Lipoprotein(a) in Diet-Induced Atherosclerosis in Nonhuman Primates

Ralph L. Nachman, Dov Gavish, Neal Azrolan, and Thomas B. Clarkson

Lipoprotein(a) (Lp[a]) is a low density lipoprotein particle that contains plasminogen-like apolipoprotein(a). Recent studies suggest an association of Lp(a) with atherosclerotic vascular disease. We have studied the accumulation of Lp(a) in atherosclerotic arteries of monkeys with diet-induced atherosclerosis. Immunohistochemistry with monospecific Lp(a) antisera revealed striking accumulations of Lp(a) in atherosclerotic coronary artery lesions. There was no Lp(a) in the normal, nonatherosclerotic arteries. Analysis of paired tissue and serum samples from 17 male hyperlipoproteinemic monkeys revealed a significant correlation between aortic wall Lp(a) and serum Lp(a) levels. The serum cholesterol level failed to correlate with either aortic Lp(a) or serum Lp(a). These results add further evidence for the potential role of Lp(a) in the pathogenesis of atherosclerosis. (Arteriosclerosis and Thrombosis 1991;11:32–38)
Cal, were fed to various animal groups for extended periods of time. The composition of a typical diet has been published previously.17

Reagents

Lp(a) and apo(a) were purified as previously described.6,18 Monospecific anti-Lp(a) was raised in horses (G.M. Kostner, University of Graz, Graz, Austria) and adsorbed to extinction as assessed by Western blot with both human plasminogen and human apo(B) insolubilized on nitrocellulose strips. Cross-reactivity with plasminogen, low density lipoprotein (LDL), and fibrinogen were all negative. The anti-Lp(a) was used at a dilution of 1:4,000. Monospecific rabbit anti-human plasminogen was kindly provided by P. Harpel (Cornell University Medical College, New York, N.Y.) and used at a dilution of 1:2,000. Monospecific rabbit antisera to apo(B), albumin, fibronec tin, and fibrinogen were obtained commercially (Cappel, Malverne, Pa.), and specificity was verified by enzyme-linked immunosorbent assay (ELISA) and Western blots.

Tissue Sampling

At the time of necropsy, the coronary arteries were perfusion fixed for 1 hour with 10% neutral buffered formalin under 100 mm Hg pressure. After perfusion, the hearts were immersion fixed in the same fixative. Five serial blocks (each 3-4 mm wide) were obtained from the left circumflex and the left anterior descending arteries. These were cut perpendicular to the long axis of each artery, originating at the ostia. Hematoxylin/eosin-stained sections were obtained from each block, and areas showing various lesions were identified. These blocks were then selected, and additional sections were cut for immunohistochemical studies.

Aortas were removed at necropsy, and blocks for immunohistochemical studies were taken immediately. Aortas were immersion fixed in 10% neutral buffered formalin, and standard blocks were taken for atherosclerosis evaluation. The extent of atherosclerosis was defined by evaluation of the microscopic pattern. Minimal lesions consisted of a slightly thickened intima with a clearly defined intact internal elastic lamina. More extensive thickening involved cellular infiltration and lipid deposition. Extensive lesions consisted of greatly thickened intima, constituting over 80% of the artery width, with extensive lipid deposition. In some arteries, this was associated with marked stenosis.

Histochemistry

Immunohistochemical staining was performed as previously described.6 Formalin-fixed, paraffin-embedded sections were dewaxed and pronase treated. Endogenous peroxidase activity was blocked by treatment with hydrogen peroxide (3%, 30 minutes). The slides were preincubated with 10% normal horse serum in buffer for 1 hour and then incubated (18 hours, 4°C) with primary antibody, horse anti-Lp(a) (1:4,000 dilution). After a brief blocking step with normal horse serum, the slides were exposed to biotinylated goat anti-horse immunoglobulin G (IgG) (30 minutes, 21°C, 1:250 dilution) and incubated with avidin/biotin peroxidase complex. For the detection of plasminogen, rabbit anti-plasminogen at a 1:2,000 dilution followed by biotinylated goat anti-rabbit IgG was used. The slides were incubated with avidin/biotin peroxidase complex, and peroxidase deposition was visualized with 3,3'-diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin. The extent of Lp(a) deposition was roughly estimated on a minimal, moderate, and extensive scale. Sections illustrating a particular lesion were representative of the general field; however, extensive serial studies were not performed, and the heterogeneity of Lp(a) deposition was not fully evaluated within a given lesion.

