Partial characterization of lipoproteins containing apo[a] in human atherosclerotic lesions

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Abstract Previously we quantified the amounts of immunoreactive apo[a] found in human atherosclerotic lesions extracted sequentially with phosphate-buffered saline (PBS) and guanidine hydrochloride (GuHCl). In this study we have attempted to characterize lipoproteins containing apo[a] in such PBS and GuHCl fractions, obtained from autopsy samples, in order to eventually determine their structure–function relationships critical for evaluating the mechanisms that make them atherogenic. Apo[a] in the PBS extracts migrated slightly ahead of plasma Lp[a] on agarose electrophoresis. Although apo[a] in extracts showed the same isoforms as in plasma in SDS-PAGE, it was also highly fragmented. When a $d < 1.10$ g/ml ultracentrifugation fraction of the PBS extract was subjected to gel filtration, a major part of the immunoreactive apo[a] in this fraction co-isolated with plasma Lp[a]. When the Lp[a]-sized fraction was further separated by density gradient ultracentrifugation, a subpopulation was isolated containing apo[a] in the $1.06 < d < 1.08$ g/ml density range that was free of lesion-derived low density lipoprotein (LDL) (A-LDL). This fraction contained immunoreactive apo[a] and apoB, had a total cholesterol to protein ratio of about 1, and demonstrated increases in fluorescence (360 ex/430 em) and conjugated dienes that were even greater than values obtained for the corresponding A-LDL sample. The void volume fraction following gel exclusion chromatography of the $d < 1.10$ g/ml fractions contained both apo[a] and apoB that comigrated on nondenaturing PAGE, suggesting that they were present on the same particle. Apo[a] in GuHCl extracts comigrated with plasma Lp[a] on agarose electrophoresis and contained apo[a] isoforms of similar molecular weights as those found in corresponding plasma samples. When the GuHCl extract was subjected directly to gel filtration in the presence of 6 M GuHCl, two included peaks of apo[a] immunoreactivity were present, one eluting slightly ahead of plasma Lp[a], the other slightly ahead of plasma LDL. Collectively, these data indicate that apo[a] is present in human atherosclerotic lesions in forms resembling intact but oxidized plasma Lp[a], as larger particles possibly representing Lp[a] complexed to itself or other plaque components, and as slightly smaller particles possibly representing degraded Lp[a].—**Hoff, H. F., J. O'Neil, and A. Yashiro.** Partial characterization of lipoproteins containing apo[a] in human atherosclerotic lesions. *J. Lipid Res.* 1993. **34:** 789–798.

Supplementary key words Lp[a] • apoB • arteries • plaques • affinity chromatography • immunoblotting

Lp[a] is a cholesterol-rich plasma lipoprotein that shares many structural and chemical properties with plasma LDL (1–3). It has attracted attention in recent years, as numerous clinical-chemical correlative studies have indicated that Lp[a] is an independent risk factor for cardiovascular diseases. Elevations of plasma Lp[a] were shown to be associated with increased atherosclerosis in the coronary arteries leading to myocardial infarction (4–7), in intra- and extracranial arteries leading to stroke (7), and in saphenous vein grafts after coronary artery bypass surgery (8). Although both LDL and Lp[a] share apoB-100, and have similar relative lipid compositions, Lp[a] also possesses a unique protein designated apo[a], whose structural and chemical characteristics have been extensively described (1–3, 9, 10).

In an effort to better understand the atherogenicity of Lp[a], we recently undertook a study to quantify the amounts of Lp[a] accumulating in human atherosclerotic lesions using immunoreactive apo[a] as a measure of Lp[a] accumulation (11). We also measured the accumulation of apo[a] and of apoB as indicators of the accumulation of Lp[a] and LDL in plaques, as had also been done by Cushing et al. (12) and Rath et al. (13). All these studies showed that plaque and plasma apo[a] contents correlated positively in contrast to plaque and plasma apoB. When normalized to equivalent plasma concentrations of Lp[a] and LDL, both we (11) and Cushing et al. (12) showed that apo[a] accumulated in plaques to a greater degree than apoB. This roughly translated into a greater accumulation of Lp[a] than LDL for equivalent plasma concentrations. We also extracted lesions sequen-

Abbreviations: Lp[a],lipoprotein[a]; LDL, low density lipoprotein; apo[a], apolipoprotein[a]; apoB, apolipoprotein B; PBS, phosphate-buffered saline; BSA, bovine serum albumin; REM, relative electrophoretic mobility; PVDF, polyvinylidene difluoride; BCA, bicinchoninic acid; TEM, transmission electron microscopy; RIA, radioimmunoassay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; GuHCl, guanidine hydrochloride.

