

# Quantification of apo[a] and apoB in human atherosclerotic lesions

Judith M. Pepin, June A. O'Neil, and Henry F. Hoff<sup>1</sup>

Department of Vascular Cell Biology and Atherosclerosis Research, Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195

**Abstract** Lipoprotein[a] or Lp[a] is a cholesterol-rich plasma lipoprotein that is associated with increased risk for cardiovascular disease. To better understand this association we determined the amount of apo[a] and apoB as possible estimates for Lp[a] and low density lipoprotein (LDL) accumulation in atherosclerotic lesions and in plasma, from patients undergoing vascular surgery, using specific radioimmunoassays for apolipoprotein[a] and apolipoprotein B. Apo[a] and apoB were operationally divided into a loosely bound fraction obtained by extracting minced samples of plaque with phosphate-buffered saline (PBS), and a tightly bound fraction obtained by extracting the residual tissue with 6 M guanidine-HCl (GuHCl). We found that 83% of all apo[a] but only 32% of all apoB in lesions was in the tightly bound fraction. When normalized for corresponding plasma levels, apo[a] accumulation in plaques was more than twice that of apoB. All fractions of tissue apo[a], loosely bound, tightly bound, and total, correlated significantly with plasma apo[a]. However, no significant correlations were found between any of the tissue fractions and plasma apoB. If all apo[a] and apoB had been associated with intact Lp[a] or LDL particles, the calculated mass of tightly bound Lp[a] would actually have exceeded that of tightly bound LDL in five cases with plasma Lp[a] levels above 5 mg apo[a] protein/dl. When PBS and GuHCl extracts of lesions were subjected to one-dimensional electrophoresis, the major band stained for lipid and immunoblotted positively for apo[a] and apoB, suggesting the presence of some intact Lp[a] in these extracts. **Key words:** These results suggest that Lp[a] accumulates preferentially to LDL in plaques, and that plaque apo[a] is directly associated with plasma apo[a] levels and is in a form that is less easily removable than most of the apoB. This preferential accumulation of apo[a] as a tightly bound fraction in lesions, could be responsible for the independent association of Lp[a] with cardiovascular disease in humans. —Pepin, J. M., J. A. O'Neil, and H. F. Hoff. Quantification of apo[a] and apoB in human atherosclerotic lesions. *J. Lipid Res.* 1991. 32: 317–327.

**Supplementary key words** Lp[a] • LDL • arterial wall • plaques • radioimmunoassay • cardiovascular disease

Lipoprotein[a], or Lp[a], like low density lipoprotein (LDL), is a cholesterol-rich plasma lipoprotein (1–3) that has been proposed to be an independent risk factor for cardiovascular and cerebrovascular disease. High levels of

plasma Lp[a] were shown to correlate positively with increased arterial stenosis resulting from atherosclerosis of coronary (4, 5), carotid (6), cerebral arteries (7), and saphenous vein grafts after coronary bypass surgery (8). Although Lp[a] and LDL have several characteristics in common, e.g., both contain apoB-100 (9) and are rich in cholesteryl esters (2, 3), they are immunochemically and physicochemically distinct (3). Lp[a] possesses, in addition to apoB-100, a unique protein called apo[a] (9) which is linked to B-100 by disulfide bridges (3, 9) and is rich in complex carbohydrates (10). Apo[a] exists as several isoforms with molecular weights ranging from 300,000 to 700,000 (2, 3, 11). This size heterogeneity has been speculated to be due to polypeptide chain polymorphism (9, 12).

The link between plasma Lp[a] levels and accelerated atherosclerosis may be related to accumulation of Lp[a] in atherosclerotic lesions and the subsequent metabolic fate of these lipoproteins. As an initial step to assess this link, we measured the accumulation of apo[a] and apoB in lesions as estimates of the accumulation of intact Lp[a] and LDL. We wished to determine whether apo[a] was retained to a greater degree than apoB, and whether positive correlations existed between plasma and tissue Lp[a] and LDL. Based on earlier studies measuring apoB accumulation in human lesions (13), we divided tissue apo[a] and apoB into loosely and tightly bound fractions, operationally defined as the fraction extracted with phosphate-buffered saline (PBS), and that extracted from the residual tissue with the dissociative agent, guanidine-HCl, respectively. Apo[a] and apoB concentrations in these studies were then measured by respective radioimmunoassays.

Abbreviations: LDL, low density lipoprotein; PBS, phosphate-buffered saline; GuHCl, guanidine hydrochloride; RIA, radioimmunoassay.

<sup>1</sup>To whom reprint requests should be addressed.

## METHODS

### Materials

Na <sup>125</sup>I was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA). Nonimmune goat serum, antisera raised in goats against human Lp[a] and LDL, and rabbit anti-goat IgG were prepared by Bethyl Laboratories (West Montgomery, TX). Plasminogen, guanidine-hydrochloride, aprotinin, leupeptin, pepstatin, vitamin E, gentamicin, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). CNBr-activated Sepharose used for the preparations of affinity chromatography gels (LDL-Sepharose and anti-apo[a]-Sepharose) and Sephacryl S-400 (HR) for gel filtration, were obtained from Pharmacia LKB Biotech. Inc (Piscataway, NJ). Pre-made agarose films for one-dimensional gel electrophoresis, Universal PHAB buffer, and Fat Red 7B lipid stain were obtained from Corning (Palo Alto, CA).

### Sample acquisition and tissue processing

Samples of atherosclerotic lesions consisting of fibrous plaques and complicated lesions but not fatty streaks or grossly normal areas derived from carotid, iliac, and femoral arteries and the abdominal aorta, and corresponding venous plasma samples were obtained from 26 patients (mean age, 69 years) undergoing vascular surgery at the Cleveland Clinic Foundation. Blood samples obtained during surgery were collected immediately in Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). Blood cells were sedimented by low speed centrifugation at 4°C within 2 h and plasma fractions were measured by radioimmunoassay (RIA). No patients were taking lipid-lowering medications prior to surgery. The project was approved by the Cleveland Clinic Foundation's Institutional Review Board.

Samples of plaques were brought to the laboratory on ice. The tunica intima was separated from the underlying tunica media along the natural cleavage plane of the internal elastic lamella, as preformed previously (13) and rinsed briefly in phosphate-buffered saline (PBS), i.e., 0.15 M NaCl, 0.1 M phosphate, 0.3 mM EDTA, pH 7.4, to remove any loosely adherent blood components. The tissue was blotted to remove excess moisture, weighed, and finely minced with tissue slicer blades. A loosely bound lipoprotein fraction was isolated from the minces by extraction without homogenization into cold PBS at a ratio of 1 g wet weight tissue to 5 ml PBS for 18 h at 4°C. As earlier studies have indicated that proteolytic activity may co-isolate with Lp[a] (14), aprotinin (100 K.I.U./ml), leupeptin (35 μM), and pepstatin (5 μM) were added to tissue extract buffers to inhibit protease activity. Furthermore, to eliminate auto-oxidation of the lipoproteins and growth of bacteria, 20 μM vitamin E and 50 μg/ml gen-

tamicin, respectively, were added to all samples during isolation. The resulting suspensions were centrifuged at 20,000 g for 1 h at 4°C (15) and the supernatant separated from the pellet.