For the purposes of simplicity and clarity, the immunohistochemically identified material in the various lesions is referred to as Lp(a) even though the specificity of the antibodies used is for the apo(a) component. In studies not yet published, we have demonstrated colocalization of apo(B) in all sections containing apo(a).

Tissue Extraction of Lipoprotein(a)

For these studies, aortas were removed before perfusion-fixation and flushed with buffer containing protease inhibitors phenylmethylsulfonyl fluoride (0.1 mM), pepstatin (0.1 mM), and EDTA (1 mM). One-centimeter portions of affected and unaffected aortas were frozen in liquid nitrogen and saved for subsequent extraction. Intima from atherosclerotic and apparently normal aortic segments was dissected from the media, rinsed twice with saline, and weighed (50–100 mg). Intimal specimens were minced into 1–2-mm² pieces and homogenized (Bio spec, Bartlesville, Okla.). The samples were incubated for 60 minutes at 37°C in Tris-buffered saline (pH 7.4) containing 0.1 M CaCl₂, 1 mM leupeptin, 1 mM pepstatin, 1 mM aprotinin, and 860 units/ml collagenase (Sigma 1A, Sigma Chemical Co., St. Louis, Mo.). Samples were centrifuged for 10 minutes at 3,000g, and the supernatant was saved for Lp(a) quantification and polyacrylamide gel electrophoresis (PAGE). Addition of exogenous standard Lp(a) to the extraction system was used to assess the recovery. In three different experiments, recovery was greater than 90%.

Lipoprotein(a) Assays

An ELISA specific for Lp(a) was developed, using a monospecific polyclonal antibody raised in rabbits against purified monkey (cytomolgus) Lp(a). The antibody was purified on a series of affinity columns containing LDL, albumin, lgs plasminogen, and apo(a). This antibody was found to be specific for apo(a) by both Ouchterlony19 and Western blot20 analyses. The assay used for Lp(a) quantification was similar to that recently described by Fless et al.21
Briefly, the specific anti-Lp(a) antibody was used as a capture antibody. Alkaline phosphatase conjugated to anti-apo B was used as a detecting antibody. Purified Lp(a) with a single-band apo(a) phenotype and an apparent molecular weight of 480 kd by sodium dodecyl sulfate (SDS)–PAGE was employed as the standard. Each plate contained 11 duplicate serial dilutions of the Lp(a) standard. Samples were
Results

Lipoprotein(a) Deposition in Atherosclerotic Coronary Arteries

Immunohistochemical analysis of the left anterior descending coronary artery of a 7-year 1-month-old male rhesus monkey fed a moderately atherogenic diet for 7 years revealed extensive Lp(a) deposition in the thickened intima (Figure 1B). All reactivity was removed on adsorption of the monospecific anti-Lp(a) with insolubilized Lp(a) (Figure 1C). Immunodetectable plasminogen was limited to the surface of the thickened intima (Figure 1D). Immunohistochemical analysis of adjacent sections showed the colocalization of apo(B) with Lp(a) in the thickened intima. Fibronectin and fibrinogen were also present, but the staining was less intense; no albumin was detected. These immunohistochemical studies were typical of the lesions seen in 10 other animals on similar hyperlipidemic diets over the same time period.

Lipoprotein(a) Deposition in Early and Advanced Atherosclerotic Lesions

Coronary arteries from three different monkeys with varying degrees of atherosclerosis were evaluated histochemically. Lp(a) deposition was increased in more extensively atherosclerotic arteries (Figure 2). There was no deposition in the coronary artery of a normal female cynomolgus macaque (9-year-old feral animal fed a control nonatherogenic diet for 3 weeks, containing 0.0 mg/Cal cholesterol) (Figure 2A). Moderate deposition of Lp(a) was detected in an 8-year-old female rhesus cynomolgus macaque monkey that had consumed a moderately atherogenic diet for over 6 years (0.49 mg/Cal cholesterol) (Figure 2B). More extensive deposition of Lp(a) was detected in a 7-year-old female rhesus monkey fed essentially the same diet for 6 years and 6 months, but more extensive coronary intimal thickening was observed (Figure 2C). Immunohistochemical analysis of adjacent sections showed colocalization of apo(B) with apo(a) in the diseased, thickened intima.