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tially with phosphate-buffered saline (PBS) and 6 M GuHCl. We found that, although on an absolute scale apoB greatly exceeded apo[a] in both the PBS extract as well as in the GuHCl extract, when assessing the relative distributions in these two fractions, major differences were found. Although 68% of the total apoB in lesions was extractable with PBS, only 18% of the total apo[a] in lesions was extractable with PBS, the remainder being extractable with GuHCl. This result suggested that Lp[a] was more tightly bound in lesions than LDL.

Because characterization of the structure-function properties of Lp[a] will be necessary to eventually elucidate the mechanisms responsible for the atherogenicity of Lp[a], we initiated a study to chemically and structurally characterize lipoproteins containing immunoreactive apo[a] in human atherosclerotic lesions. We now report initial results on the partial characterization of such particles. Our results indicate that modified forms of intact Lp[a]-like particles are present in lesions, some chemically altered such as by oxidation, some possibly degraded, and some possibly aggregated.

MATERIALS AND METHODS

Carrier-free $Na^{125}I$ was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA). Guanidine HCl, aprotinin, leupeptin, pepstatin, vitamin E, BHT, EDTA, NaBr, and NaN_3 were purchased from Sigma Chemical Co. (St. Louis, MO). Plasminogen and bovine serum albumin (fatty acid-free) were purchased from Boehringer Mannheim (Indianapolis, IN). CNBr-activated Sepharose used for affinity chromatography, Sephacryl 400 HR used for gel filtration, and premade 4–15% gradient polyacrylamide Phast Gel for SDS PAGE were obtained from Pharmacia LKB Biotech Inc. (Piscataway, NJ). SDS gradient polyacrylamide gels (3–8%) were prepared using diacrylylpiperazine (Integrated Separation Systems, Hyde Park, MA) as crosslinker. Pre-made 1% agarose gels and Fat Red 7B were purchased from Corning (Palo Alto, CA). Nitrocellulose membranes were obtained from Schleicher and Schuell, (Keene, NH), Immobilon-P from Millipore Corp. (Bedford, MA), and Spectrapor 2 and 3 dialysis tubing from Spectrum Medical Industries Inc. (Los Angeles, CA). Goat anti-human LDL and goat anti-human Lp[a], normal goat and rabbit sera, and rabbit anti-goat IgG were purchased from Bethyl Laboratories (West Montgomery, TX). Gold-conjugated rabbit anti-goat IgG, as well as the silver enhancement kit, were purchased from Amersham (Arlington Heights, IL). Silver stain for PAGE was obtained from Daiichi (Tokyo, Japan). Bicinchoninic acid (BCA) protein kit was from Pierce (Rockford, IL), while the microprotein kit Quantagold was from Diversified Biotech (Newton Centre, MA).

Isolation of plasma LDL and Lp[a]

LDL was isolated from fresh plasma, obtained from the Cleveland Clinic Blood Bank, by sequential ultracentrifugation as a $1.019 < d < 1.063$ g/ml fraction using the procedure of Hatch and Lees (14) and stored in 0.15 M NaCl containing 0.5 mM EDTA, pH 8.5. Lp[a] was isolated by plasmaphoresis from fresh plasma of healthy donors with Lp[a] levels of > 30 mg/dl. Aprotinin (100 K.I.U./ml), leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), EDTA (1 mg/ml), and NaN_3 (0.01%) were added immediately after plasmaphoresis. Lp[a] was then isolated by sequential ultracentrifugation as a $1.050 < d < 1.120$ g/ml fraction, concentrated by dialyzing against 30% PEG (MW 20,000) using a Spectropor 3 membrane (3500 MW) in 10 mM Tris, pH 7.2, containing 1 mg/ml EDTA. It was further separated by gel filtration chromatography on Sephacryl 400 HR (2.5 × 95 cm) in 10 mM Tris, pH 7.2, containing 1 mg/ml EDTA and 0.01% NaN_3. Only those fractions completely free of LDL and HDL were pooled. Purity of Lp[a] was evaluated by using the Phast-Gel Automated System (Pharmacia-LKB Biotech) and premade SDS polyacrylamide 4–15% gradient under nonreducing conditions followed by silver staining for visualization of protein. Lp[a] was stored in 10 mM Tris, pH 7.2, containing 1 mg/ml EDTA.

