Interaction of Low Density Lipoproteins With Human Aortic Elastin

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Interaction between lipoproteins and elastin in the arterial wall may play an important role in atherosclerotic lipid deposition, but binding affinities and other characteristics of the interaction have not been determined previously. Elastin was isolated by hot alkali treatment of human aortic tissue. At 4°C, radioiodinated human low density lipoprotein (LDL) bound to more than one class of binding sites on elastin. Sites of highest affinity had an apparent dissociation constant of $3.6 \times 10^{-10}$ M. Total binding at an LDL concentration of 50 $\mu$g/ml ranged from 4 to 50 ng LDL protein/mg elastin. The binding was relatively specific, since binding was competitively inhibited by LDL and apo E-containing high density lipoprotein (HDL) but only modestly by HDL3. Atherosclerotic elastin exhibited a twofold to fourfold higher capacity for binding LDL, but a reduced affinity. At 37°C, normal elastin exhibited an initial rapid binding of LDL, with a slower linear phase of binding over a 15-hour period, indicating an additional complex process at this temperature. Consideration of the expected LDL concentrations in the arterial intima, in comparison with binding affinities, suggests that LDL binding to elastin probably occurs in the intima and may foster atherosclerotic lipid deposition. (Arteriosclerosis and Thrombosis 1991;11:116-122)

Considerable evidence suggests that elastic fibers may play a role in arterial lipid accumulation. Cholesteryl ester droplets have been localized to elastic lamellae by histochemical techniques, and intravenously administered [3H]cholesterol has shown similar localization by radioautography. Electron microscopy has recently confirmed a striking tendency for lipid droplets to reside adjacent to and within elastic fibers. Large quantities of lipid–protein complexes containing apolipoprotein (apo) B can be released from atherosclerotic tissue after elastase treatment.

Binding of radioiodinated low density lipoprotein (LDL) to isolated elastin has been studied in several laboratories. However, these studies often involved prolonged incubation periods (18–24 hours) at 21°C or 37°C, and a complex interaction was indicated by continued uptake of LDL by elastin throughout the incubation period. An affinity constant for LDL–elastin interaction has not been determined previously.

In this article, we report studies of the interaction of radioiodinated human plasma LDL with human aortic elastin, focusing especially on temperature/time dependence and affinity. We have also investigated the specificity of the interaction and the binding of LDL to elastin isolated from atherosclerotic plaques. Elastin preparations were characterized by amino acid analysis, electron microscopy, and measurement of calcium, hexosamine, and sulfate.

Methods

Elastin

Elastin was prepared from human aortas obtained at autopsy approximately 18 hours post mortem. Normal elastin was obtained from aortas with either no atherosclerosis or minimal fatty streaking. The adventitia was separated by blunt dissection and discarded. Intima–medial tissue was homogenized in 0.1 M NaCl, 0.025 M Tris, pH 7.3, 5 ml/g tissue, using a Tekmar Tissumizer, Tikmar Co., Cincinnati, Ohio. Buffer was removed by centrifugation at 3,000 rpm for 20 minutes. Elastin residue was prepared by a modified Lansing procedure. The tissue was incubated in 0.1N NaOH at 100°C for 1 hour. The insoluble residue was centrifuged and then washed three times in NaOH for 20 minutes each, three times in deionized water, and finally in 2:1 chloro-
form/methanol (vol/vol) at 4°C for 12 hours. The residue was filtered, dried in a desiccator under vacuum, and ground to 60-mesh size in a ceramic mortar. The powdered elastin was further delipidated by three washes for 3 hours each in 2:1 chloroform/methanol (vol/vol). Atherosclerotic elastin was similarly prepared from aortic intima with raised fibrous plaques. Extractable cholesterol, measured enzymatically, was reduced to background levels by this technique.

Amino acid analysis of elastin preparations was performed on a Beckman System 6300 Amino Acid Analyzer, Beckman, Palo Alto, Calif. To measure their calcium contents, duplicate samples of elastin were dried to constant weight at 70°C and then hydrolyzed by incubation in concentrated HNO₃ at 100°C for 5 minutes. Calcium concentrations were then determined on an atomic absorption spectrometer (model Fastac II, Instrumentation Laboratory, Lexington, Mass.).