The resulting pellet was extracted at 4°C for several sequential 24-h periods with 6 M guanidine-HCl (GuHCl) at a ratio of 1 g tissue wet weight to 3 ml GuHCl, which included all of the inhibitors listed above. As up to six sequential 24-h extraction periods were needed to remove all tissue apo[a], we ultimately used one 6-day extraction procedure for subsequent studies. Preliminary studies showed that the total amount of apo[a] extractable from tissue in six sequential 24-h extraction periods gave values comparable to one 6-day extraction period (data not shown). This GuHCl-extracted fraction was designated the tightly bound fraction.

### Lipoprotein isolation

Human LDL was isolated from plasma within 24 h of drawing of blood obtained from the Cleveland Clinic Foundation Blood Bank, using sequential ultracentrifugation, ( $1.019 < d < 1.063$  g/ml) according to the procedure of Hatch and Lees (16) and was stored at 4°C in 0.15 M NaCl containing 0.54 mM EDTA (pH 8.5). Plasma samples from donors with concentrations of Lp[a] mass > 30 mg/dl, were selected for the isolation of Lp[a] which was performed by sequential ultracentrifugation at  $d$  1.063 and  $d$  1.10 g/ml. The isolated  $1.063 < d < 1.10$  g/ml fraction was subjected to gel filtration chromatography using a Sephacryl S 400 HR column (1.5 cm × 90 cm) eluted with PBS. The column eluate was monitored for absorbance at 280 nm and for immunoreactivity of apo[a]. Individual fractions were also subjected to one-dimensional gel electrophoresis to determine whether Lp[a] fractions were contaminated with HDL or LDL. Two protein peaks were resolved by gel filtration chromatography, one peak containing HDL and the other immunoreactive apo[a]. The fractions under the faster eluting peak were pooled for subsequent studies requiring purified Lp[a].

### Lipoprotein quantification and preparation of antibodies

Competitive binding RIAs were used to measure the apo[a] and apoB in plasma and tissue extracts. Briefly, phase separation was accomplished with rabbit anti-goat IgG and facilitated with 3% PEG 3500. Data were calculated as  $B/B_0$ ; where  $B$  represented the amount of bound tracer in the pellet, and  $B_0$  represented the maximum binding of the tracer in the absence of the competitor, which was approximately 30% of the total counts originally placed in the tube. Nonspecific binding using nonimmune goat serum in place of the primary antibody was determined and subtracted from sample values.

Sample data were calculated using a standard curve plotted as  $B/B_0$  against a log transformation of the sample protein content. The amount of radioactivity in the pellet, which is inversely proportional to the amount of antigen in the sample, was linear over two orders of magnitude.

A purified sample of Lp[a] was quantified gravimetrically to calibrate a serum sample to be used as an assay standard. Plasma and plaque Lp[a] values were expressed in terms of their apo[a] protein moiety, calculated on the assumption that approximately 50% of the protein in Lp[a] is apo[a] and the remainder is apoB. If one also makes the assumption that the apo[a] measured in the plasma and plaque samples is present in intact lipoproteins, the data may be converted from apo[a] protein to lipoprotein mass by multiplying by a factor of 6.7. Plaque apo[a] and apoB contents were also described in a normalized fashion by dividing each plaque lipoprotein concentration by its respective plasma concentration.

Freshly prepared LDL ( $1.019 < d < 1.063$  g/ml) was subjected to affinity chromatography using an anti-apo[a]-Sepharose column to remove contaminating Lp[a]. The protein contents of several LDL standards were determined and then used to calibrate a standard serum sample. The RIA quantifying apoB measures apoB in both LDL and Lp[a]. ApoB concentrations in plasma and tissue was also presented as LDL-apoB, obtained by subtracting the apoB derived from Lp[a] from the total apoB, making the assumptions stated previously.

#### Radioimmunoassay for apo[a] and apoB

A polyclonal antibody was raised in goats against Lp[a], and a combination of DEAE Affigel blue and precipitation by 50% saturated ammonium sulfate was used to isolate a protease-free IgG fraction (Bio-Rad Bulletin #1062). Anti-apo[a] was separated from contaminating anti-apoB using affinity chromatography on LDL-Sepharose 4B according to the manufacturer's instructions (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The purified anti-apo[a] IgG was used at a 1 to 115,000 final dilution for quantitation of Lp[a] by RIA. Freshly prepared LDL free of contaminating Lp[a] (removed using anti-apo[a]-Sepharose) was also used to elicit in goats polyclonal antibodies against apoB. The IgG fraction was used at a dilution of 1 to 200,000 for quantitation of apoB by RIA. Under our assay conditions, both RIAs were sensitive to levels below 4 ng of protein.

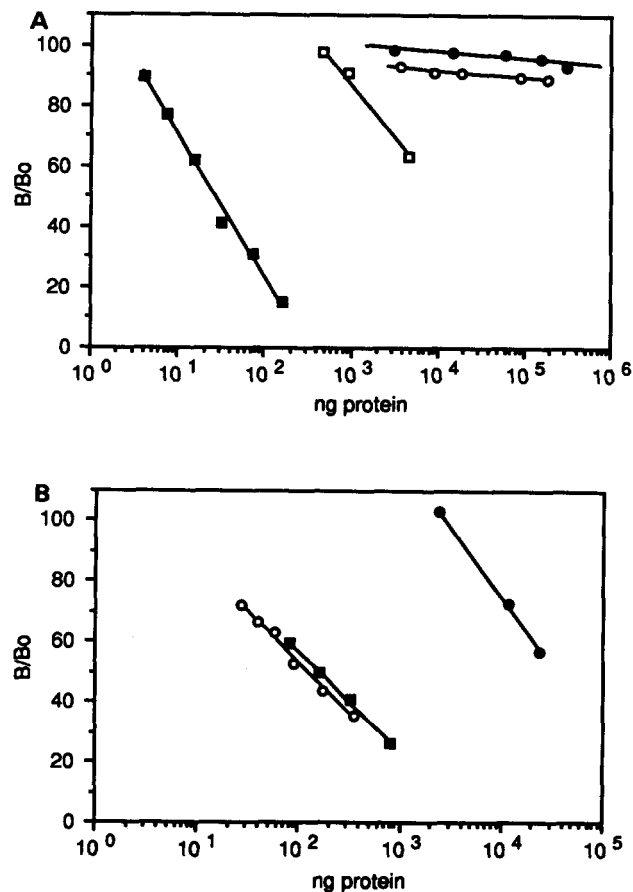
#### Quantification of Lp[a] and LDL in plasma

Since the amino acid sequence of one apo[a] isoform has been reported to be highly homologous to the kringle 4 of plasminogen (17), we determined whether plasminogen cross-reacted with apo[a] in our RIA. When we compared the abilities of apo[a] and plasminogen to displace