Isolation of Lp[a] from aortic plaques

Lp[a] was extracted from aortic plaques as described earlier (11) except that human aortas with advanced atherosclerotic plaques and their corresponding plasma were obtained at autopsy within 12 h of death and only from lesions derived from individuals with plasma levels > 20 mg/dl Lp[a]. The tunica intima was stripped from the underlying media at a natural cleavage plane. The data shown in this report were obtained from six representative cases, although an additional eight autopsy cases and four surgery cases were used to compare the relative electrophoretic mobility (REM) on agarose electrophoresis of LDL-like and Lp[a]-like fractions in plaque extracts and corresponding plasma. A loosely bound lipoprotein fraction was isolated from minces of the tunica intima by extraction at 4°C with PBS, (0.1 M phosphate, 0.3 mM EDTA, 0.15 M NaCl, pH 7.4), at a ratio of 1 g wet weight tissue to 5 ml PBS for 18 h at 4°C (11). Additives were included as described for Lp[a] isolated from plasma except that 20 μM vitamin E and 50 μg/ml gentamicin were added instead of NaN_3. The resulting suspensions were centrifuged at 20,000 *g* for 1 h at 4°C to obtain a supernatant fraction. The remaining pellet fraction was extracted at 4°C for 6 days with 6 M GuHCl at a ratio of 1 g tissue wet weight to 3 ml GuHCl which included all the additives present in the PBS buffer. The supernatant fraction of the GuHCl extract after centrifugation at 20,000 *g* for 1 h at 4°C was then chromatographed on

Sephacryl 400 HR (1.5 × 95 cm) in the presence of 6 M GuHCl.

A portion of the supernatant fraction of PBS extracts was subjected to ultracentrifugation at 15°C for 18 h at 300,000 *g* at a density of d 1.10 g/ml. The floating fraction of lipoproteins was then subjected to gel filtration on a Sephacryl 400 HR column of dimensions 2.5 × 95 cm. An Lp[a]-sized fraction containing immunoreactive apo[a] was then further fractionated by density gradient ultracentrifugation according to Redgrave et al. (15) with minor modifications. Fractions in the $1.06 < d < 1.08$ g/ml density range were monitored for increases in conjugated dienes and fluorescence (360 ex/430 em) as described previously (16). In select experiments a GuHCl tissue extract was dialyzed against 20 mM phosphate, 0.15 mM NaCl, 0.3 mM EDTA, pH 7.4, for 12 h and then applied to anti-apoB-Sepharose at a ratio of 1 ml of extract to 0.5 ml of anti-apoB–Sepharose. The anti-apoB used for this chromatography was derived from goat anti-Lp[a] as described earlier (11). Elution was performed using saline EDTA adjusted to pH 11 with NH_4OH as previously described (17).

Protein content of lipoproteins was measured by the BCA assay as described by Smith et al. (18) except that a 60-min, 60°C heating step was used with BSA as a standard. A microprotein assay was also used to measure bound and eluted lipoprotein after affinity chromatography (Procedure Sheet, Diversified Biotech, Newton Centre, MA). Transmission electron microscopy (TEM) was performed on negatively stained lipoprotein samples as reported earlier (19). Measurements of conjugated dienes and fluorescence at 360 ex/430 em were performed as described previously (16, 20).

Polyclonal antibodies raised in goats against human plasma Lp[a] and plasma LDL were obtained as described earlier (11) and used for radioimmunoassays (RIAs) and immunoblotting for apo[a] and apoB. Because anti-Lp[a] contained both anti-apo[a] and anti-apoB, we quantitatively removed all anti-apoB by applying an IgG fraction of anti-Lp[a] on an LDL-Sepharose column as reported earlier (17). Iodination of LDL and Lp[a] for RIAs was performed using the iodine monochloride (21) method. A brief description of the RIAs used in this study for Lp[a] and LDL has been previously made by Pepin, O'Neil, and Hoff (11). Electrophoretic mobility of Lp[a] relative to LDL (REM) was determined on premade 1% agarose gels following the manufacturer's instructions except that electrophoresis was performed at 90 V for 75 min. BSA (1%) was added to ensure reproducible migration distances. Gels were stained for lipid using 0.025% Fat Red 7B in 60% methanol. Immunoblotting was performed after transferring lipoproteins to nitrocellulose by simple diffusion which required only 2–4 h. After blocking nitrocellulose membranes with nonfat dry milk, immunoblotting was performed using a goat anti-human apo[a] or apoB at a dilution of 1 to 5000. Rabbit anti-goat IgG conjugated to 5 nm particles of gold was used at 1 to 250 dilution. Gels were then treated with silver enhancer. Under these conditions of immunoblotting, no cross-reactivity occurred with plasma concentrations of plasminogen. When nonimmune serum was used in place of anti-apo[a] or anti-apoB for the detection of plasma Lp[a], no staining was seen.