For electron microscopy, samples of powdered elastin were fixed initially with 2.5–3% glutaraldehyde and then with 2% cacodylate-buffered OsO₄ for 5 hours at 21°C. Specimens were dehydrated in an ethanol series and embedded in Epon resin. Thin sections were stained with uranyl and lead salts and examined at 80–100 keV in a JEOL 200CX electron microscope (JEOL, Peabody, Mass.).

Hexosamine and sulfate contents of the elastin preparations were determined to assess the presence of contaminating glycopeptides and glycosaminoglycans. Elastin samples were hydrolyzed for 4 hours (to release hexosamine) or 6 hours (to release sulfate) in 4 M HCl at 100°C. Hexosamine was assayed as described by Davidson, including preliminary chromatography on Dowex-50 resin, Sigma Chemical Co., St. Louis, Mo., to remove interfering substances. Glucosamine (added to elastin before hydrolysis) was fully recovered. Sulfate arations were determined to assess the presence of sulfate added before elastin hydrolysis.

Lipoproteins

Protease inhibitors, 0.01 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.), 0.001 mM 6-phenylalanyl-L-proplyl-L-arginine chloromethylketone (Calbiochem, La Jolla, Calif.), 50,000 IU/ml aprotinin (Sigma), 0.01 mM Na₂EDTA, and 1 mM sodium azide were added to donor plasma immediately after separation. Ultracentrifugation was performed at 14°C, 45,000 rpm, in a Beckman 60 Ti rotor for 18 hours (LDL) or 45 hours (high density lipoprotein subfraction 3 [HDL₃]). LDL was isolated by sequential spins at KBr-adjusted densities of 1.030 and 1.055 g/ml and then washed by repeated ultracentrifugation at the same densities. The narrow density range and washing procedures help to eliminate easily transferred apolipoproteins, which could interfere with binding studies. The presence of only apo B in the LDL preparation was confirmed by gradient gel electrophoresis with 5% sodium dodecyl sulfate (SDS). HDL₃ was isolated in the density range of 1.20–1.210 g/ml.

LDL was radioiodinated by the iodine monochloride method of McFarlane, as modified by Bilheimer et al, and dialyzed. Greater than 98% of the radioactivity was precipitated by cold 5% trichloroacetic acid. Protein contents of labeled and unlabeled lipoproteins were determined by a modification of the method of Lowry. Total cholesterol assay of LDL preparations revealed an approximate 1.4:1 ratio of cholesterol to protein.

High density lipoprotein cholesterol (HDL₃) from hypercholesterolemic dogs was kindly provided by Thomas Innerarity. These lipoproteins had been purified from the d = 1.006–1.02 g/ml ultracentrifugal fraction of canine plasma by Pevikon block electrophoresis. The presence of apo E as the only protein constituent in HDL₃ was confirmed by SDS gradient gel electrophoresis. HDL₃ was radioiodinated by the iodine monochloride method as described above.

Binding Studies

Thirty milligrams elastin was placed in capped 12×75-mm polystyrene tubes. Varying concentrations of labeled and unlabeled lipoproteins in phosphate buffer (13.5 mM KH₂PO₄, 46.6 mM Na₂HPO₄, pH 7.35) were prepared in a total volume of 0.4 ml generally containing 0.5% bovine serum albumin (Sigma). Lipoproteins were filtered through a 0.22-μm syringe filter just before they were mixed. Lipoproteins were added to the elastin and incubated in duplicate tubes for the indicated times and temperatures. Control tubes without elastin were also prepared in duplicate for each lipoprotein concentration. The supernatant was removed after centrifugation at 3,000 rpm for 10 minutes, and the pellet was washed with 3 ml buffer for three washes, then with water repetitively until supernatant radioactivity was less than twice the background level. The washing process often required 10 or more repetitions. Gamma emissions from the pellet were quantified on a Model 100 Nucleus spectrometer, Oak Ridge, Tenn. The retained protein per milligram elastin was calculated after correction for binding to polystyrene tubes without elastin. Binding was linear with respect to the elastin content of the tubes.