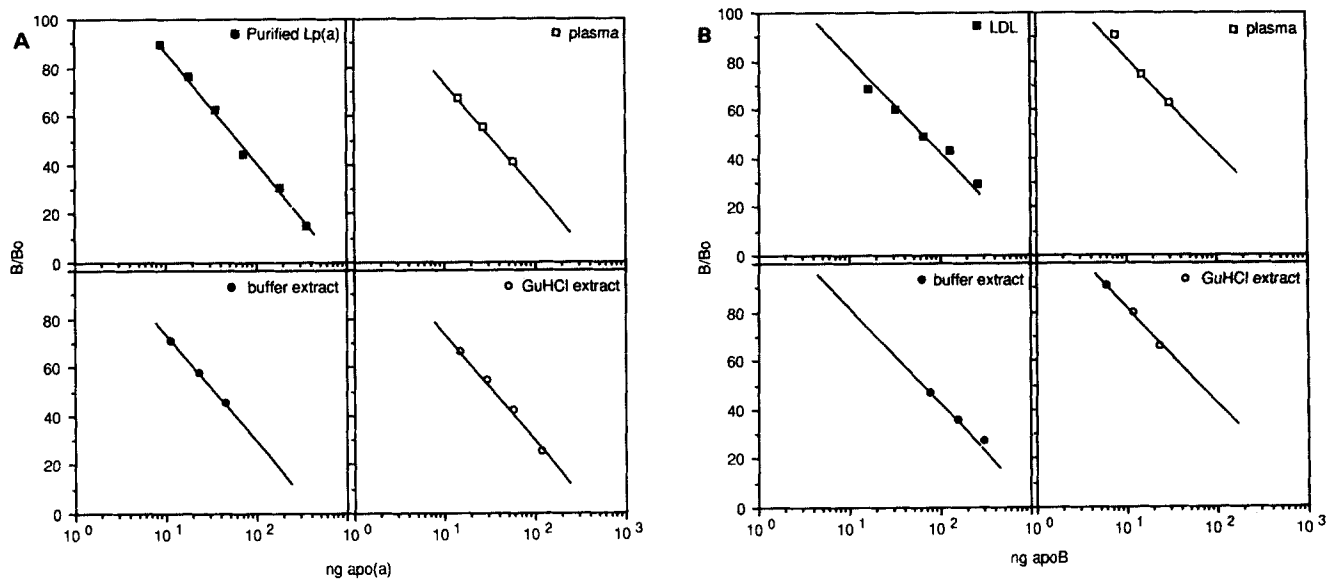
$^{125}\text{I}$ -labeled Lp[a] binding to anti-apo[a] at an antibody dilution of 1 to 115,000, the results suggested that the contribution of plasminogen to the measurement of apo[a] by our RIA was negligible (Fig. 1A). LDL and HDL also showed no reactivity with the anti-apo[a]. The RIA used to measure apoB was found to detect apoB in Lp[a] as well as in LDL (Fig. 1B). HDL showed no significant immunoreactivity with anti-apoB. Thus, both RIAs appear to be highly specific.

#### Immunological identity between tissue and plasma apoproteins

Parallel displacement curves are considered a characteristic of immunological identity (18). Parallelism was demonstrated by performing serial dilutions on representative samples of purified lipoprotein, patient plasma, and PBS and GuHCl tissue extracts using at least three different dilutions per sample to demonstrate



**Fig. 1.** Determination of cross-reactivity in radioimmunoassays (RIAs) measuring apo[a] (Fig. 1A) and apoB (Fig. 1B). A: Displacement curves for plasminogen (□), LDL (○), and HDL (●) compared to plasma Lp[a] (■) based on absolute amounts of protein added. B: Displacement curves for Lp[a] (■) and HDL (●) compared to plasma LDL (○) based on absolute amounts of protein added. At least three different dilutions were used per sample to demonstrate linearity.  $B/B_0$  was plotted against the protein content in each of the dilutions.



**Fig. 2.** Apo[a] and apoB parallelism in respective RIAs. A: Apo[a] displacement curves for Lp[a] (■), plasma (□), PBS tissue extracts (●), GuHCl (○) tissue extracts. B: ApoB displacement curves for LDL (■), plasma (□), PBS (●), and GuHCl (○) tissue extracts. An apolipoprotein value determined by RIA from one sample dilution was used to calculate the expected value for each subsequent dilution and plotted against  $B/B_0$  using at least three different dilutions per sample to demonstrate linearity.

linearity as described by Feldcamp and Smith (18). The data plotted on a graph should, ideally, result in a straight line that is parallel to that determined for the standard curve for each RIA. If the slopes are not parallel, disparate final concentrations would be observed at different dilutions. As seen in **Fig. 2A**, the displacement curves of apo[a] in purified Lp[a], apo[a] in plasma, and apo[a] in the PBS- and GuHCl-extracted fractions were parallel. When the same assessment was made for apoB in LDL, plasma, and in the PBS- and GuHCl-extracted fractions, again all four were parallel (**Fig. 2B**). These results suggest that plasma and tissue apo[a], and plasma and tissue apoB were immunologically identical.

#### Preliminary studies to develop procedures for quantifying of apo[a] and apoB in plaques

In initial studies, we extracted apo[a] with PBS for 24 h at 4°C to isolate a loosely bound fraction. An additional extraction of the residual pellet for 24 h with PBS yielded less than 5% of the apo[a] in the first extraction. The residual pellet was subsequently extracted with 1% Triton X-100 for 24 h at 4°C to obtain a tightly bound fraction, as we had used previously for extracting apoB from human lesions (13). To assess whether any additional apo[a] could be removed from the residual tissue, we first extracted tissue minces with PBS, divided the residual pellet minces into two equal aliquots, and then compared the amount of apo[a] that could be extracted using a 1% Triton X-100, with the amount that could be extracted with 6 M GuHCl. Triplicate observations were made from one sample per condition. The PBS-extracted fraction con-

tained  $2.9 \pm 0.4 \mu\text{g apo[a]}/\text{g tissue wet weight}$  (mean  $\pm$  SE). GuHCl extracted almost four times more apo[a] ( $12.6 \pm 2.2$ ) as Triton X-100 ( $3.4 \pm 0.3$ ) from the residual tissue.

#### Effect of extraction procedures on RIA

Since GuHCl in residual tissue extracts is added to the RIA at final concentrations of 12 mM to 48 mM, we tested the effects of these concentrations of GuHCl on the measurements of apo[a] and apoB in Lp[a] and LDL in respective RIAs. GuHCl, at these final concentrations, did not significantly interfere with the measurements of apo[a] and apoB in Lp[a] and LDL, respectively. We considered the possibility that some material extracted from plaques and present in the PBS extract or in the GuHCl extract might interfere with the measurement of apo[a] or apoB by RIA. However, we found that the measurement of immunoreactive apo[a] and apoB in Lp[a] and LDL, after the addition of aliquots of PBS- or GuHCl extracts, was additive. In another study, we quantitatively removed immunoreactive apo[a] or apoB from separate aliquots of representative samples of one PBS extract and three GuHCl extracts by affinity chromatography using anti-apo[a]-Sepharose or anti-apoB-Sepharose. The respective immunoabsorbed tissue extracts were then added to assay tubes containing either Lp[a] or LDL. We then measured the apo[a] or apoB concentration by respective RIAs in the presence and absence of tissue extracts. Again, no significant differences were found. Since extractions of residual tissue with GuHCl were performed for up to 6 days, we also determined whether the im-



munoreactivity of endogenous Lp[a] or of LDL would be affected by this long extraction period, i.e., by protease digestion or masking of immunoreactive sites on apo[a] or apoB by tissue components. Plasma Lp[a] and LDL were, therefore, incubated for periods of 0, 2, and 6 days with a GuHCl extract from which all endogenous apo[a] or apoB immunoreactivity had been removed as described above. However, no effect on the plasma Lp[a] or LDL measurements by RIA could be detected by incubating with corresponding extracts.