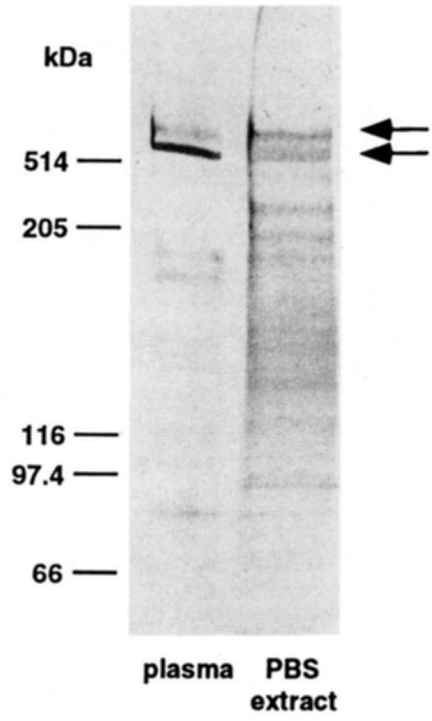

Fig. 1. SDS-PAGE plus immunoblotting for apo[a] of a PBS plaque extract and its corresponding plasma sample. Samples of a PBS extract and the corresponding plasma sample at about equal apo[a] contents were applied concurrently to SDS-PAGE using a 3–8% acrylamide gel under reducing conditions. The gels were subsequently immunoblotted for apo[a] as described in Materials and Methods. The arrows indicate the positions of the two isoforms of apo[a]. The numbers on the left side of the gel represent the positions of molecular weight standards run on a parallel gel.

The protein components of reduced, denatured, and delipidated lipoproteins were evaluated on SDS-PAGE using a 3–8% polyacrylamide gradient gel (22) and PIP-diacrylylpiperazine as crosslinker. Nondenaturing PAGE with a 2.5–16% polyacrylamide gradient gel was used to identify the size of lipoprotein particles and determine the presence of apo[a] and apoB on the same particles (Isolabs, Akron, OH) by immunoblotting for apo[a] and apoB. Proteins were immediately electrotransferred to PVDF as described (23), and immunoprobed as described above, or fixed overnight in the gel with 50% methanol followed by detection of proteins using the silver technique of Daiichi according to manufacturer's instructions.

RESULTS

Apo[a] in PBS extracts

We had previously shown that when atherosclerotic lesions were extracted with PBS and applied to agarose electrophoresis, a major band was seen staining for apo[a], apoB, and lipid and migrating slightly ahead of the corresponding band in plasma from the same individual (11). When the PBS extract and its corresponding plasma were subjected to SDS-PAGE under reducing conditions using a 3–8% gradient gel followed by immunoblotting for apo[a], two isoforms with essentially the same molecular weights were seen in both (**Fig. 1**). However, the lower molecular weight isoform in the PBS extracts was stained more weakly. In addition, numerous bands of different molecular weights immunostained for apo[a]. It appeared that the lower molecular weight apo[a] isoform was preferentially undergoing fragmentation. Similar patterns were observed from extracts of plaques obtained at surgery (not shown). Parallel apoB blots also showed fragmentation of apoB in the PBS extract (not shown). A low level of apo[a] fragmentation was also present in the corresponding plasma sample obtained at autopsy (Fig. 1). However, these fragments were also seen in antemortem plasma samples.

Characteristics of an Lp[a]-like fraction in PBS extracts

To isolate an Lp[a]-like fraction from PBS extracts, we first ultracentrifuged this fraction at d 1.10 g/ml and then chromatographed the floating fraction on a Sephacryl 400 HR column. Although apoB exceeded apo[a] in all fractions by over 20 to 1, the elution profile of apo[a] showed an included peak that co-eluted with plasma Lp[a] (**Fig. 2a**), suggesting the presence in PBS extracts of particles resembling Lp[a] in size. This peak was at the leading edge of the major peak containing apoB, previously defined as lesion-derived LDL or A-LDL (22). These observations which were made on three separate plaque samples gave similar findings. When the total apo[a] in the Lp[a]-sized fraction was compared to the total apoB in the LDL-sized fraction after excluding the fractions containing apo[a], we obtained an LDL-apoB to Lp[a]-apo[a] ratio of 26. If one assumes equal molecular weights, for apoB and apo[a], equal lipid compositions and Lp[a] contains one apoB and one apo[a] molecule, this result would suggest that there was about 26 times more LDL particles than Lp[a] particles in the PBS extract. Because this Lp[a]-sized fraction was not clearly separated from A-LDL by gel filtration, we subjected a

