg/ml) or chondroitin sulfate type B (dermatan sulfate, 0.1–1.0 mg/ml), diethylenetriaminepentaacetic acid (DTPA, 5.2–26 mM), or ethylenediaminetetraacetic acid (EDTA, 5.2–26 mM) were added to the lipoproteins just prior to incubation of the lipoproteins with matrix preparations.

Selective enzymatic degradation

Components of SMC matrix preparations were selectively digested with DNase, trypsin, pronase, chondroitin ABC lyase, heparinase I and II, collagenase, or elastase. Treatment with DNase (1,000 U/ml) was carried out in 0.1 M sodium acetate, pH 5.0, for 1 h at room temperature. Trypsin (0.05%; Gibco) digestions were carried out in 0.025 M Tris, pH 7.4, with 0.01 M calcium acetate for 5 or 20 min at 37°C. Pronase (300 μg/ml; Boehringer Mannheim) treatment was carried out in the same buffer for 3 h at 37°C. Matrices were digested with 1.2 U/ml chondroitin ABC lyase (ICN, Costa Mesa, CA) alone or in combination with 12.5 U/ml heparinase I and II in 0.3 M Tris-HCI, pH 8.0, with 0.6 mg/ml BSA and 18 mM sodium acetate for 3 h at 37°C. Digestion with 500 U/ml collagenase form III from *Clostridium histolyticum* (Advance Biofactures Corporation, Lynbrook, NY) was carried out in 0.025 M Tris, pH 7.4, with 0.01 M calcium acetate for 2 h or overnight at 37°C. Digestion with 1 U/ml human leucocyte elastase in 0.01 M sodium phosphate, pH 7.5, was performed for 4 h at 37°C. For all enzyme treatments, control wells were incubated in the appropriate digestion buffer without enzyme. All control and enzyme-digested matrices were washed briefly with EBSS/BSA prior to incubation with lipoproteins.

[^{35}S]SO_4^- and ^{3}H-labeled matrices were harvested at completion of the binding studies using either 8 M urea/0.5% Triton X-100 or 5 N NaOH. Aliquots were analyzed by scintillation counting to assess the effectiveness of the various digestion protocols or analyzed by SDS-PAGE with 2 M urea (35) to assess collagen digestion.

Statistical analyses

The significance of differences in mean values was determined by Student's *t*-test.

RESULTS

Characterization of native and oxidized LDL

Native LDL was modified by a variety of techniques to prepare oxidized LDL with different degrees of lipid and protein modification. Native LDL and LDL oxidized by AAPH, SLO, HOC1, or Cu^{2+} were characterized to determine the extent of modification. AAPH and SLO modifications generated relatively mildly oxidized LDL particles that contained some of the early measurable products of oxidation such as lipid peroxides and conjugated dienes. Only slight changes in the indices of oxidation such as reactive aldehyde formation (TBARS), reduced positive charge (increased electrophoretic mobility), lysine amino group modification (TNBS reactivity; **Table 1**), or protein fragmentation (not shown) could be detected in AAPH-LDL or SLO-LDL compared with native LDL. HOCl modification generated LDL particles with more extensive protein modification, as indicated by markedly reduced positive charge, decreased free lysine amino groups (Table 1), and extensive protein fragmentation (not shown). Finally, Cu^{2+} modification generated extensive modification of both the lipid and protein components of LDL, as indicated by a high amount of conjugated diene formation, the highest level of reactive aldehyde formation (TBARS), markedly reduced positive charge, a high degree of lysine amino group modification (Table 1), and extensive protein fragmentation (not shown). Lipid peroxides, an early unstable oxidation product of Cu^{2+} oxidation, were not detected in this extensively oxidized Cu^{2+}-LDL.

Negative staining electron microscopy also was performed to examine lipoprotein particle size and the state of aggregation before and after oxidation. Native LDL particles were round and relatively uniform in size with monomeric particle diameters of 22.2–33.3 nm (**Fig. 1A**). AAPH-LDL particles also were round and relatively uniform in size with monomeric particle diameters of 22.2–33.3 nm (Fig. 1B). SLO-LDL ranged from monomeric spheres to large aggregates of fused lipoprotein particles with particle diameters ranging from 22.2 to 166.7 nm (Fig. 1C). HOCl-LDL was even more extensively aggregated, with particle diameters ranging from monomers of 22.2 nm to very large fused aggregates of 333.3 nm (Fig. 1D). By comparison, Cu^{2+}-LDL was only moderately aggregated with particle diameters ranging from 22.2 to 61.1 nm (Fig. 1E).

Thus, oxidation of LDL by these different methods had varying effects on the degree of lipid and protein modifications, as well as on state of aggregation of the LDL particles.

SMC matrix binds oxidized LDL with higher capacity, but with similar affinity as native LDL

The binding of native LDL to SMC matrices was compared with the binding of LDL oxidized by a variety of methods. Oxidized LDL prepared by any of these methods bound to SMC matrices with high affinity and in a saturable manner, as did native LDL (**Fig. 2**). LDL oxidized by AAPH, SLO, HOCl, or Cu^{2+} all exhibited 15- to 45-fold higher binding capacity than native LDL at concentrations of LDL ranging from 1 to 25 μg/ml. There was some variability in the order of binding of the AAPH-LDL, SLO-

TABLE 1. Characterization of native and oxidized LDL

LDL	Lipid Peroxides	Conjugated Dienes	TBARS	Relative Electrophoretic Mobility	TNBS Reactivity
	A_{265}	A_{234}	*nmol/mg protein*	R_f	*% Native-LDL*
Native	0.11 ± 0.09	0.25 ± 0.02	1.79 ± 0.23	1.0	100
AAPH	0.32 ± 0.03	1.07 ± 0.11	8.65 ± 0.88	1.5 ± 0.1	98.0 ± 6.4
SLO	0.37 ± 0.04	1.55 ± 0.13	5.45 ± 0.50	1.2 ± 0.2	121.0 ± 16.0
HOCl	0.65 ± 0.06	0.41 ± 0.03	2.77 ± 0.29	2.0 ± 0.1	54.6 ± 6.2
Cu^{2+}	0.07 ± 0.01	1.24 ± 0.11	47.55 ± 1.21	2.4 ± 0.1	52.2 ± 1.9

Values given as means ± SEM; n = 12.