Since Rath et al. (19) had recently measured the accumulation of apo[a] in homogenates of human atherosclerotic plaques extracted with 1% Tween-20, we tested whether 6 M GuHCl would remove any additional apo[a] from tissue initially extracted with Tween. We mimicked Rath's procedure by first homogenizing minces of plaque in the presence of 1% Tween-20 using both a Polytron (Brinkman Instruments, Westbury, NY) or a glass tissue homogenizer, pelleting the insoluble residual tissue by centrifugation, and then extracting the residual tissue with 6 M GuHCl. In two studies using plaques from different cases, we found that GuHCl extracted an additional amount of apo[a] from the residual tissue after Tween extraction amounting to 30% and 200%, respectively, of the amount of apo[a] extracted by Tween alone. Furthermore, comparisons of total apo[a] extracted with and without homogenization suggested that homogenization destroyed apo[a] immunoreactivity. We concluded from these preliminary studies that sequential extraction of nonhomogenized plaque minces with PBS and with GuHCl was the procedure of choice.

#### Other methods

Protein content of lipoprotein samples was determined by the bicinchoninic acid (BCA) macro-protein assay (Pierce, Rockford, IL) according to the method described by Smith et al. (20) except that a 60-min, 60°C heating step was used to enhance the sensitivity of the assay. Bovine plasma albumin was used as the standard. This technique is simpler to perform and much more sensitive than the Lowry procedure (21). However, we found that values of LDL protein measured by the method of Lowry et al. (21) gave values consistent with independent molecular weight estimates, while the BCA method gave values 1.3 times higher. We therefore, chose to convert our BCA measurements to Lowry measurement using a factor of 0.77. Lipoproteins were iodinated for RIA tracer using a modification of Bilheimer, Eisenberg, and Levy's (22) alteration of the iodine monochloride procedure of McFarlane (23), in order to produce tracer with a high specific activity. One-dimensional gel electrophoresis using 1% agarose, which was used to verify the column chromatography purification of Lp[a], was performed according to the manufacturer's instructions (Corning, Palo Alto, CA) except that the electrophoresis time was in-

creased to 75 min to allow for greater separation between LDL and Lp[a]. To eliminate differences in migration due to protein concentration, 60  $\mu$ g BSA was added to each well prior to application of the sample. GuHCl extracts of lesions were first dialyzed against 4 M urea prior to electrophoresis. The samples were visualized by lipid-staining with Fat Red 7B in methanol. Parallel agarose gels were subjected to immunoblotting by capillary transfer to nitrocellulose and immunostained for apo[a] or apoB using primary antibodies at dilutions of 1 to 10,000 and/or secondary antibody (rabbit anti-goat IgG) coupled to colloidal gold (1 to 350 dilution) followed by silver enhancement (Janssen Biologicals NV, distributed by Amersham International, U.K.).

Each RIA analysis was performed in triplicate. Comparisons of the differences between the means of two equal sized groups were performed using nonpaired Student's *t*-test and statistical significance was designated for values of  $P < 0.05$ . Linear regression analysis was performed on correlation data in order to determine correlation coefficients ( $r$ ), and statistical significance of regression lines was determined using the method of least squares.

## RESULTS

### Quantification of apo[a] and apoB in human plasma and corresponding plaques

The distribution of plasma apo[a] and apoB levels in 26 individuals (Fig. 3A and 3B) showed a wide spectrum ranging from 0.3 to 17.3 mg apo[a] protein/dl (corresponding to 1.8 and 114.0 mg Lp[a] mass/dl, respectively); and from 32.1 to 181.5 mg apoB protein/dl. Mean levels of plasma apo[a] and apoB were 4.3 mg/dl (29 mg/dl Lp[a] mass) and 95.2 mg/dl (Table 1). Using this assay we had previously found that Lp[a] levels in a group of 32 cases with no angiographically determined stenosis of coronary arteries had a mean Lp[a] level of 17.3 mg/dl (8), a value that was consistent with levels found by others for a normal population (5). Plasma Lp[a] mass levels of 30 mg/dl (4.5 mg apo[a]/dl) or higher were shown to be associated with significantly greater risk for developing coronary atherosclerosis (5) or vein graft stenosis (8). This value was close to the mean levels of Lp[a] in this study.

When the accumulations of plaque apo[a] and apoB, expressed as loosely bound, tightly bound, and total apolipoprotein (Table 1), were measured in the lesions of these 26 individuals whose plasma levels were just described, only 18% of the total plaque apo[a] was in a loosely bound form, i.e., extractable with PBS, whereas 68% of the total plaque apoB was in this form ( $P < 0.001$ ). Since some apoB might be associated with apo[a] as intact particles in plaques, we also calculated the amount not associated with apo[a] in Lp[a]. This was measured by subtracting

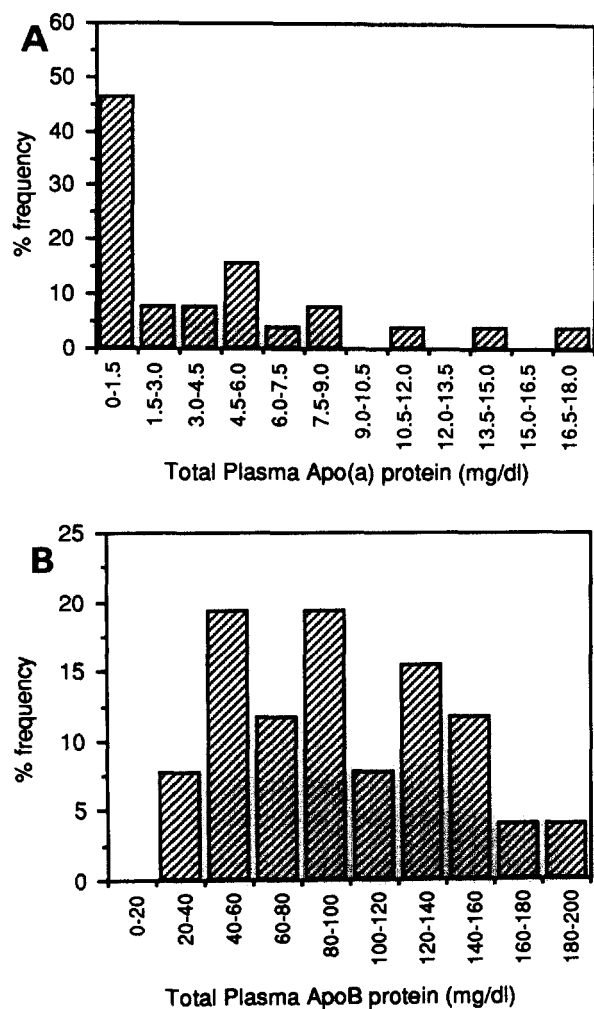


Fig. 3. Distribution of total plasma apo(a) (A) and apoB (B) levels in the 26 patients undergoing vascular surgery.

the amount of apoB in Lp[a] from total apoB, assuming that this amount of apoB is equal to the total tissue apo[a]. Making these calculations we found again that only 28% of plaque apoB that was not associated with Lp[a] was in a tightly bound form.