Emphasis was given to binding studies performed at 4°C for 2 hours. To distinguish low- from high-affinity binding, unlabeled LDL was added at a concentration of 500–1,000 μg protein/ml. Because the region of greatest interest in the binding curve was under 50 μg/ml of labeled LDL protein, at least a 10-fold excess of unlabeled ligand was present in this concentration range of labeled ligand. The lowest concentrations of usable labeled LDL were in the range of 5–10 μg/ml because of the necessity of subtracting counts that bound nonspecifically to the plastic tube without elastin. At these low concentrations, counts retained in the tubes with elastin were generally fourfold to eightfold higher than counts retained in tubes without elastin.
Results

Elastin Characterization

Amino acid analyses for normal and atherosclerotic elastin preparations, as well as comparative values from the literature,7 are shown in Table 1. The purity of these preparations is confirmed by their very low contents of histidine, cysteine, and methionine. Amino acid analyses of two additional preparations of normal elastin and one additional preparation of atherosclerotic elastin gave very similar results. A moderate increase in charged residues was evident in the atherosclerotic elastin. Current results closely resembled literature values.7

Electron microscopy of four preparations of normal elastin and one preparation of atherosclerotic elastin showed complete digestion of tissue structures other than elastin. One other preparation of atherosclerotic elastin not used in this study revealed occasional areas of collagen fibrils among the elastic fibers. Microscopic sites of calcification were evident, especially in atherosclerotic elastin preparations. The mean calcium content of six preparations of normal elastin was 2.2±0.8 μg/ml elastin (mean±SD). Among these six preparations, there was no consistent correlation between calcium content and either affinity or capacity for LDL binding. Two preparations of atherosclerotic elastin showed calcium contents of 31.9 and 27.8 μg/mg elastin, respectively.

Assay for hexosamine showed a content of 0.09–0.11% for five separately isolated samples of elastin from normal intima–media, 0.48% for another sample of normal elastin, and 0.15% for one sample of atherosclerotic elastin. Sulfate analysis revealed contents ranging from 0.03% to 0.05% for three samples of normal elastin and two of atherosclerotic elastin. By these criteria, glycosaminoglycan contamination of the elastin preparations was judged minimal. The preparation with the highest hexosamine content showed LDL binding characteristics similar to those of other preparations.

Effect of Albumin

Albumin was added to LDL just before incubation with elastin. This led to a concentration-dependent decrease in LDL binding to elastin (Figure 1). Since most of the decrement occurred at a concentration of 0.5% albumin, all subsequent experiments were per-

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Line plot of effect of bovine serum albumin concentration (gm/dl) on 125I-low density lipoprotein (LDL) binding (μg prot/mg elastin) to normal elastin. Lipoproteins were incubated at the indicated concentrations with elastin at 37°C for 15 hours, followed by extensive washing.
formed in the presence of 0.5% albumin to reduce nonspecific binding.

Time and Temperature Dependence

Determination of the time course of binding at temperatures of 4°C, 21°C, and 37°C revealed at least two phases of binding of LDL to elastin (Figure 2). The first consisted of a rapid binding that was complete within 2 hours; this was similar at 4°C and 21°C and was somewhat greater at 37°C. A second, slower phase of binding followed; this was minimal at 4°C, but at 37°C it accounted for more than half of total binding at 15 hours.

Binding at Physiological Temperature

To provide comparison with published results, incubations of LDL with normal and atherosclerotic elastin were performed at varying LDL concentrations at 37°C for 16 hours. The atherosclerotic elastin bound approximately five times as much LDL as normal elastin at every comparable concentration (Figure 3).

It was considered that the binding of LDL to elastin might be due to a subtraction of LDL containing apo E. A small amount of apo E may not have been detected by gel electrophoresis of LDL apo. Therefore, HDL₄, a lipoprotein shown to contain only apo E by electrophoresis, was incubated with normal elastin in direct comparison with LDL (Figure 3). Binding was only modestly increased with HDL₄. Thus, it seems unlikely that the binding of LDL to normal elastin could be due largely to a minor subtraction containing apo E.