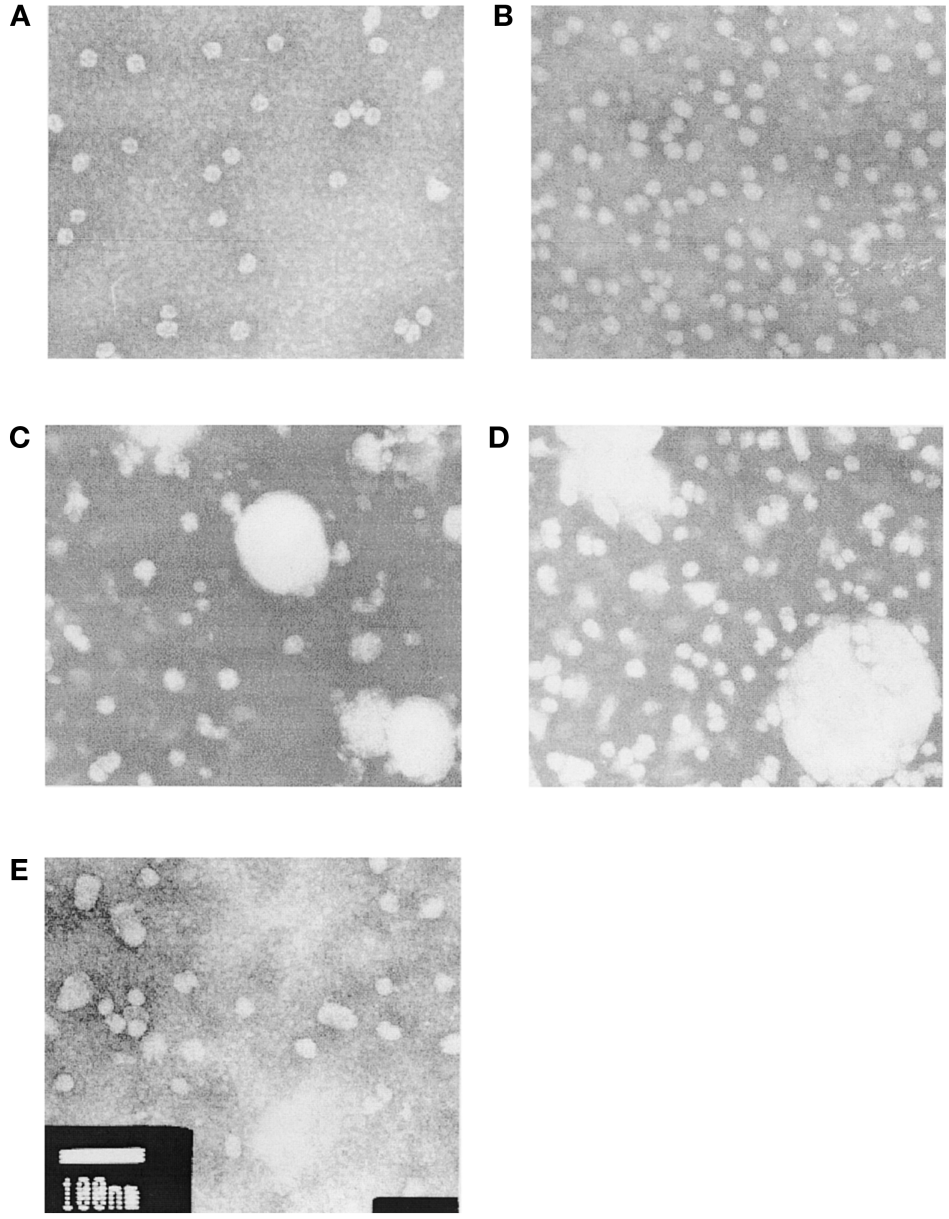

Fig. 1. Characterization of native and oxidized LDL. A: native LDL. B: AAPH-LDL. C: SLO-LDL. D: HOCl-LDL. E: Cu^{2+}-LDL. Preparations were examined by negative staining electron microscopy (bar = 100 nm).

LDL, and HOCl-LDL due to variability in these preparations. For example, in some experiments, AAPH-LDL bound better than SLO-LDL, whereas in other experiments, SLO-LDL bound better than AAPH-LDL. However, Cu^{2+}-LDL always had the highest amount of binding, and native LDL always had the lowest amount of binding. Maximum total binding capacity (B_{max}) of the oxidized LDL preparations ranged from 10.3 ng for AAPH-LDL to 32.9 ng for Cu^{2+}-LDL compared with only 0.7 ng for native LDL (**Table 2**). All of the LDL preparations bound to the matrix with high affinity and binding constants (K_a) ranging from $2.82 \times 10-9$ M to $7.32 \times 10-9$ M (Table 2). The total binding capacity and the binding constants could not be correlated with any of the physical or chemical characteristics such as overall charge or state of aggregation of the LDL particles. For example, the least negatively charged oxidized LDL (SLO-LDL) did not exhibit the lowest binding to SMC matrix, and the oxidized LDL with the highest binding capacity (Cu^{2+}-LDL) was not the most aggregated. Binding of native and oxidized LDL to control wells without SMC matrices was markedly lower than binding to wells with SMC matrices (data not shown).

Thus, SMC extracellular matrix has a high binding capacity and high affinity for oxidized LDL. The binding capacity for all forms of oxidized LDL tested was considerably greater than for native LDL. Because similar binding properties were observed for all of the different forms of oxidized LDL, yet not all forms were as reproducible in their preparation, Cu^{2+}-LDL was chosen for additional studies that attempted to identify the matrix compo-

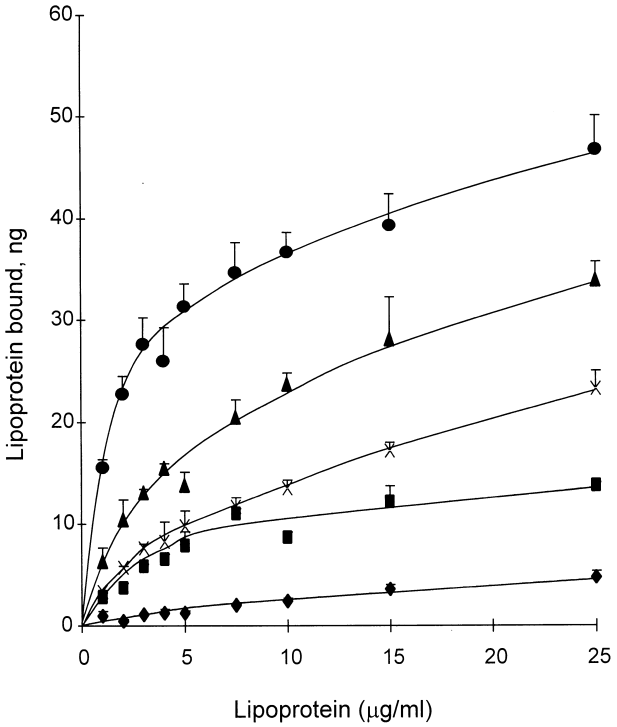

Fig. 2. SMC extracellular matrix binds oxidized LDL with high affinity and high capacity. Native LDL (solid diamond), AAPH-LDL (solid square), HOCl-LDL (X), SLO-LDL (solid triangle), and Cu^{2+}-LDL (solid circleL) were incubated with SMC extracellular matrices and detected by ELISA. Absorbance units were converted into nanograms of lipoprotein bound to the matrix using a standard curve of ^{125}I-LDL of known specific activity. Values are expressed as the means ± SEM (n = 4–7).