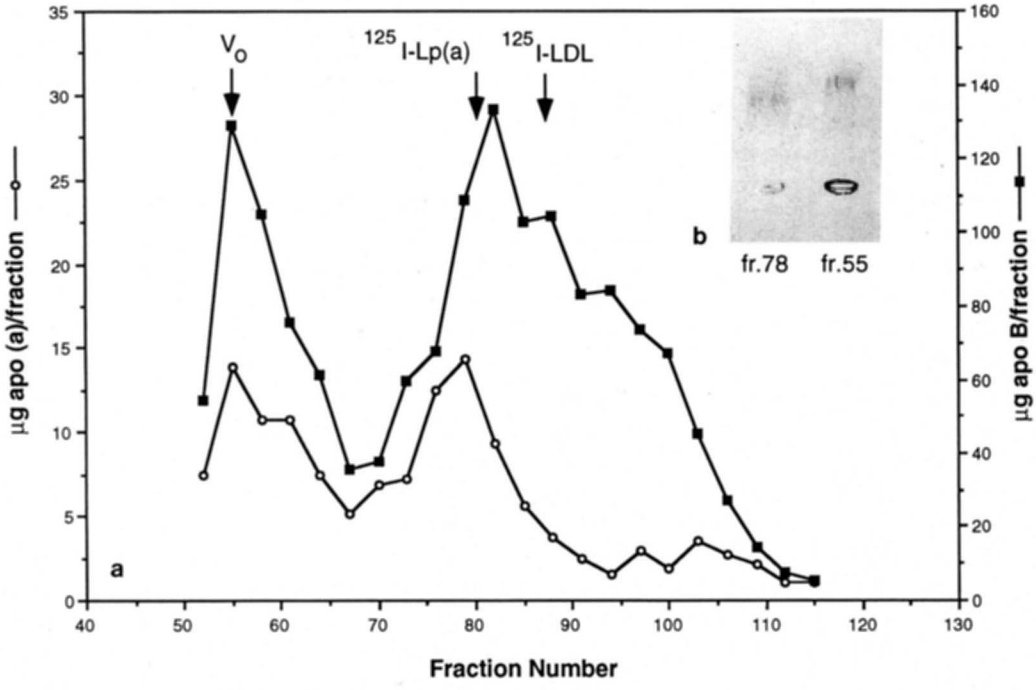

Fig. 2. (a) Apo[a] and apoB elution profiles of a d < 1.10 g/ml fraction of a PBS plaque extract after gel exclusion chromatography. A PBS extract of aortic plaques was first subjected to ultracentrifugation at d 1.10 g/ml. The floating fraction (d < 1.10 g/ml) was then applied to a Sephacryl 400 HR column, (2.5 × 95 cm), 4-ml fractions were collected, and each third tube assayed for apo[a] (○) and apoB (■) content by appropriate RIAs as described in Methods. Arrows indicate the elution positions of the void volume fraction (V_o), ^{125}I-labeled Lp[a], and ^{125}I-labeled LDL. (b) Agarose gel electrophoresis plus immunoblotting for apo[a] of fractions isolated by gel exclusion chromatography described in Fig. 2a. The Lp[a]-sized fraction (fr. 78) and the void volume fraction (fr. 55) were applied to 1% agarose, subjected to electrophoresis, and subsequently immunoblotted for apo[a] as described in Materials and Methods.

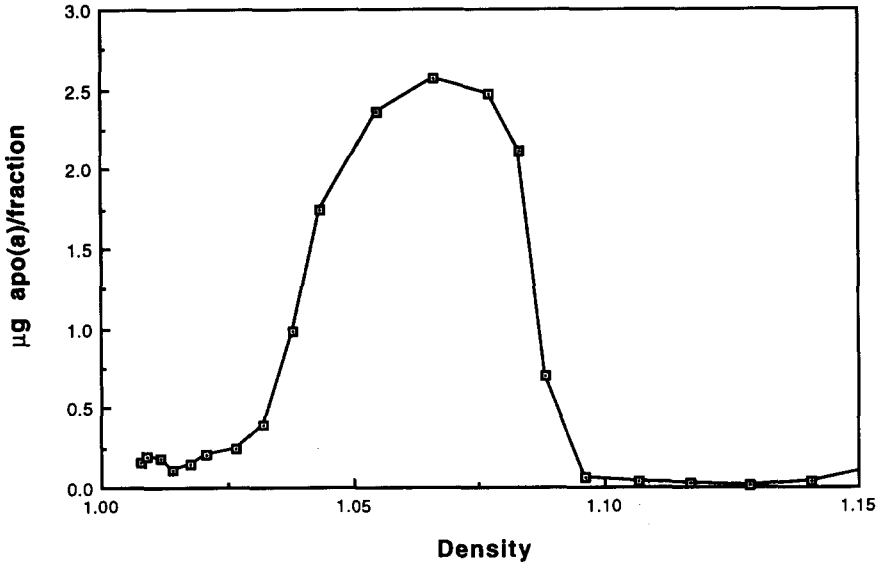

Fig. 3. Profile of apo[a] immunoreactivity after density gradient ultracentrifugation of a pool of fractions containing immunoreactive apo[a] after gel filtration of a d < 1.10 g/ml fraction of a PBS extract, as described in Materials and Methods.

pool of tubes containing immunoreactive apo[a] from a PBS-extract to density gradient ultracentrifugation. Immunoreactive apo[a] was present in a density range of 1.03 < d < 1.09 (**Fig. 3**). Tubes in the density fraction 1.06 < d < 1.08 g/ml showed only the presence of an apo[a]-apoB complex and no apoB-100 band in SDS-PAGE under nonreducing conditions indicating no contamination with A-LDL (not shown).