Binding at 4°C

Incubations were performed at 4°C for 2 hours to study the more-rapid initial phase of binding. The concentration dependence of LDL binding to normal nonatherosclerotic elastin was determined on eight occasions with seven different preparations of LDL and elastin. Binding of moderately high affinity can be distinguished in Figure 4 as the difference (dashed curve) between binding of labeled LDL in the absence and presence of a large excess of unlabeled LDL. Even after subtracting the low-affinity binding, Scatchard analysis performed on the dashed curves (Figure 4, insert) generally showed more than one class of binding sites. Apparent dissociation constants for the linear portion of the curve (including at least four points) showing the highest binding affinity ranged from $1.3 \times 10^{-8}$ M to $1.44 \times 10^{-7}$ M. The geo-
Concentration (ng prot/ml) dependence of [125I]-low density lipoprotein (LDL) binding (ng prot/mg elastin) to atherosclerotic elastin after incubation for 2 hours at 4°C under the same conditions as shown in Figure 4. Results are representative of two experiments.

metric mean was 3.6x10^{-8} M. Total LDL binding to elastin at an LDL concentration of 50 μg/ml (approximately 9x10^{-8} M) ranged from 0.004 to 0.05 μg LDL protein/mg elastin. For preparations that exhibited the highest binding affinities, the amounts of LDL bound were near the low end of this range, and vice versa.

The concentration dependence of LDL binding to atherosclerotic elastin was determined with two preparations of LDL and elastin. Scatchard analysis revealed apparent dissociation constants of 8.7x10^{-8} M and 9.9x10^{-8} M in the two preparations, respectively (Figure 5). At a concentration of 50 μg/ml in the incubating medium, total LDL binding to atherosclerotic elastin averaged 0.12 μg/mg elastin. Thus, the capacity for LDL binding of atherosclerotic elastin was approximately two to four times higher than that of nonatherosclerotic elastin although the binding to atherosclerotic elastin was of weaker affinity.

Specificity of the LDL-elastin interaction at 4°C is shown in Figure 6. HDL containing apo E, inhibited [125I]-LDL binding as well as unlabeled LDL, while HDL3, lacking apo E, competed poorly for LDL binding sites. The experiment with HDLc was performed once; a relative lack of competition by HDL3, compared with unlabeled LDL, was confirmed in three experiments.

Discussion

The most important result of this study is the demonstration of an interaction between LDL and elastin with moderately high affinity. For nonatherosclerotic elastin derived mostly from the aortic media, the apparent dissociation constant was approximately 3.6x10^{-8} M.

The significance of the apparent dissociation constant can be ascertained by comparing it with the estimated concentration of LDL in human arterial intima. This concentration has been estimated to approximate that in lymph, about 3x10^{-7} M, or in plasma, about 3x10^{-6} M. At either concentration, one might expect LDL to bind to elastin protein readily if elastin is exposed to interstitial fluid (see below). The affinity of LDL for elastin appears to be approximately one order of magnitude less than that of LDL for its cellular receptor (Kd = 2.8x10^{-8} M). Nevertheless, it seems unlikely that the binding of LDL to elastin is a normal physiological phenomenon.

A reasonable hypothesis can be formulated for a pathogenic role of the LDL–elastin interaction. Early lipid deposits in human arterial intima are often remarkably associated with elastic fibers. Perifibrous lipid consists of small droplets of mostly cholesteryl ester, found in extracellular locations in the deeper intimal sublayers of large arteries in all adult humans. Using a new electron microscopic technique that prevents extraction of neutral lipid, we demonstrated that more than 75% of perifibrous lipid appeared in droplets adjacent to or within elastic fibers. In early atherosclerotic fibrous plaques, dense infiltration of elastic fibers by lipid can be seen. It seems possible that an LDL–elastin interaction could be an initial step in the formation of these deposits. It is not yet known whether LDL is specifically localized to the surfaces of elastic fibers in the arterial intima because immunocytochemical procedures of sufficient specificity and sensitivity have not yet been applied.

The binding of LDL to elastin was relatively specific, since HDL3 gave little inhibition. Nonspecific binding was minimized by performing all studies in the presence of 0.5% albumin, a concentration approximately equal to that suggested by Smith for albumin concentration in normal human aortic intima. The apo E–containing HDLc from hypercholesterolemic dogs did inhibit LDL binding to elastin. Labeled HDLc also exhibited binding of a similar degree to that of LDL. LDL and HDLc are known to compete for binding to the cellular LDL...
receptor, with HDL₄ having an even higher affinity than LDL.²⁴ The similar results with elastin raise the question of whether a similar mechanism of binding may be operative. The present results, however, do not allow an assessment of the mechanism of binding. Further studies are needed to address this issue.