Correlation of Serum Lipoprotein(a) Levels With Coronary Artery Lipoprotein(a) Among Hyperlipidemic Animals

Among animals with equivalent degrees of intimal thickening, different patterns of Lp(a) deposition were seen (Figures 3A and 3B). These animals were approximately 11-year-old male cynomolgus monkeys of Indonesian origin, both of which had been fed a high-cholesterol, atherogenic diet for 1 year. After this period, animal A was switched to a low-cholesterol diet (0.05 mg/Cal cholesterol), while animal B was maintained on a high-cholesterol diet (0.19 mg/Cal cholesterol). Both animals were necropsied after 24 months on the second-phase diet. In one animal maintained on the low-cholesterol regression diet, Lp(a) was preferentially deposited at the endothelial surface of the thickened intima (Figure 3A), while in the other animal maintained on the higher-cholesterol atherogenic diet,

Figure 2. Photomicrographs showing immunohistochemical identification of lipoprotein(a) (Lp[a]) in early and advanced coronary atherosclerosis. Immunoperoxidase staining of coronary arteries with anti-Lp(a) adsorbed with plasminogen from A: Normal monkey; B: Monkey fed an atherogenic diet (at necropsy, total plasma cholesterol=200 mg/dl and high density lipoprotein [HDL] cholesterol=45 mg/dl); C: Monkey fed an atherogenic diet (at necropsy, total plasma cholesterol=400 mg/dl and HDL cholesterol=65 mg/dl). ×150

added to the plate in four dilutions. Aorta extracts were diluted 10- to 40-fold and plasma samples 100- to 10,000-fold. Lp(a) measurements were valid for all phenotypes, since the detection system was directed against apo B (i.e., apo[a] was used only to capture Lp[a] particles). Apo(a) isoform size was determined by SDS-PAGE of whole plasma and subsequent Western blotting as recently described.
there was more extensive Lp(a) deposition throughout the entire thickened intima (Figure 3B). The latter animal with the greater amount of Lp(a) in the coronary artery also had increased amounts of Lp(a)-immunoreactive material extracted from normal-appearing as well as atherosclerotic-appearing aorta.

Paired tissue and serum samples from 17 male hyperlipoproteinemic cynomolgus monkeys with coronary atherosclerosis in various stages of progression and regression were examined. These monkeys were fed an atherogenic diet (1 mg/Cal cholesterol) for 1 year. For the next 24 months, the monkeys were fed a prudent diet (0.05 mg/Cal cholesterol). The necropsied animals ranged in age from 11 to 14 years. There was a significant correlation between aortic wall and serum Lp(a) levels (Figure 4a). Serum

<table>
<thead>
<tr>
<th>CHOL</th>
<th>Lp(a)</th>
<th>AORTA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>HDL</td>
<td>Serum</td>
</tr>
<tr>
<td>131</td>
<td>43</td>
<td>15.6</td>
</tr>
<tr>
<td>197</td>
<td>66</td>
<td>25</td>
</tr>
</tbody>
</table>

FIGURE 3. Photomicrographs showing patterns of lipoprotein(a) (Lp[a]) deposition in atherosclerotic lesions in two hyperlipoproteinemic male cynomolgus monkeys (A and B). Immunoperoxidase staining of coronary arteries with anti-Lp(a) adsorbed with plasminogen. Serum cholesterol (CHOL) and Lp(a) levels (mg/dl) were obtained at necropsy. Aortic Lp(a) extracted from diseased-(D) or normal-(N) appearing aorta is expressed as nanograms per gram wet tissue weight. HDL, high density lipoprotein. ×80

FIGURE 4. Scatterplots showing comparison of serum lipoprotein(a) (Lp[a]) levels (mg/dl) and Lp(a) extracted from aortic tissue (µg/g). a: Serum Lp(a) concentration versus extracted aortic wall lesion Lp(a) (r=0.54, p<0.02). b: Serum total cholesterol (mg/dl) versus aortic Lp(a) (r=0.12, p=NS). c: Serum Lp(a) versus serum total cholesterol (r=0.20, p=NS).
cholesterol failed to correlate with either aortic Lp(a) (Figure 4b) or serum Lp(a) (Figure 4c).