We wished to determine whether plaque apo[a] content correlated with plasma apo[a] levels. As seen in Fig. 4A, using linear regression analysis, not only did total plaque apo[a] correlate with plasma apo[a] levels ( $r = 0.82$ ,  $P < 0.001$ ), but so did the loosely bound fraction of apo[a] ( $r = 0.78$ ,  $P < 0.001$ ) and tightly bound apo[a] ( $r = 0.82$ ,  $P < 0.001$ ). These results suggest that there is a strong association between tissue and plasma apo[a] in human plaques. When similar correlations (Fig. 4B) were performed between plasma apoB and total plaque apoB, loosely and tightly bound apoB fractions,  $r$  values of 0.29 ( $P = 0.12$ ), 0.19 ( $P = 0.34$ ), and 0.31 ( $P = 0.10$ ), respectively, were obtained. Thus, no strong association was found between tissue and the plasma apoB. When total plaque apo[a] was correlated with total plaque apoB, no correlation was found ( $r = 0.18$ ,  $P = 0.386$ ).

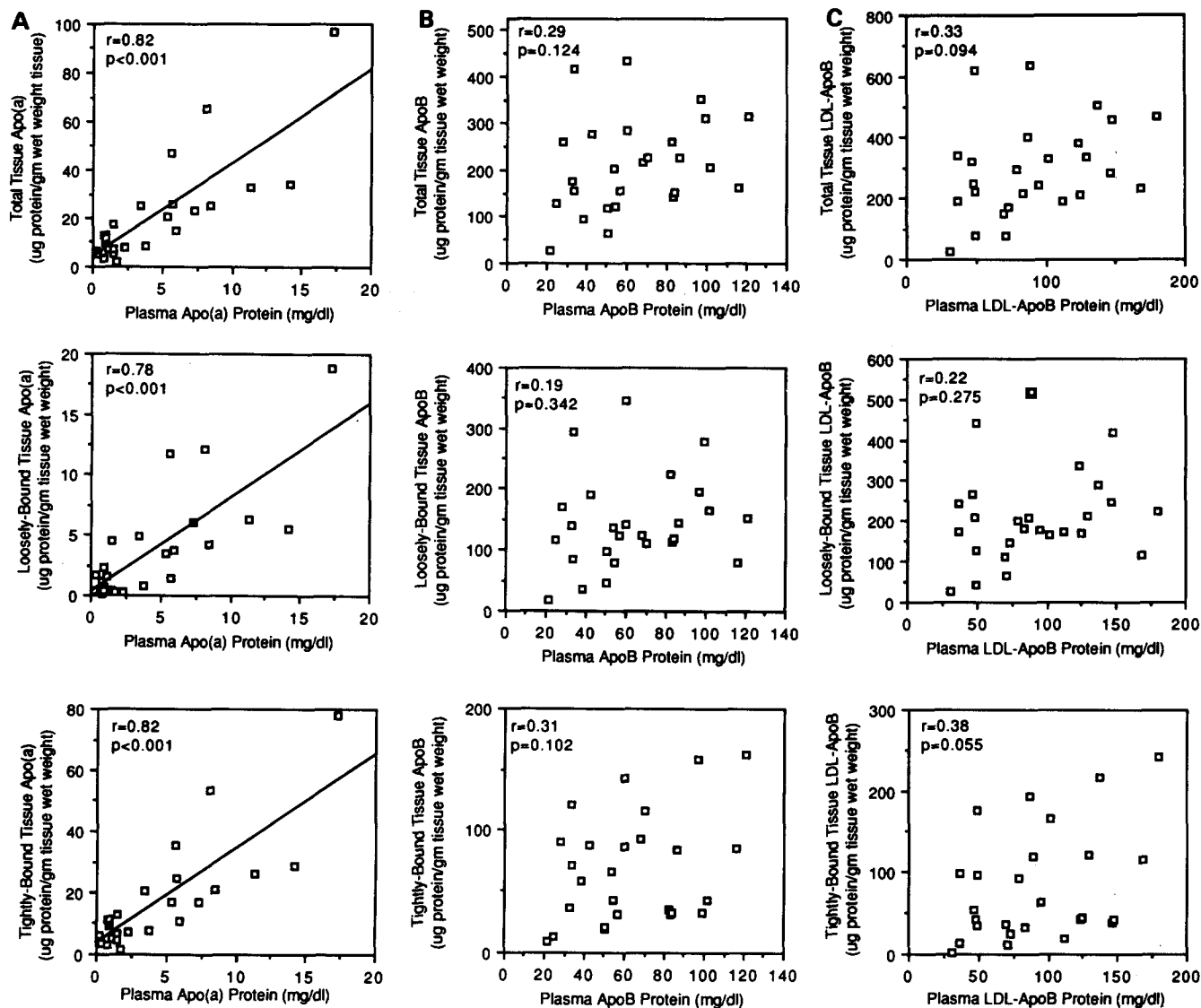
On an absolute scale, the total amount of apoB that accumulated in plaques was greater than the total amount of apo[a] (Table 1). However, when these values were normalized to equal plasma concentrations by dividing each plaque apolipoprotein value by its corresponding plasma concentration, we found that in 20 out of 26 cases, total normalized apo[a] accumulation was actually higher than total normalized apoB accumulation. The ratio of the amounts of plaque apo[a] to plaque apoB, when normalized by their respective plasma concentrations, should be greater than one if the former exceeds the latter. Twenty out of 26 cases studied had ratios greater than one (Fig. 5), with a mean ratio of  $2.35 \pm 2.05$ . Similar ratios were found for normalized values of plaque apo[a] to the calculated value of apoB not associated with Lp[a], with a mean ratio of  $2.63 \pm 2.74$ .

To assess whether in some individuals tightly bound Lp[a] expressed as particle mass might have actually accumulated to a greater degree than tightly bound LDL mass, we converted our measurements of apo[a] and apoB into Lp[a] mass and LDL mass making the calculations previously described in Methods. When the calculated amounts of Lp[a] mass and LDL mass that were tightly bound were compared in our 26 cases studied, 5 cases had

TABLE 1. Apo[a] and apoB in plasma and plaques

	Plasma Apolipoprotein Concentration	Plaque Apolipoprotein Content				
		Loosely Bound	(%)	Tightly Bound	(%)	Total
	mg/dl	$\mu\text{g/g tissue wet wt}$		$\mu\text{g/g tissue wet wt}$		$\mu\text{g/g tissue wet wt}$
Apo[a]	$4.3 \pm 4.5$	$3.6 \pm 4.5$	(17.5)	$17.0 \pm 17.0$	(82.5)	$20.6 \pm 21.3$
ApoB	$95.2 \pm 43.1$	$215.0 \pm 116.0$	(68.3)	$99.6 \pm 66.9$	(31.7)	$314.6 \pm 152.3$

Results are expressed as mean  $\pm$  SD of the values in the lesions from the 26 cases studied. Plaques were extracted sequentially with PBS and with GuHCl as described in Methods. Total plasma, plaque apo[a], and apoB were measured by individual RIAs as described in Methods; %, the percent of total extracted plaque-derived apo[a] or apoB found in the loosely bound and tightly bound fractions.