Because the immunoreactive apo[a] fraction from all PBS-extracts studied demonstrated a slight increase in REM, it was possible that Lp[a] in lesions had undergone oxidation. We, therefore, measured two determinants of lipoprotein oxidation, conjugated dienes, and fluorescence at 360 ex/430 em (16) in a subpopulation of lipoproteins containing apo[a] in the density range 1.06 < d < 1.08 g/ml which we have designated A-Lp[a]. We also measured these parameters in its corresponding A-LDL fraction, e.g., the right side of the LDL-sized fraction obtained after gel filtration chromatography. We found that both parameters were at least 10-fold higher in the A-Lp[a] than in a typical plasma Lp[a] sample (**Table 1**). As we had previously documented that A-LDL underwent oxidation (20), we asked whether the degree of oxidation was the same in A-Lp[a] and in A-LDL isolated from the same case. When the two were directly compared at the same concentration, both the conjugated dienes and fluorescence values were higher for A-Lp[a] than for A-LDL (Table 1), especially the conjugated dienes, suggesting a greater accumulation of hydroperoxides in the former. When the total cholesterol to total protein ratio was also determined in this A-Lp[a] fraction and in plasma Lp[a], both gave a ratio around 1 (Table 1).

As oxidation was measured in A-Lp[a] isolated from a PBS extract of plaque obtained at autopsy, we could not rule out the possibility that this oxidation was a post-mortem artifact. Because insufficient amounts of plaques were available from surgery to isolate enough A-Lp[a] to assess oxidation, we estimated this oxidation in several PBS extracts of plaques obtained at surgery and at autopsy by determining the increases in REM relative to those for plasma Lp[a] by agarose electrophoresis. These two parameters were shown previously to be associated in A-LDL (20). If this assessment of oxidation had been a post-mortem artifact, we would have expected to find a greater mean increase in REM for the apo[a]-staining band from PBS extracts of plaques obtained at autopsy when compared to those from extracts obtained at surgery. However, as seen in **Table 2,** the mean REM of both

TABLE 1. Measurement of oxidation in lesion-derived Lp[a]

Fraction	Conjugated Dienes Absorbance at 234 nm	Fluorescence (360 ex/430 em)	Total Cholesterol to Total Protein Ratio
	× 10^3		
Plasma LDL	23	2	
Plasma Lp[a]	14	4	1.06
A-LDL	43	25	
A-Lp[a]	274	36	0.97

Samples of lesion-derived Lp[a] (A-Lp[a]) and lesion-derived LDL (A-LDL) were isolated from a PBS extract of lesion minces by combinations of flotation during ultracentrifugation at d 1.10 g/ml followed by gel filtration. Fractions containing apo[a] from gel filtration were pooled and further fractionated by density gradient ultracentrifugation. Fractions from the gel filtration column containing apoB but not apo[a] were pooled and designated A-LDL. Samples of LDL and Lp[a] unrelated to the case whose aorta was used for isolation of A-Lp[a] and A-LDL were isolated as described in Materials and Methods. Each sample (10 µg protein/ml) was then subjected to determination of conjugated diene, fluorescence, expressed in arbitrary units, and total cholesterol and total protein for equal concentrations by procedures described in Materials and Methods.

TABLE 2. Relative electrophoretic mobility of plasma Lp[a], lesion-derived Lp[a], and lesion-derived LDL obtained at surgery or at autopsy

Sample	Relative Electrophoretic Mobility
Plasma Lp[a]	
Surgery (n = 4)	1.42 ± 0.19
Autopsy (n = 6)	1.49 ± 0.17
A-Lp[a]	
Surgery (n = 4)	1.97 ± 0.24
Autopsy (n = 4)	1.89 ± 0.26
A-LDL	
Surgery (n = 8)	1.62 ± 0.20
Autopsy (n = 8)	1.49 ± 0.23

The indicated samples of plasma Lp[a], lesion-derived Lp[a], designated A-Lp[a], and lesion-derived LDL, designated A-LDL, were isolated from plaques obtained either immediately at surgery or within 12 h after death at autopsy. Each sample was then subjected to electrophoresis on 1% agarose as described in Materials and Methods.