The lack of inhibition of LDL binding by HDL₃ differs from results reported by Noma et al.¹¹ Those results were based on an 18-hour incubation at 37°C without albumin, rather than the 2-hour incubation at 4°C in the presence of albumin used in our inhibition experiments. Moreover, Noma and co-workers¹¹ found a far greater amount of LDL binding to elastin than we observed, indicating that different mechanisms might be operative.⁹

The reason for the slower phase of LDL binding to elastin, evident at temperatures above 4°C, is unclear. In some of the studies previously reported, which were generally performed over 24 hours, a majority of the LDL binding appears to have occurred during this slower phase.⁷⁻⁹ LDL does have a tendency to form aggregates slowly on standing, particularly at higher temperatures, and the slower phase could reflect binding of these aggregates to the elastin. If LDL is not filtered to remove aggregates before performing an incubation, increases in binding of twofold to 10-fold may be seen, even when one corrects for the decreased LDL content of the filtrate (E.J. Podet, unpublished observations). Unless the mechanism of the slower phase of binding can be better defined, its pathophysiological significance will remain doubtful.

The maximal capacity for LDL binding to elastin observed in this study was 0.37 μg LDL protein/mg atherosclerotic elastin and 0.11 μg LDL protein/mg normal elastin, attained after 16 hours of incubation at 37°C. Using similar LDL concentrations and a 24-hour incubation at 37°C, Kramsch and Hollander⁷ reported 21 and 14 μg LDL protein binding/mg atherosclerotic and normal elastin, respectively. Noma et al.¹⁹ reported somewhat lower levels, but levels that were still 20-fold higher than the present results. These investigators did not report filtering their LDL before use, and their incubations were performed without albumin in the incubation medium. Even with these differences in technique, the discrepancy is large and remains to be explained. The binding of LDL to elastin clearly will be a function of the surface area of the insoluble elastin particles. Since our elastin was ground to a fine powder, it seems unlikely that its surface area to mass ratio was much less than that of the elastin prepared by previous investigators.

As have previous investigators,⁷⁻⁸ we found markedly increased binding of LDL to elastin prepared from atherosclerotic aortic intima. The relative binding capacity of atherosclerotic elastin was even more striking than previously reported, perhaps due to the fact that this study focused on high-affinity binding. LDL bound to atherosclerotic elastin with apparently lower affinity (Kd approximately 1×10⁻⁷ M) than to normal elastin, but it is possible that binding sites of higher affinity may have been obscured in the analysis by a greatly increased capacity for binding at the lower affinity. Kramsch and Hollander⁷ found that atherosclerotic elastin prepared in a manner similar to that used in this study had an increased content of polar amino acids compared with those of normal elastin. In this study, atherosclerotic elastin showed only a slight tendency for increases in polar amino acids, much less than that described by Kramsch and Hollander,⁷ who considered that the formation of a new but defective elastin replacing degenerated elastic tissue was a more likely explanation for the altered amino acid content than the presence of contaminating peptides. However, the possibility of covalent attachment of contaminating peptides or other moieties to atherosclerotic elastin that are resistant to alkaline hydrolysis must be considered. We found, as expected, a strikingly increased content of calcium in atherosclerotic elastin. However, among six samples of normal elastin in which calcium content varied by more than a factor of three, no consistent relation between calcium content and binding parameters emerged. The increased capacity for LDL binding to atherosclerotic elastin, whatever its nature, could reflect an increased capacity for LDL binding in atherosclerotic lesions, thereby facilitating lipid deposition.

Whether LDL normally have access to elastin protein within the arterial intima is uncertain. Microfibrillar proteins, including fibrillin,²⁵ might prevent the binding from occurring. Alternatively, proteoglycans could prevent the diffusion of LDL to the surface of elastic fibers. It seems possible that these extracellular tissue components might be degraded during atherogenesis, allowing contact between LDL and elastin. Furthermore, degradation of elastin itself occurs during atherogenesis.²⁶,²⁷ Piecemeal degradation could make a vastly increased surface area of elastin available for binding LDL, possibly accelerating the atherogenic process.

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References


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