**Fig. 4.** A: Correlation of plasma apo[a] with total tissue apo[a], loosely bound tissue apo[a], and tightly bound tissue apo[a] in the 26 cases studied. B: Correlation of plasma apoB with total tissue apoB, loosely bound tissue apoB, and tightly bound tissue apoB in the 26 cases studied. C: Correlation of plasma and tissue LDL-apoB protein (total apoB minus apoB assumed to be present in Lp[a] and equal to total apo[a]).

a higher calculated Lp[a] mass than a calculated LDL mass in this fraction. When the case that had extraordinarily low plasma and tissue apoB levels (sample at far right of Fig. 5) was omitted, we found a significant positive correlation ( $r = 0.72$ ,  $P = 0.001$ ) for the ratio of Lp[a] mass to LDL mass with plasma Lp[a] concentrations (Fig. 6). This result suggested that the absolute amount of Lp[a] could have actually exceeded LDL in some patients with high plasma Lp[a] levels, e.g., above 5 mg apo[a] protein/dl, if all apo[a] and apoB had been present as intact Lp[a] and LDL particles.

To assess whether some of the plaque-derived apo[a] and apoB could be present within intact Lp[a] particles, we subjected the PBS extract, the GuHCl extract, and the

corresponding serum sample from one case to one-dimensional agarose electrophoresis, and then stained a gel for lipid and immunostained parallel gels for apo[a] and apoB. Fig. 7 demonstrates a montage of such gels, illustrating that in both the PBS extract and the GuHCl extract the major band co-stained for lipid, apo[a], and apoB, suggesting the presence of some intact Lp[a]. Also noteworthy are the observations that the bands staining positively for apo[a] and apoB in the PBS extract migrate further than the corresponding band in plasma, and that these bands in the GuHCl extract migrate even further than those in the PBS extract. Still unexplained is the strong immunostaining at the origin for apo[a] and for apoB, but not for lipid from plasma and PBS extracts.



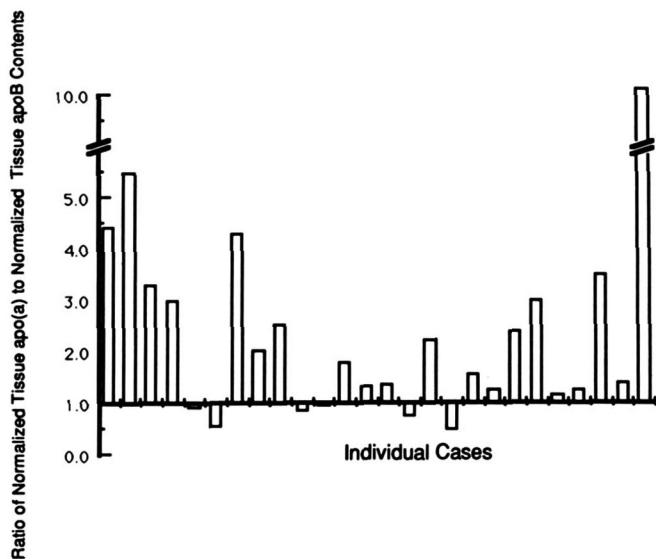


Fig. 5. Relative ratios of plaque apo[a] to plaque apoB, both normalized for corresponding plasma concentrations in the 26 cases studied. A ratio  $> 1$  indicates that, relative to their corresponding plasma concentrations, more apo[a] has accumulated in the artery wall than apoB.

## DISCUSSION

In this study we have documented the accumulation of apo[a] and apoB in human atherosclerotic lesions using a procedure that sequentially extracts nonhomogenized minces of tissues with PBS and with 6 M GuHCl. The two fractions obtained were operationally defined as loosely and tightly bound forms of apo[a] and apoB, respectively. Plaque and plasma apo[a] and apoB showed parallel displacement curves in respective RIAs, suggesting identical immunoreactivities. Neither GuHCl nor plaque-derived components that co-extracted with these

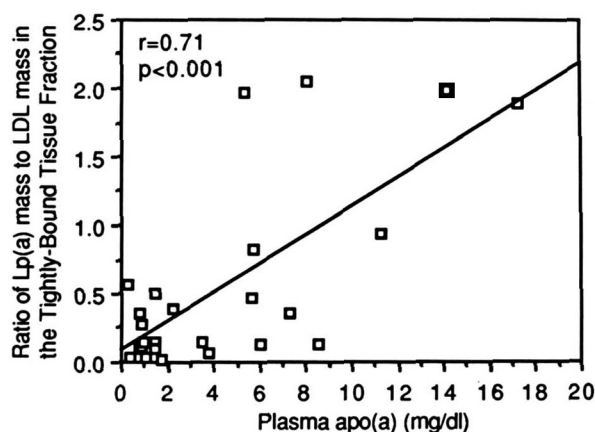


Fig. 6. Correlation of the ratio of the calculated values of tightly bound Lp[a] mass to the calculated values of tightly bound tissue LDL mass with plasma Lp[a] levels in 25 of the 26 cases studied. Apo[a] levels and apoB levels were converted to Lp[a] mass and LDL as described in the text.

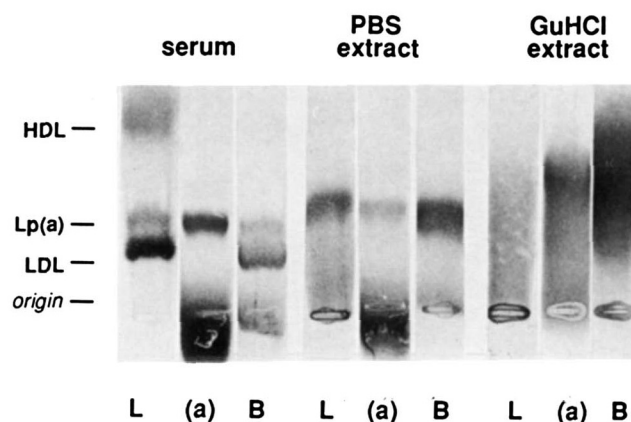


Fig. 7. One-dimensional electrophoresis patterns of a plaque PBS extract, a plaque GuHCl extract, and its corresponding serum, all from one individual. Minces of plaques were sequentially extracted with PBS and GuHCl as described in Methods. Samples of the serum, PBS extract, and GuHCl extract were applied to a 1% agarose gel at equivalent concentrations of apo[a] and apoB as measured by RIAs (0.5 to 1.0  $\mu\text{g}/\text{well}$ ). Prior to application, the sample of the GuHCl extract was dialyzed against 4 M urea. Samples were electrophoresed for 75 min at 90 volts. Serum and plasma gave the same results.

apolipoproteins perturbed their measurement in respective assays. Finally, at the dilutions of antibody used in the apo[a] RIA, neither plasminogen nor other lipoproteins demonstrated significant cross-reactivity.

Our results confirm and extend on those recently reported by Cushing et al. (24) and Rath et al. (19), although major procedural differences exist among these studies. Using the technique described by Cushing et al. (24), e.g., electrophoresis of apo[a] directly from tissue minces, we had previously found that only about one-half of total apoB in plaques from hypercholesterolemic monkeys (25) or swine (26, 27) could be quantified by this procedure. Subsequent treatment with detergents (13) or hydrolytic enzymes (28) was needed to remove the remaining apoB. Based on data from the present study, it is likely that apo[a] would be at least as refractory to extraction by electrophoresis as apoB. We found that a significant amount of apo[a] could be removed with GuHCl from tissue that had been originally extracted by the procedure described by Rath et al. (19). These results suggest that the published data might be underestimates of tissue apo[a] content. However, direct comparisons of the absolute numbers in these studies, with appropriate conversions, indicate that the actual apo[a] values reported were higher than reported by us. Although the reasons for this apparent discrepancy are not clear, they may reflect some aspect of the immunochemical measurements, i.e., Rath et al. (19) used monoclonal rather than polyclonal antibodies for apolipoprotein detection, and neither of the published studies reported on the immunochemical identity between tissue and plasma apo[a].