A-Lp[a] and corresponding A-LDL (apoB-staining band migrating behind the band staining for both apo[a] and apoB) were actually slightly lower in extracts obtained at autopsy than those obtained at surgery but the differences were not significantly different. REM values for plasma obtained at autopsy and surgery did not differ significantly. Thus, based on observations of REM, we believe that it is unlikely that demonstration of oxidation of A-Lp[a] is a post-mortem artifact.

Apo[a] in particles larger than Lp[a]

Both apo[a] and apoB immunoreactivity were also found in the void volume fraction following gel filtration of a PBS extract (Fig. 2a). When the electrophoretic migration of apo[a] in the Lp[a]-sized fraction and the void volume fraction were assessed on 1% agarose that was immunoblotted for apo[a], we found that both the void volume fraction (fr. 55) and the Lp[a]-sized fraction (fr. 78) migrated from the well (Fig. 2B). This result suggested that apo[a] in the void volume fraction was associated with a particle still small enough to enter into 1% agarose. When the void volume fraction was subjected to nondenaturing PAGE and the gel was immunoblotted for apo[a] and apoB, we observed a band that immunostained for both apo[a] and apoB (**Fig. 4**), suggesting the possibility that Lp[a] was present, either in a self-associated state or complexed to other material.

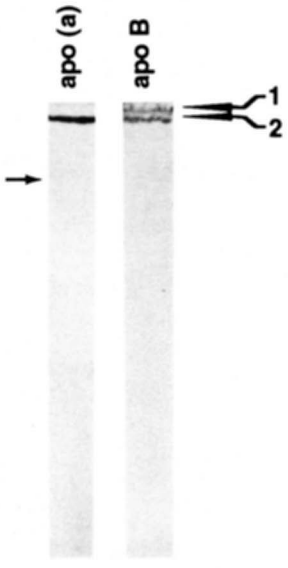

Fig. 4. Nondenaturing PAGE (2.5–16% acrylamide gradient) of the void volume fraction isolated as described in Fig. 2a immunoblotted with anti-apo[a] and anti-apoB. Band 1 staining only for apoB can be seen at the top of the resolving gel, whereas band 2 staining for both apo[a] and apoB just enters into the resolving gel. Lp[a] migrated substantially ahead of this band (arrow).

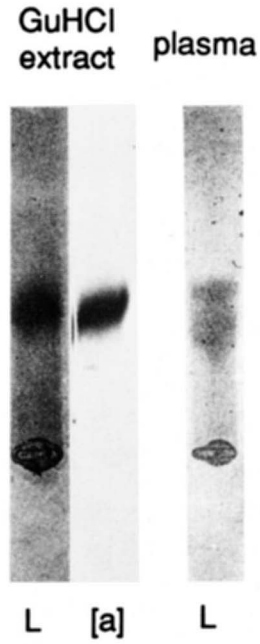

Fig. 5. Agarose electrophoresis of a GuHCl plaque extract and the individual's plasma sample stained for lipid or immunoblotted for apo[a]. Plaque tissue that had been extracted with PBS was re-extracted with 6 M GuHCl. The extract was dialyzed against PBS to remove GuHCl, applied to parallel 1% agarose gels, and electrophoresed as described in Materials and Methods. One gel of the extract was stained with Fat Red 7B (L) while another was subjected to immunoblotting for apo[a] as described in Methods.

Lp[a] in GuHCl extracts

When GuHCl extracts were subjected to agarose electrophoresis and then immunoblotted for apo[a], a major band staining for both lipid and apo[a] (**Fig. 5**) was seen as well as apoB (not shown). This band co-migrated with the lipid-stained band from its corresponding plasma sample, suggesting the presence of intact Lp[a] in the GuHCl extract. However, in other samples this band migrated slightly ahead of the Lp[a] band in plasma. No differences in migration were found between plasma Lp[a] sham-treated with GuHCl and untreated Lp[a] (not shown). When a GuHCl extract was subjected to

SDS-PAGE under reducing conditions and immunoblotted for apo[a], two bands were seen with isoforms of molecular weight similar to those in its corresponding plasma. Even though the lower molecular weight isoform from extracts appeared to migrate slightly ahead of its plasma counterpart in this particular sample (**Fig. 6**), this was not a consistent observation.