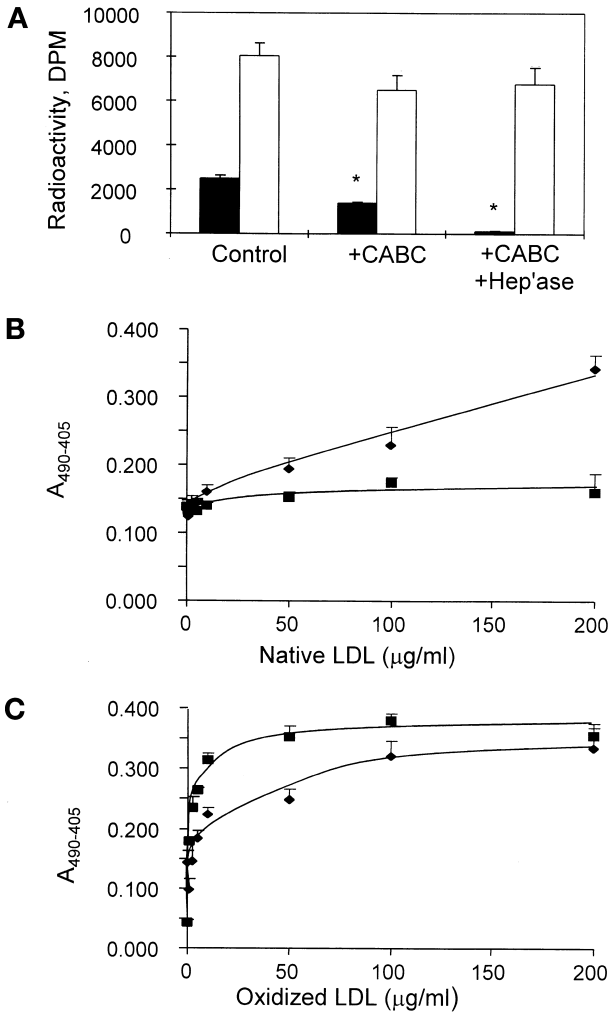

Fig. 3. The binding of Cu^{2+}-LDL to SMC extracellular matrices is not chondroitin ABC lyase or heparinase sensitive. A: Extracellular matrices prepared from [^{35}S]SO_4-labeled (solid bars) or [^{3}H]leucine-labeled (open bars) SMC were left intact (Control) or treated with chondroitin ABC lyase (CABC) alone, or in combination with heparinase I and II (Hep'ase), and monitored for loss of radioactivity. * Statistically significant differences ($P < 0.05$) between the amount of radioactivity remaining after enzyme digestion compared with control. B and C: 0–200 μg/ml of native LDL (B) or Cu^{2+}-LDL (C) were incubated with intact (solid diamond) or digested (solid square) matrices. Bound lipoproteins were detected by ELISA. Values are expressed as the means ± SEM (n = 3).

nent(s) responsible for the high affinity and high capacity binding of oxidized LDL.

Cu^{2+}-LDL does not bind to the glycosaminoglycan chains of extracellular matrix proteoglycans

Extracellular matrix proteoglycans are hypothesized to contribute most significantly to LDL accumulation within the arterial wall (1–4). The lipoprotein-proteoglycan interaction is thought to occur between positively charged amino acids of the apoB protein moiety of LDL and negatively charged residues on the glycosaminoglycan chains of proteoglycans (5, 6). Therefore, selective enzymatic digestion experiments and competition experiments with free glycosaminoglycans were performed to determine whether native or oxidized LDL were binding to proteoglycans within the SMC extracellular matrix.

Treatment of [^{35}S]SO_4-labeled or [^{3}H]leucine-labeled extracellular matrices prior to the binding assay with chondroitin ABC lyase alone or in combination with heparinase I and II selectively removed the chondroitin and heparan sulfate components of the matrix, as evidenced by the loss of [^{35}S]SO_4-labeled matrix, but did not result in loss of matrix protein, as evidenced by the recovery of [^{3}H]leucine (**Fig. 3A**). Removal of the glycosaminoglycan chains from the matrix had no effect on the binding of low concentrations of native LDL (0–10

TABLE 2. Binding constants for the interaction of native and oxidized LDL with SMC extracelluar matrix

LDL	B_{max}	K_a
	ng/well	*M*
Native (n = 3)	0.7	2.82×10^{-9}
AAPH (n = 3)	10.3*	6.65×10^{-9}*
SLO (n = 3)	16.4*	7.32×10^{-9}*
HOCl (n = 3)	10.4*	3.97×10^{-9}
Cu^{++} (n = 3)	32.9*	6.71×10^{-9}*

* Significant differences ($P < 0.05$) for B_{max} or K_a for these forms of oxidized LDL compared with native LDL.

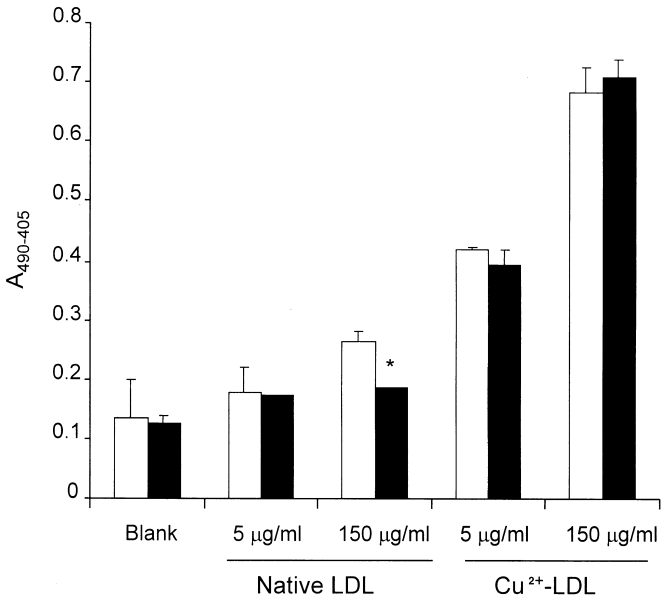

Fig. 4. Free glycosaminoglycans do not compete for the binding of Cu^{2+}-LDL to SMC extracellular matrices. Native LDL (5 or 150 μg/ml) or Cu^{2+}-LDL (5 or 150 μg/ml) was preincubated without (open bars) or with (solid bars) 1 mg/ml chondroitin-6-sulfate for 1.5 h at room temperature prior to incubation with matrices. Bound lipoproteins were detected by ELISA. Values are expressed as the means ± SEM (n = 3). Where no error bars are shown, the SEM are too small to be visible. *A statistically significant difference between the binding of native LDL (150 μg/ml) preincubated with chondroitin-6-sulfate compared with native LDL (150 μg/ml) not preincubated with chondroitin-6-sulfate was observed ($P < 0.05$).