The concentration of apoB estimated from lesions in this study was consistent with those values published by us earlier from tissue homogenates (29, 30). However, these values were about five times greater than the values reported by Cushing et al. (24) and Rath et al. (19). It is possible that this discrepancy could be due to differences in the degree of lesion pathology in the two studies, since Rath et al. (23) used aortic specimens obtained at bypass surgery that were probably not as involved as the fibrous plaques and complicated lesions used in this study. This discrepancy could also have resulted from the lack of immunological identity between tissue and plasma apoB in the recently published studies (19, 24), since no assessment of such identity was given.

Notwithstanding the differences in absolute values of apo[a] and apoB found in this study and the previously published two studies, all three studies found a significant positive correlation between plasma and plaque apo[a] contents, but not between plasma and plaque apoB. It is noteworthy that such high correlations for plaque and plasma apo[a] levels were obtained using plasma samples obtained directly at surgery, even though numerous factors could have resulted in perturbation of these plasma values. The lack of correlation for plasma and plaque apoB might have been due to the greater degradation of LDL than Lp[a] by vascular cells (31) or to a greater influence of plasma LDL than Lp[a] on dietary fluctuations (32), precluding accurate measurement in cross-sectional studies. In both this study and the report of Cushing et al. (24), apo[a] accumulation exceeded apoB accumulation in plaques when the values were normalized for corresponding plasma concentrations.

The current study extends the previous studies by demonstrating that tissue-derived apo[a] and apoB can be operationally divided into loosely bound and tightly bound fractions, and that most tissue-derived apo[a] is found in the latter, while most tissue-derived apoB is found in the former (Table 1). Furthermore, in a few cases with high plasma Lp[a] levels, the absolute mass of Lp[a] accumulating in the tightly bound fraction would have been greater than the accumulation of LDL in that fraction if one had assumed that apo[a] and apoB were entirely present as intact Lp[a] and LDL. Although we have no evidence that all apo[a] and apoB are in the form of intact Lp[a] or LDL, we have demonstrated by one-dimensional agarose electrophoresis that some apo[a] is associated with apoB and with lipid in both loosely and tightly bound fractions of apo[a], suggesting the presence of intact Lp[a]. In addition to this electrophoresis data, we have obtained several lines of preliminary data suggesting the presence of intact Lp[a] in both PBS extracts and GuHCl extracts. Particles in the size range of LDL and Lp[a] could be seen in these extracts by electron microscopy. Immunoreactive apo[a] and apoB were present in a fraction that co-eluted with Lp[a] when fractions

of PBS and GuHCl extracts were subjected to gel filtration chromatography. Finally, when plaque extracts were subjected to affinity chromatography on Sepharose anti-apoB, the bound and eluted fraction gave a band on one-dimensional agarose electrophoresis in which immunoblots of apoB and apo[a] co-migrated. Furthermore, particles in the size range of LDL and Lp[a] were seen in such purified samples. The data from Rath et al. (19) also suggested that much of the apo[a] appears to be associated with apoB, possibly within an intact Lp[a] particle.

One explanation for the tight binding of apo[a] is that Lp[a] interacts more avidly with some tissue component(s) than LDL does to form insoluble complexes that require dissociative agents for solubilization. Whether this component is arterial proteoglycan, as was shown *in vitro* to interact more avidly with Lp[a] than with LDL to form insoluble complexes (33, 34), or other components still needs to be determined. Such tight binding of apo[a] could also be the result of aggregation of Lp[a], since Lp[a] was shown to be more sensitive than LDL to aggregation following interaction with  $Ca^{2+}$  ions (35). It is possible that the large accumulation of Lp[a] in the tightly bound form is, in some way, responsible for the independent association of the lipoproteins with accelerated atherosclerosis. Perhaps this fraction represents aggregated Lp[a] which is then internalized by tissue macrophages to induce lipid-loading, e.g., foam cell formation, a characteristic of the early fatty streak lesions (36).

Although we have provided evidence that much of the plaque apo[a] is probably still present in an intact Lp[a] and have highlighted the impact of such an accumulation with regards to subsequent lipid loading of vascular cells and the resultant foam cell formation, it needs to be stressed that other lines of evidence also suggest the presence of apo[a] in forms other than intact Lp[a]. The data of Rath et al., (19) suggest that some apo[a] may be dissociated from apoB. Our unpublished gel filtration data of GuHCl extracts in the presence of this dissociative agent demonstrate the presence of a smaller peak eluting after Lp[a]. Given that apo[a] from Lp[a] has recently been shown to inhibit the fibrinolytic system in numerous ways, i.e., competing for plasminogen-binding sites on fibrin (37, 38) or plasminogen receptors on endothelial cells and monocyte-macrophages (39), free apo[a] may play a role in the vessel wall during the atherosclerotic-thrombotic process that has hereto not been considered. Further studies on the structural and functional properties of free apo[a] and/or apo[a] associated with intact Lp[a] isolated from human atherosclerotic lesions are still needed. ■■

The authors thank Elizabeth Gordon, Kathy Osborne, and Gary Smejkal for technical assistance, James Lang for photographic services, Dr. Leonard Krajewski for assisting in the acquisition of tissue and plasma samples at surgery, Dr. Michael Cressman

for assisting in the acquisition of plasma samples for the isolation of Lp[a], and Drs. Guy Chisolm, Paul DiCorleto, Allen Ehrhart, and Richard Morton for helpful suggestions in the preparation of the manuscript. This work was supported by a fellowship from the American Heart Association, Northeast Ohio Affiliate and NIH Grants HL-43339 and HL-29582.

Manuscript received 23 August 1990 and in revised form 31 October 1990.