Discussion

This study presents the first immunologic identification of Lp(a) in lesions associated with diet-induced atherosclerosis in nonhuman primates. Deposition of Lp(a) in coronary lesions was striking, and, to a reasonable degree, mirrored the Lp(a) deposition described previously in human atherosclerotic lesions. In general, the amount of intramuscular Lp(a) deposition reflected the extent of intimal thickening and associated atherosclerotic changes (Figure 2). There was no clear relation between the phenotype size of the apo(a) protein and the extent of tissue penetration and deposition (Figure 3). The amount of Lp(a) extracted from adjacent atherosclerotic aortic tissue was greater in animals with more extensive coronary Lp(a) deposition. This probably reflected the greater extent of atherosclerosis in a given animal. These findings indicate that Lp(a) deposition in most animals reflected the extent of vascular damage.

In all the histochemical studies with adjacent sections, there appeared to be full colocalization of apo(B) with apo(a) in the same lesion. In some areas, apo(B) was detectable without the concomitant presence of apo(a), suggesting the presence of LDL alone. The colocalization data strongly suggest that at least a major portion of the apo(a) identification in the diseased vessel reflects the presence of the apo(B)-containing Lp(a) molecule. The extraction data support this conclusion.

Correlative necropsy studies in 17 chronically hyperlipoproteinemic animals with coronary atherosclerosis demonstrated greater amounts of aortic Lp(a) deposition in animals with higher serum Lp(a) levels (Figure 4a). There was no significant correlation between serum cholesterol and aortic Lp(a) levels or between serum Lp(a) and serum cholesterol levels. Similar observations have been reported recently in human atherosclerosis.7

One of the major unresolved issues raised by this study relates to the factor or factor(s) determining Lp(a) tissue entry. Our previous studies have demonstrated endothelial cell Lp(a) binding, presumably at a membrane plasminogen-binding site(s).6 It is of some interest that in certain lesions, Lp(a) appeared to be deposited on or within the endothelial layer (Figure 3A). It was not possible from these studies to determine whether, in fact, the immunochromically reactive protein was intracellular. Whether this “surface” Lp(a) localization represents plasminogen receptor–bound lipoprotein remains to be determined. It is striking that in some atherosclerotic arteries, there was extensive deep intimal deposition (Figure 3B). No Lp(a) was detected in arterial media or adventitia. Lp(a) binds with high avidity to plasminogen-modified fibrinogen.22 Recent studies have shown that Lp(a) colocalizes in human tissues at intravascular sites of fibrin deposition.23 Since some atherosclerotic lesions contain significant amounts of fibrin,24 the possibility exists that some of the Lp(a) in lesions represents lipoprotein bound to tissue fibrin. By immunohistology, fibrinogen was seen in most of the lesions observed in these monkeys; however, in several lesions using adjacent sections, it did not appear that there was direct colocalization of Lp(a) and fibrinogen in the same region.

The identification of Lp(a) in coronary atherosclerotic lesions from nonhuman primates with diet-induced atherosclerosis and previous studies suggesting an antifibrinolytic effect of Lp(a) at the endothelial cell surface add further evidence for the role of Lp(a) as a molecular bridge between local thrombogenesis and atherosclerosis.

References

1. Eaton DL, Fless GM, Kohr WJ, McLean JW, Xu QT, Miller CG, Lawn RM, Scamu AM: Partial amino acid sequence of apolipoprotein(a) shows that it is homologous to plasminogen. Proc Natl Acad Sci USA 1987;84:3224–3228
15. Laplaud PM, Beaubatie I, Rail SC, Luc G, Saboureau M: Lipoprotein(a) is the major apo B–containing lipoprotein in the plasma of a hibernator, the hedgehog (Erinaceus europaeus). J Lipid Res 1988;29:1157–1170


18. Gavish D, Azrolan N, Breslow JL: Plasma Lp(a) concentration is inversely correlated with the ratio of Kringel II/Kringle V-encoding domains in the apo(a) gene. *J Clin Invest* 1989;84:2021–2027


**KEY WORDS** • lipoprotein(a) • atherogenic diet • nonhuman primates
Lipoprotein(a) in diet-induced atherosclerosis in nonhuman primates.
R L Nachman, D Gavish, N Azrolan and T B Clarkson

doi: 10.1161/01.ATV.11.1.32
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/11/1/32

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/