μg/ml), but decreased the binding of higher concentrations of native LDL (50–200 μg/ml) by 20–50% ($P < 0.05$; Fig. 3B). In contrast, removal of the glycosaminoglycan chains actually increased the matrix binding of Cu^{2+}-LDL at concentrations from 1 to 100 μg/ml ($P < 0.05$; Fig. 3C).

Competition experiments were performed by preincubating native LDL or Cu^{2+}-LDL with chondroitin-6-sulfate for 1.5 h at room temperature prior to incubation with matrices. Preincubation of the lipoproteins with chondroitin-6-sulfate at 1 mg/ml, a concentration that was 10-fold greater than that reported to be required for 100% inhibition of LDL-proteoglycan interactions (36), had no effect on the matrix binding of a low concentration of native LDL (5 μg/ml). However, the binding of a higher concentration of native LDL (150 μg/ml) was decreased 30% by preincubation with 1 mg/ml of chondroitin-6-sulfate (**Fig. 4**). Binding of either low (5 μg/ml) or high (150 μg/ml) concentrations of Cu^{2+}-LDL to matrix was not inhibited by chondroitin-6-sulfate at 1 mg/ml (Fig. 4). Similar results were obtained for preincubation of native LDL or Cu^{2+}-LDL with dermatan sulfate (not shown).

Thus, the glycosaminoglycan chains of proteoglycans do not appear to be responsible for the high affinity/high capacity binding of Cu^{2+}-LDL. However, the binding of high concentrations of native LDL is mediated in part by glycosaminoglycan chains within the extracellular matrix.

Chelators do not affect the extracellular matrix binding of Cu^{2+}-LDL

The role of divalent cations in the matrix binding of native LDL and Cu^{2+}-LDL was investigated further with use of the metal ion chelators diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA). Addition of EDTA (in a 2-fold molar excess relative to the metal ion content of EBSS) to the lipoproteins had no effect on the matrix binding of either native LDL (1–25 μg/ml) (**Fig. 5A**) or Cu^{2+}-LDL (1–25 μg/ml; Fig. 5B). Further addition of EDTA or DTPA at up to 10-fold molar excess still had no effect on the binding of either native LDL or Cu^{2+}-LDL (data not shown). Thus, the interactions of native LDL and oxidized LDL with the extracellular matrix are not mediated by divalent cation-bridging molecules.

Enzymatic digestion of matrix by trypsin, pronase, and elastase, but not DNase or collagenase, reduces binding of Cu^{2+}-LDL

To identify or rule out other components of the matrix as being responsible for the binding of Cu^{2+}-LDL, digestion

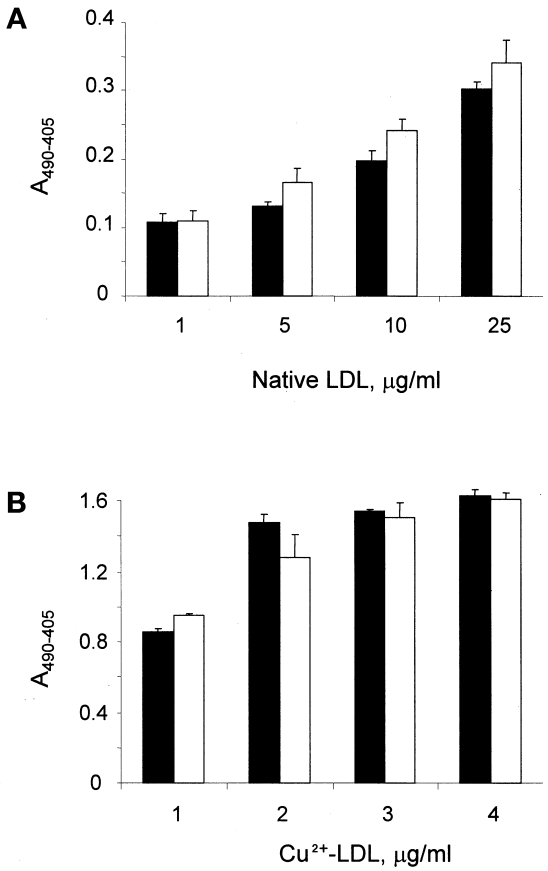

Fig. 5. EDTA has no effect on the matrix binding of native LDL or Cu^{2+}-LDL. Native LDL (A) or Cu^{2+}-LDL (B) was preincubated with no chelators (solid bars) or a 2-fold molar excess of EDTA relative to the metal ion content of EBSS (open bars) prior to incubation with matrices. Bound native LDL and Cu^{2+}-LDL were detected by ELISA. Values are expressed as means ± SEM (n = 3). At no concentration of either native LDL or Cu^{2+}-LDL were there statistically significant differences in binding as a result of preincubation with chelators.