## REFERENCES

1. Utermann, G. 1989. The mysteries of lipoprotein [a]. *Science*. **246**: 904-910.
2. Fless, G. M., M. E. ZumMallen, and A. M. Scanu. 1986. Physicochemical properties of apolipoprotein[a] and lipoprotein[a-] derived from the dissociation of human plasma lipoprotein[a]. *J. Biol. Chem.* **261**: 8712-8718.
3. Gaubatz, J. W., C. Heideman, A. M. Gotto, J. D. Morrisett, and G. H. Dahlen. 1983. Human plasma lipoprotein[a]. Structural properties. *J. Biol. Chem.* **258**: 4582-4589.
4. Armstrong, V. W., P. Cremer, E. Eberle, A. Manke, F. Schulze, H. Wieland, H. Kreuzer, and D. Seidel. 1986. The association between serum Lp[a] concentrations and angiographically assessed coronary atherosclerosis. Dependence on serum LDL levels. *Atherosclerosis*. **62**: 249-257.
5. Dahlen, G. H., J. R. Guyton, M. Attar, J. A. Farmer, J. A. Kautz, and A. M. Gotto. 1986. Association of levels of lipoprotein Lp[a], plasma lipids, and other lipoproteins with coronary artery disease documented by angiography. *Circulation*. **74**: 758-765.
6. Koltringer, P., and G. Jürgens. 1985. A dominant role of lipoprotein[a] in the investigation and evaluation of parameters indicating the development of cervical atherosclerosis. *Atherosclerosis*. **58**: 187-198.
7. Murai, A., T. Miyahar, N. Fujimoto, M. Matsuda, and M. Kameyama. 1986. Lp[a] lipoprotein as a risk factor for coronary heart disease and cerebral infarction. *Atherosclerosis*. **59**: 199-204.
8. Hoff, H. F., G. J. Beck, C. I. Skibinski, G. Jürgens, J. A. O'Neil, J. Kramer, and B. Lytle. 1988. Serum Lp[a] levels as a predictor of vein graft stenosis after coronary artery bypass surgery in patients. *Circulation*. **77**: 1238-1244.
9. Fless, G. M., C. A. Roli, and A. M. Scanu. 1984. Heterogeneity of human plasma lipoprotein[a]. Isolation and characterization of the lipoprotein subspecies and their apoproteins. *J. Biol. Chem.* **254**: 11470-11478.
10. Enhalm, C., H. Garoff, O. Renkonen, and K. Simons. 1972. Protein and carbohydrate composition of Lp[a] lipoprotein from human plasma. *Biochemistry*. **11**: 3229-3232.
11. Utermann, G., H. J. Menzel, H. G. Kraft, H. C. Duba, H. G. Kemmler, and C. Seitz. 1987. Lp[a] glycoprotein phenotypes. *J. Clin. Invest.* **80**: 458-465.
12. Gaubatz, J. W., M. V. Chari, M. L. Nava, J. R. Guyton, and J. D. Morrisett. 1987. Isolation and characterization of the two major apoproteins in human lipoprotein[a]. *J. Lipid Res.* **28**: 69-79.
13. Hoff, H. F., C. L. Heideman, J. W. Gaubatz, D. W. Scott, and A. M. Gotto. 1978a. Detergent extraction of tightly bound apoB from extracts of normal aortic intima and plaques. *Exp. Mol. Pathol.* **28**: 290-300.
14. Jürgens, G., E. Marth, G. M. Kostner, and A. Holasek. 1977. Investigation of the Lp[a] lipoprotein: lipoprotein aggregation and enzymatic activity associated with the Lp[a] polypeptide. *Artery*. **3**: 13-26.
15. Hoff, H. F., and B. A. Clevidence. 1987. Uptake by mouse peritoneal macrophages of large cholesteryl ester-rich particles isolated from human atherosclerotic lesions. *Exp. Mol. Pathol.* **46**: 331-344.
16. Hatch, F. T., and R. S. Lees. 1968. Practical method for plasma lipoprotein analysis. *Adv. Lipid Res.* **6**: 2-63.
17. McLean, J. W., S. E. Tomlinson, W. J. Kuang, D. L. Eaton, E. Y. Chen, G. M. Fless, A. M. Scanu, and R. M. Lawn. 1987. cDNA sequence of human apolipoprotein[a] is homologous to plasminogen. *Nature*. **330**: 132-137.
18. Feldkamp, C. S., and S. W. Smith. 1987. Practical guide to immunoassay method evaluation. In *Immunoassay - A Practical Guide*. D. W. Chan and M. T. Perlstein, editors. Academic Press/San Diego, CA. 73-75.
19. Rath, M., A. Niendorf, T. Reblin, M. Dietel, H. J. Krebber, and U. Beisiegel. 1989. Detection and quantification of lipoprotein[a] in the arterial wall of 107 coronary bypass patients. *Arteriosclerosis*. **9**: 579-592.
20. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**: 76-85.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
22. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta*. **260**: 212-221.
23. McFarlane, A. S. 1958. Efficient trace-labeling of proteins with iodine. *Nature*. **182**: 53-57.
24. Cushing, G. L., J. W. Gaubatz, M. L. Nava, B. J. Burdick, T. M. A. Bocan, J. R. Guyton, D. Weilbaecher, M. E. DeBakey, G. M. Lawrie, and J. D. Morrisett. 1989. Quantitation and localization of apolipoproteins [a] and B in coronary artery bypass vein grafts resected at re-operation. *Arteriosclerosis*. **9**: 593-603.
25. Hoff, H. F., Y. Yamauchi, and M. G. Bond. 1985. Reduction in tissue LDL accumulation during coronary artery regression in cynomolgus macaques. *Atherosclerosis*. **56**: 51-60.
26. Yamauchi, Y., and H. F. Hoff. 1984. Apolipoprotein B accumulation and development of foam cell lesions in coronary arteries of hypercholesterolemic swine. *Lab. Invest.* **51**: 325-332.
27. Hoff, H. F., R. G. Gerrity, H. K. Naito, and D. M. Dusek. 1983. Quantitation of apolipoprotein B in aortas of hypercholesterolemic swine. *Lab. Invest.* **48**: 492-504.
28. Hoff, H. F., and J. W. Gaubatz. 1979. Residual apoB in aortic plaques extracted with hydrolytic enzymes. *Artery*. **6**: 89-107.
29. Hoff, H. F., C. L. Heideman, J. W. Gaubatz, A. M. Gotto, E. E. Erickson, and R. L. Jackson. 1977. Quantification of apoB in grossly normal human aorta. *Circ. Res.* **40**: 56-63.
30. Hoff, H. F., C. L. Heideman, J. W. Gaubatz, J. L. Titus, and A. M. Gotto. 1978. Quantitation of apoB in human aortic fatty streaks. *Atherosclerosis*. **30**: 263-272.
31. Armstrong, V. W., A. K. Walli, and D. Seidel. 1985. Isolation, characterization, and uptake in human fibroblasts of an apo[a]-free lipoprotein obtained on reduction of lipoprotein[a]. *J. Lipid Res.* **26**: 1314-1323.
32. Kostner, G. M., D. Gavish, B. Leopold, K. Bolzano, M. S. Weintraub, J. L. Breslow. 1988. HMG-CoA reductase inhibitors lower LDL cholesterol without reducing Lp[a] levels. *Circulation*. **80**: 1313-1319.

33. Bihari-Varga, M., E. Gruber, M. Rotheneder, R. Zechner, and G. M. Kostner. 1988. Interaction of lipoprotein[a] (Lp[a]) and low density lipoprotein with glycosaminoglycans from human aorta. *Arteriosclerosis*. **8**: 851-857.
34. Dahlen, G. H., C. Ericson, and K. Berg. 1978. In vitro studies of the interaction of isolated Lp[a] lipoprotein and other serum lipoproteins with glycosaminoglycans. *Clin. Genet.* **14**: 36-42.
35. 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.
36. McGill, H. C., Jr. 1968. Fatty streaks in the coronary arteries and aorta. *Lab. Invest.* **18**: 560-564.
37. Loscalzo, J., M. Weinfeld, G. M. Fless, and A. M. Scanu. 1990. Lipoprotein[a], fibrin binding and plasminogen activation. *Arteriosclerosis*. **10**: 240-245.
38. Harpel, P. C., B. R. Gordon, and T. S. Parker. 1989. Plasmin catalyzes binding of lipoprotein[a] to immobilized fibrinogen and fibrin. *Proc. Natl. Acad. Sci. USA.* **86**: 3847-3851.
39. Gonzalez-Gronow, J., J. M. Edelberg, and S. V. Pizzo. 1989. Further characterization of the cellular plasminogen binding site: evidence that plasminogen 2 and lipoprotein[a] compete for the same site. *Biochemistry.* **28**: 2374-2377.