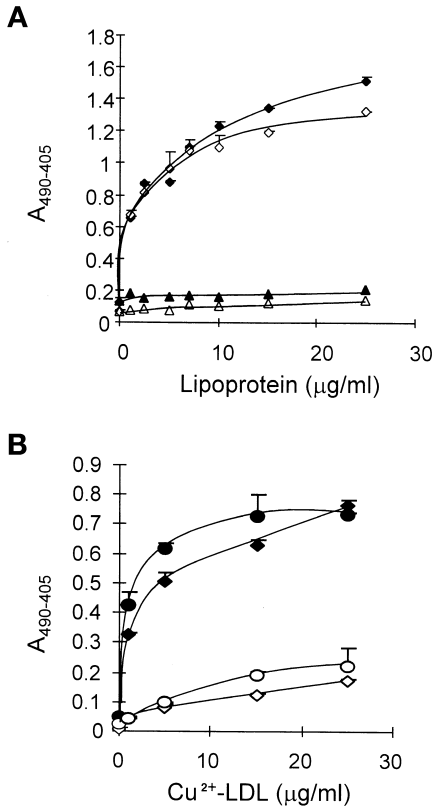

Fig. 6. The binding of Cu^{2+}-LDL to SMC extracellular matrices is not DNase sensitive, but is trypsin and pronase sensitive. A: Extracelluar matrices were prepared from [^{3}H]thymidine-labeled SMC and treated with a no-enzyme control buffer (solid diamond, solid triangle) or DNase (open diamond, open triangle) prior to incubation with native LDL (solid triangle, open triangle) or Cu^{2+}-LDL (solid diamond, open diamond). B: SMC extracellular matrices were prepared and treated with trypsin (open diamond), pronase (open circle), or the appropriate no-enzyme control buffer for trypsin (solid diamond) and pronase (solid circle) prior to incubation with Cu^{2+}-LDL. Bound lipoprotein was detected by ELISA. Values are expressed as the means ± SEM (n = 3).

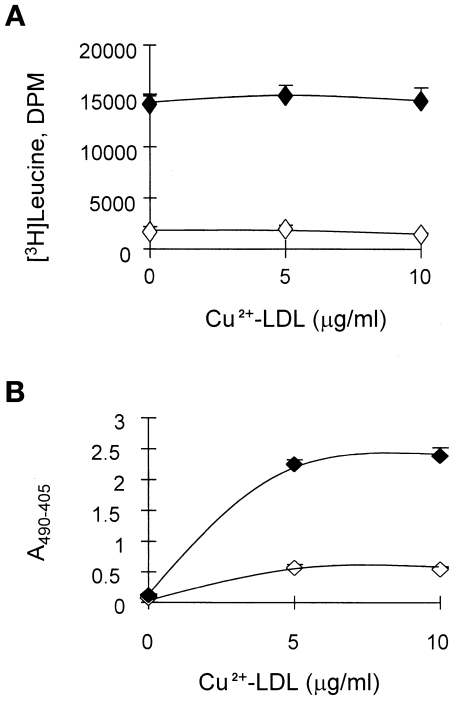

Fig. 7. The binding of Cu^{2+}-LDL to SMC extracellular matrices is elastase sensitive. A: Extracellular matrices were prepared from [^{3}H]leucine-labeled SMC, treated with a no-enzyme control buffer (solid diamond) or with human leucocyte elastase (open diamond) prior to incubation with Cu^{2+}-LDL, and monitored for loss of radioactivity. B: Cu^{2+}-LDL was incubated with the control matrix (solid diamond) or the elastase-treated matrix (open diamond) and detected by ELISA. Values are expressed as the means ± SEM (n = 3).

of the matrices with a variety of enzymes was performed prior to incubation with lipoproteins.

Matrices were digested with DNase to remove any residual DNA (known to bind to a variety of proteins) that remained after lysing and washing the cell layer. Digestion of matrices from [^{3}H]thymidine-labeled SMC with DNase completely removed DNA from the matrix preparations, as evidenced by loss of radioactivity after digestion (data not shown), while having no effect on the matrix binding of Cu^{2+}-LDL or native LDL (**Fig. 6A**).

The enzymes pronase and trypsin were used to establish that a protein component of the matrix indeed was responsible for binding Cu^{2+}-LDL. Treatment of matrices with pronase, which cleaves nonspecifically at any peptide bond, or trypsin, which cleaves at the carboxyl terminal of arginine and lysine residues, dramatically reduced the matrix binding of both Cu^{2+}-LDL (Fig. 6B; $P < 0.05$ at all concentrations of Cu^{2+}-LDL for both pronase and trypsin) and native LDL (data not shown).

A highly purified and specific form of collagenase, which cleaves the X-glycine bond of collagen and synthetic peptides at segments that contain the sequence -proline-X-glycine-proline was used to investigate whether matrix collagen was responsible for binding Cu^{2+}-LDL. Treatment with collagenase effectively digested matrix collagen, as determined by SDS-PAGE, but did not liberate [^{3}H]proline from the matrices, as determined from scintillation counting of the harvested matrix at completion of the binding study (not shown). Collagenase treatment also did not affect the binding of Cu^{2+}-LDL to the matrices (not shown). These results suggest that although collagenase effectively cleaved matrix collagen, it did not result in release of digested collagen from the matrix. This digested collagen potentially is involved in the matrix binding of Cu^{2+}-LDL.

Human leucocyte elastase also was used to examine the role of matrix elastin in the binding of oxidized LDL. Treatment with elastase resulted in loss of both [^{3}H]leucine from the matrices ($P < 0.05$ at all concentrations of Cu^{2+}-LDL; **Fig. 7A**) and Cu^{2+}-LDL binding to the matrix ($P < 0.05$ at all concentrations of Cu^{2+}-LDL; Fig. 7B). These results demonstrate that the binding of Cu^{2+}-LDL is elastase sensitive.

Thus, a protein component(s) of the SMC extracellular matrix that is pronase-, trypsin-, and elastase-sensitive, but

has not been definitively identified, appears to be responsible for the high affinity/high capacity binding of Cu^{2+}-LDL.

DISCUSSION

This study examined the direct binding of native and oxidized LDL to a complex smooth muscle extracellular matrix and the role of ionic charge interactions with matrix proteoglycans in this binding. Native LDL was oxidized by several methods that resulted in different degrees of lipid and protein modification. This allowed for a correlation to be made between the extent of modification and the extent of binding. The different forms of oxidized LDL exhibited 15- to 45-fold greater binding to SMC extracellular matrix than did native LDL, with Cu^{2+}-oxidized LDL having the greatest binding capacity. However, the affinity of the different forms of oxidized LDL for the matrix did not differ from that of native LDL. The increased binding capacity of oxidized LDL could not be directly correlated with overall charge of the molecule, state of aggregation, or generation of reactive aldehydes, lipid peroxides, or conjugated dienes. Furthermore, the binding of high, but not low, concentrations of native LDL was found to be mediated by the glycosaminoglycan chains of extracellular matrix proteoglycans; however, the high affinity/high capacity binding of oxidized LDL was not.

The retention of oxidized LDL by the extracellular matrix potentially is an important step in atherogenesis (8–13). Therefore, a potential mechanism for this interaction was explored. Many studies have described both in vitro and in vivo associations between LDL and matrix proteoglycans that occur as a result of electrostatic interactions between positively charged amino groups of LDL apoB and negatively charged sulfate and carboxyl residues of the glycosaminoglycan chains of proteoglycan molecules (1–6). However, oxidation by AAPH, SLO, HOCl, or copper all reduce the overall positive charge of LDL. If overall charge was the determining factor for binding of LDL to matrix proteoglycans in this system, then oxidized LDL would not be predicted to have higher matrix binding capacity than native LDL. Nevertheless, the possibility that oxidation exposed novel sites on apoB capable of binding negatively charged glycosaminoglycans was explored. Thus, competition binding studies and selective enzymatic digestion of the matrix were used to identify the contribution of proteoglycans to the retention of native and oxidized lipoproteins by the complex extracellular matrix. Although it is not known which form of oxidized LDL may be most physiologically relevant, all of the different forms of oxidized LDL had similar matrix-binding properties. Cu^{2+}-LDL was chosen for the additional studies, in part, because of the greater reproducibility of the final preparation. Further, antibodies generated against copper-oxidized LDL detect epitopes in atherosclerotic lesions of experimental animals and humans (14–17). Selective enzymatic degradation studies with chondroitin ABC lyase and heparinase showed that the glycosaminoglycan chains of matrix proteoglycans are involved in the binding of high, but not low, concentrations of native LDL. This phenomenon previously has been described as "non-receptor-mediated LDL binding" that occurs especially at high LDL concentrations (37). However, selective enzymatic degradation or competition studies could not demonstrate the involvement of glycosaminoglycans in the binding of either low or high concentrations of oxidized LDL. In fact, digestion with chondroitin ABC lyase and heparinase increased the binding of oxidized LDL, suggesting that removal of glycosaminoglycans from the matrix resulted in increased exposure of oxidized LDL binding sites. Thus, glycosaminoglycans do not mediate the binding of oxidized LDL to the extracellular matrix.

The absence of a role for glycosaminoglycans in the retention of oxidized LDL is supported further by the use of metal ion chelators. Divalent cations are reported to facilitate the interaction between LDL and glycosaminoglycans by acting as bridging molecules (38). Thus, the inability of the chelators DTPA or EDTA to diminish the binding of oxidized LDL to the extracellular matrix argues against a divalent cation bridging interaction between oxidized lipoproteins and glycosaminoglycans.

The low level of native LDL binding to the SMC extracellular matrix in this system is consistent with the findings of others (39, 40) who report very little binding of native LDL to cell-derived matrix preparations without the addition of another bridging molecule such as lipoprotein lipase (40). These findings do not contradict a role for proteoglycans as the major lipoprotein binding site within atherosclerotic lesions in vivo (1–4) because others have shown that bridging molecules such as lipoprotein lipase (39–41) greatly enhance the interaction of lipoproteins with proteoglycans in the extracellular matrix. It should be mentioned that our finding of high capacity binding of oxidized LDL to the extracellular matrix is not likely to be due to the presence of lipoprotein lipase in the matrix preparations. If lipoprotein lipase were present, then the binding of native LDL should not be at such a low level, but rather significantly elevated (42).

The association of oxidized LDL with endothelial cell matrix in the presence and absence of lipoprotein lipase also has been examined by others (39). In those studies, LDL was mildly oxidized by long-term storage and very little binding of this mildly oxidized LDL to the endothelial cell matrix was observed without the addition of lipoprotein lipase. In our studies, mildly oxidized LDL, prepared by the same method of long-term storage, similarly exhibited very low binding to the SMC extracellular matrix (data not shown). The binding profile of this mildly oxidized LDL was identical to that of native LDL. Thus, only the more extensively oxidized forms of LDL were examined further.

In addition to proteoglycans, a number of other matrix molecules have lipoprotein binding capacities. Thus, the low level retention of native LDL and the much greater retention of oxidized LDL are likely to be due to interactions with these other molecules. Collagen types I and III, the predominant vascular collagens (43), both have been shown to bind native LDL under physiological conditions

(44, 45). The extent of this binding was found to be related to the net negative charge on LDL so that oxidized LDL, in which derivatization of amino groups increases its negative charge, exhibited even greater collagen binding than did native LDL (45). This is in contrast to proteoglycan-lipoprotein complex formation, which is believed to be due to interactions between positively charged LDL residues and negatively charged proteoglycan side groups (5, 6, 41). However, other studies have shown that the interaction between LDL and collagen is dependent on the presence of decorin, a collagen-binding small proteoglycan (46, 47).

An association between elastin and lipid also has been demonstrated (48–50). Electron microscopy has been used to visualize lipid droplets adjacent to elastic fibers in normal and atherosclerotic human aortas (48), and apoB-containing lipoproteins can be released from atherosclerotic tissue by elastase treatment (50). In vitro, the interaction between elastin and LDL has been found to be of moderately high affinity, with increased binding of LDL to elastin isolated from atherosclerotic versus normal arteries (50).

With these other potential lipoprotein-matrix interactions in mind, additional enzymatic degradation studies were performed to examine the roles of elastin and collagen in the binding of oxidized LDL to this complex matrix. Oxidized LDL binding was sensitive to elastase treatment, but also to trypsin treatment of the matrix. Because elastin is not trypsin digestible, it is most likely that elastase liberated some other protein component of the matrix that was involved in LDL binding. The inability of collagenase treatment to reduce binding of oxidized LDL is inconclusive because [^{3}H]proline was not liberated from the matrix. Although it was determined by SDS-PAGE that the collagen was effectively digested, the collagen fragments remained trapped in the matrix and could potentially still play a role in binding oxidized LDL. However, in interpreting these results, it must be pointed out that the use of elastase, trypsin, and collagenase all are relatively nonspecific enzymatic treatments.

Our use of this complex SMC matrix for binding studies was an attempt to determine whether a physiological presentation of proteoglycans was involved in the extracellular retention of oxidized LDL. Other studies have utilized similar matrix preparations to evaluate the role of the extracelluar matrix in the binding of lipoprotein [a] (51) and lipoprotein lipase-mediated LDL binding (40). The difficulties with identifying a specific component(s) of this matrix responsible for the oxidized LDL retention was a limitation of this system. No doubt, multiple nonproteoglycan components are responsible for oxidized LDL retention by the extracellular matrix. Nevertheless, the enhanced binding of oxidized LDL is consistent with both in vitro and in vivo studies that have demonstrated a role for oxidized LDL in the atherosclerotic process (8–10). Oxidation-specific epitopes have been demonstrated in the extracellular matrix of lesions in both humans and animal models of atherosclerosis (14–17, 52).

On the basis of these findings, extracellular oxidation of LDL in the subendothelial space may lead to greater and prolonged retention by one or more matrix components, perhaps as a result of exposure of additional or novel binding sites on the LDL molecule. We suggest that one potential pathway by which oxidized LDL becomes retained in the vascular extracellular matrix involves a series of steps that occur subsequent to the initial retention and modification of native LDL. As described by the response-to-retention hypothesis of atherogenesis, lipoproteins initially are retained by proteoglycans in the arterial wall by ionic interactions and become modified by processes such as oxidation (4). The initial retention of native LDL may involve lipoprotein lipase (40). The decrease in positive charge of the LDL as a result of such oxidative modification would disrupt the ionic interaction and lead to release of the lipoprotein particle from matrix proteoglycans. The liberated particles then are available for binding and retention by other matrix molecules that have high binding capacity for oxidized LDL. The enhanced retention of oxidized LDL could contribute to the development of atherosclerosis via modulation of a variety of functions in vascular wall cells (8, 9, 53).

This work was supported in part by NIH grants DK02456 (NIDDK) and HL18645 (NHLBI). The authors wish to thank Raina Bulota and Stephanie Lara for technical assistance, and Dr. Hugh Barrett for assistance with calculation of binding constants.

Manuscript received 26 October 2000 and in revised form 5 February 2001.

REFERENCES

1. Camejo, G., E. H. Camejo, U. Olsson, and G. Bondjers. 1993. Proteoglycans and lipoproteins in atherosclerosis. *Curr. Opin. Lipidol.* **4:** 385–391.
2. Galis, Z. S., M. Z. Alavi, and S. Moore. 1993. Co-localization of apolipoprotein B and chondroitin sulfate in an injury model from human atherosclerotic lesions. *Am. J. Pathol.* **142:** 1432–1438.
3. Dalferes, E. R., B. Radhakrishnamurthy, H. A. Ruitz, and G. S. Berenson. 1987. Composition of proteoglycans from human atherosclerotic lesions. *Exp. Mol. Pathol.* **47:** 363–376.
4. Williams, K. J., and I. Tabas. 1995. The response-to-retention hypothesis of early atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **15:** 551–561.
5. Camejo, G., B. Rosengren, U. Olson, F. Lopez, S. O. Olofson, C. Westerlund, and G. Bondjers. 1990. Molecular basis of the association of arterial proteoglycans with low density lipoproteins: its effect on the structure of the lipoprotein particle. *Eur. Heart J.* **11:** 164–173.
6. Cardin, A. D., and H. J. R. Weintraub. 1989. Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis.* **9:** 21–32.
7. Oorni, K., O. Pentikainen, A. Annila, and P. T. Kovanen. 1997. Oxidation of low density lipoprotein particles decreases their ability to bind to human aortic proteoglycans. *J. Biol. Chem.* **272:** 21303–21311.
8. Berliner, J. A., and J. W. Heinecke. 1996. The role of oxidized lipoproteins in atherogenesis. *Free Radic. Biol. Med.* **20:** 707–727.
9. Chait, A., and J. W. Heinecke. 1994. Lipoprotein modification: cellular mechanisms. *Curr. Opin. Lipidol.* **5:** 365–370.
10. Witztum, J. L. 1994. The role of oxidized LDL in the atherogenic process. *J. Arterioscler. Thromb.* **1:** 71–75.
11. Cushing, S. D., J. A. Berliner, A. J. Valente, M. C. Territo, M. Navab, F. Parhami, R. G. Gerrity, C. J. Schwartz, and A. M. Fogelman. 1990. Minimally modified low density lipoprotein induces monocyte-chemotactic protein-1 in human endothelial cells and SMC. *Proc. Natl. Acad. Sci. USA.* **87:** 5134–5138.
12. Frostegard, J., J. Nilsson, A. Haegerstrand, A. Hamsten, H. Wigzell, and M. Gidlund. 1990. Oxidized low density lipoprotein induces

differentiation and adhesion of human monocytes and monocytic cell line. *Proc. Natl. Acad. Sci. USA.* **87:** 904–908.
13. Rajavashisth, T. B., A. Andalibi, M. C. Territo, J. A. Berliner, M. Navab, A. M. Fogelman, and A. J. Lusis. 1990. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified LDL. *Nature.* **344:** 254–257.
14. Ylä-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. W. Butler, J. L. Witztum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84:** 1086–1095.
15. Boyd, H. C., A. M. Gown, G. Wolfbauer, and A. Chait. 1989. Direct evidence for a protein recognized by a monoclonal antibody against oxidatively modified low density lipoprotein in atherosclerotic lesions from a Watanabe heritable hyperlipidemic rabbit. *Am. J. Pathol.* **135:** 815–825.
16. Chang, M. Y., M. Sasahara, A. Chait, E. W. Raines, and R. Ross. 1995. Inhibition of hypercholesterolemia-induced atherosclerosis in the nonhuman primate by probucol. II. Cellular composition and proliferation. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1631–1640.
17. Rosenfeld, M. E., W. Palinski, S. Yla-Herttuala, S. Butler, and J. L. Witztum. 1990. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arteriosclerosis.* **10:** 336–349.
18. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait. 1986. Superoxide-mediated free radical modification of low density lipoprotein by arterial smooth muscle cells. *J. Clin. Invest.* **77:** 757–761.
19. Chung, B. H., T. Wilkinson, J. C. Geer, and J. P. Segrest. 1980. Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* **21:** 284–291.
20. Esterbauer, H., G. Striegl, H. Puhl, and M. Rotheneder. 1989. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radic. Res. Commun.* **6:** 67–75.
21. Noguchi, N., N. Gotoh, and E. Niki. 1994. Effects of ebselen and probucol on oxidative modifications of lipid and protein of low density lipoprotein induced by free radicals. *Biochim. Biophys. Acta.* **1213:** 176–182.
22. Cader, A., F. M. Steinberg, T. Mazzone, and A. Chait. 1997. Mechanisms of enhanced macrophage apo E secretion by oxidized LDL. *J. Lipid Res.* **38:** 981–991.
23. Parhami, F., Z. T. Fang, A. M. Fogelman, A. Andalibi, M. C. Territo, and J. A. Berliner. 1993. Minimally modified low density lipoprotein-induced inflammatory responses in endothelial cells are mediated by cyclic adenosine monophosphate. *J. Clin. Invest.* **92:** 471–478.
24. Buege, J. A., and S. D. Aust. 1978. Microsomal lipid peroxidation. *Methods Enzymol.* **52:** 302–310.
25. El-Saadani, M., H. Esterbauer, M. El-Sayed, M. Goher, A. Y. Nassar, and G. Jurgens. 1989. A spectrophotometric assay for lipid peroxides in serum lipoprotein using a commercially available reagent. *J. Lipid Res.* **30:** 627–630.
26. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1983. Enhanced macrophage degradation of biologically modified low density lipoproteins. *Arteriosclerosis.* **3:** 149–159.
27. Kawamura, M., J. W. Heinecke, and A. Chait. 1994. Pathophysiological concentrations of glucose promote oxidative modification of LDL by superoxide-dependent pathway. *J. Clin. Invest.* **94:** 771–778.
28. Forte, T. M., and R. W. Nordhausen. 1986. Electron microscopy of negatively stained lipoproteins. *Methods Enzymol.* **128:** 442–457.
29. McFarlane, A. 1958. Efficient trace labeling of proteins with iodine. *Nature.* **182:** 53–56.
30. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. *Biochim. Biophys. Acta.* **260:** 212–221.
31. Schonherr, E., H. T. Jarvelainen, L. J. Sandell, and T. N. Wight. 1991. Effects of platelet-derived growth factor and transforming growth factor, beta1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. *J. Biol. Chem.* **66:** 17640–17647.
32. Aggeler, J. 1988. Three-dimensional organization of the extracellular matrix secreted by cultured rat smooth muscle cells. *In Vitro Cell. Dev. Biol.* **24:** 633–638.
33. Young, S. G., J. L. Witztum, D. C. Casal, L. K. Curtiss, and S. Bernstein. 1986. Conservation of the low density lipoprotein receptor-binding domain of apoprotein B. Demonstration by a new monoclonal antibody, MB47. *Arteriosclerosis.* **6:** 178–188.
34. Valentinova, N. V., Z. W. Gu, M. Yang, E. V. Yanushevskaya, I. V. Antonov, J. R. Guyton, C. V. Smith, A. M. Gotto, Jr., and C. Y. Yang. 1994. Immunoreactivity of apolipoprotein B-100 in oxidatively modified low density lipoprotein. *Biol. Chem. Hoppe. Seyler.* **375:** 651–658.
35. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–686.
36. Christner, J. E., and J. R. Baker. 1990. A competitive assay of lipoprotein:proteoglycan interaction using a 96-well microtitration plate. *Anal. Biochem.* **184:** 388–394.
37. Galeano, N. F., M. Al-Haideri, F. Keyserman, S. C. Rumsey, and R. J. Deckelbaum. 1998. Small dense low density lipoprotein has increased affinity for LDL receptor-independent cell surface binding sites: a potential mechanism for increased atherogenicity. *J. Lipid Res.* **39:** 1263–1273.
38. Dahlen, G., C. Ericson, and K. Berg. 1978. In vitro studies of the interaction of calcium ions and other divalent cations with the Lp[a] lipoprotein and other isolated serum lipoproteins. *Clin. Genet.* **14:** 115–124.
39. Auerbach, B. J., C. L. Bisgaier, J. Wolle, and U. Saxena. 1996. Oxidation of low density lipoproteins greatly enhances their association with lipoprotein lipase anchored to endothelial cell matrix. *J. Biol. Chem.* **271:** 1329–1335.
40. Saxena, U., M. G. Klein, T. M. Vanni, and I. J. Goldberg. 1992. Lipoprotein lipase increases low density lipoprotein retention by subendothelial cell matrix. *J. Clin. Invest.* **89:** 373–380.
41. Olin, K. L., S. Potter-Perigo, H. R. Barrett, T. N. Wight, and A. Chait. 1999. Lipoprotein lipase enhances the binding of native oxidized low density lipoproteins to versican and biglycan synthesized by cultured arterial smooth muscle cells. *J. Biol. Chem.* **274:** 34629–34636.
42. Saxena, U., E. Ferguson, B. J. Auerbach, and C. L. Bisgaier. 1993. Lipoprotein lipase facilitates very low density lipoprotein binding to the subendothelial cell matrix. *Biochem. Biophys. Res. Commun.* **194:** 769–774.
43. Wight, T. N. 1996. The vascular extracellular matrix. *In* Atherosclerosis and Coronary Artery Disease. V. Fuster, R. Ross, and E. Topol, editors. Lippincott-Raven, Philadelphia. 421–440.
44. Hoover, G. A., S. McCormick, and N. Kalant. 1988. Interaction of native and cell-modified low density lipoprotein with collagen gel. *Arteriosclerosis.* **8:** 525–534.
45. Jimi, S., N. Sakata, A. Matunaga, and S. Takebayashi. 1994. Low density lipoproteins bind more to type I and III collagens by negative charge mechanisms than to type IV and V collagens. *Atherosclerosis.* **107:** 109–116.
46. Pentikainen, M. O., K. Oorni, R. Lassila, and P. T. Kovanen. 1997. The proteoglycan decorin links low density lipoproteins with collagen type I. *J. Biol. Chem.* **272:** 7633–7638.
47. Kovanen, P. T., and M. O. Pentikainen. 1999. Decorin links low-density lipoproteins (LDL) to collagen: a novel mechanism for retention of LDL in the atherosclerotic plaque. *Trends Cardiovasc. Med.* **9:** 86–91.
48. Guyton, J. R., T. M. Bocan, and T. A. Schifani. 1985. Quantitative ultrastructural analysis of perifibrous lipid and its association with elastin in nonatherosclerotic human aorta. *Arteriosclerosis.* **5:** 644–652.
49. Kramsch, D. M., C. Franzblau, and W. Hollander. 1971. The protein and lipid composition of arterial elastin and its relationship to lipid accumulation in the atherosclerotic plaque. *J. Clin. Invest.* **50:** 1666–1677.
50. Podet, E. J., D. R. Shaffer, S. H. Gianturco, W. A. Bradley, C. Y. Yang, and J. R. Guyton. 1991. Interaction of low density lipoproteins with human aortic elastin. *Arterioscler. Thromb.* **11:** 116–122.
51. Pillarisetti, S., L. Paka, J. C. Obunike, L. Berglund, and I. J. Goldberg. 1997. Subendothelial retention of lipoprotein [a]. Evidence that reduced heparan sulfate promotes lipoprotein binding to subendothelial matrix. *J. Clin. Invest.* **100:** 867–874.
52. O'Brien, K. D., C. E. Alpers, J. E. Hokanson, S. Wang, and A. Chait. 1996. Oxidation-specific epitopes in human coronary atherosclerosis are not limited to oxidized low-density lipoprotein. *Circulation.* **94:** 1216–1225.
53. Witztum, J. L., and D. Steinberg. 1991. Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* **88:** 1785–1792.