To determine whether apo[a] was bound to apoB in GuHCl extracts, we applied an aliquot of the extract to Sepharose–anti-apoB. Immobilized anti-apoB was chosen rather than Sepharose–anti-apo[a] to avoid the binding of any plasminogen or plasmin possibly present in tissue extracts, given the cross-reactivity of plasminogen and apo[a] observable when anti-apo[a] is in a undiluted state, as on an affinity column. Although a decrease in apo[a] immunoreactivity of 33% was obtained after removal of the GuHCl by dialysis, a subsequent loss of only 17% was obtained by binding the dialyzed fraction to Sepharose anti-apoB, and then eluting with 0.2 M glycine at pH 2.4 (**Table 3**). This procedure resulted in over a 1000-fold purification when proteins were measured before and after affinity chromatography. The REM of the apo[a] in the eluted fraction was identical to that in the crude GuHCl extract, and particles in the size range of Lp[a] and LDL could be observed by TEM after negative staining (not shown). GuHCl did not appear to disrupt Lp[a] particles, as plasma Lp[a] treated with GuHCl under similar conditions showed Lp[a]-like particles by TEM (not shown).

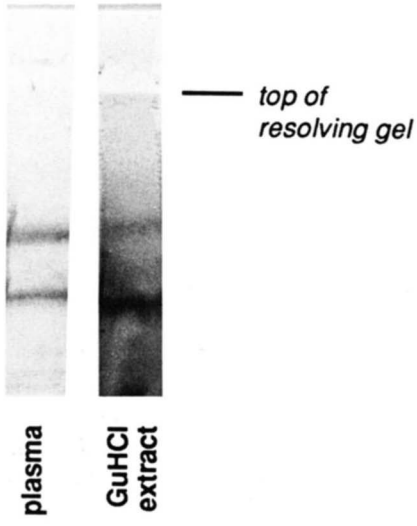

Fig. 6. SDS-PAGE plus immunoblotting for apo[a] of a GuHCl extract and the individual's plasma. Aliquots at about equal apo[a] contents, based on RIA measurement, were applied concurrently to SDS-PAGE under reducing conditions using a 3–8% acrylamide gradient gel and subsequently immunoblotted for apo[a] as described in Materials and Methods.

Size of Lp[a]-like particles in GuHCl extracts

When aliquots of GuHCl extracts were subjected to gel filtration chromatography on Sephacryl 400 HR in the presence of 6 M GuHCl to assess the size of apo[a]-containing particles, immunoreactive apo[a] was present in two included peaks, one eluting slightly ahead of ^{125}I-labeled Lp[a] and one slightly ahead of ^{125}I-labeled LDL (**Fig. 7**). When the same sample was first dialyzed against PBS and then chromatographed on the same gel filtration column using PBS as the running buffer, all apo[a] immunoreactivity now appeared in the void volume fraction (not shown). This suggested that under associative conditions apo[a] in this extract tends to associate with other components in the sample.

TABLE 3. Purification of apo[a]-containing particles from GuHCl extracts of human atherosclerotic lesions by affinity chromatography on Sepharose–anti-apoB

Sample	Immunoreactive Apo[a]	% Recovery	Total Protein
	μg		*μg*
Original GuHCl extract	12		
After dialysis	8	67	27,800
After anti-apoB Sepharose affinity chromatography	6	50	17

Minces of plaques that had been previously extracted by PBS were subsequently extracted with 6 M GuHCl. A sample of this extract was dialyzed against PBS to remove the GuHCl, applied to Sepharose anti-apoB, and a fraction was eluted with 0.2 M glycine, pH 2.4. Apo[a] immunoreactivity in each fraction was determined by RIA, while protein content was determined by the quanta gold assay.

DISCUSSION

Our results from chromatographing a d < 1.10 g/ml fraction of PBS extracts on Sephacryl 400 HR and monitoring of apo[a] and apoB immunoreactivity suggest the presence of apo[a] in the size range of Lp[a], albeit with some modification based on the increased electrophoretic mobility. When this Lp[a]-sized fraction was subsequently subjected to density gradient ultracentrifugation, a subpopulation with the same density as plasma Lp[a] could be separated from lesion-derived LDL (A-LDL) (19). Rath et al. (13) also described a fraction from lesion extracts containing apo[a] in the plasma Lp[a] density fraction. In addition to similarities in molecular weight and density, A-Lp[a] resembled plasma Lp[a] on nonreduced SDS-PAGE, showing the presence of an apo[a] and apoB complex, but no apoB-100 band. Furthermore, both plaque and plasma Lp[a] had a total cholesterol to total protein content of about 1. However, based on increases in electrophoretic mobility as well as analysis of the conjugated diene content and fluorescence at 360 ex/430 em, A-Lp[a] appeared to be oxidized. Of further note was the observation that A-Lp[a] was more oxidized than A-LDL from the same case. This was particularly striking in that, under in vitro conditions, LDL was shown to be more readily oxidized than Lp[a], based

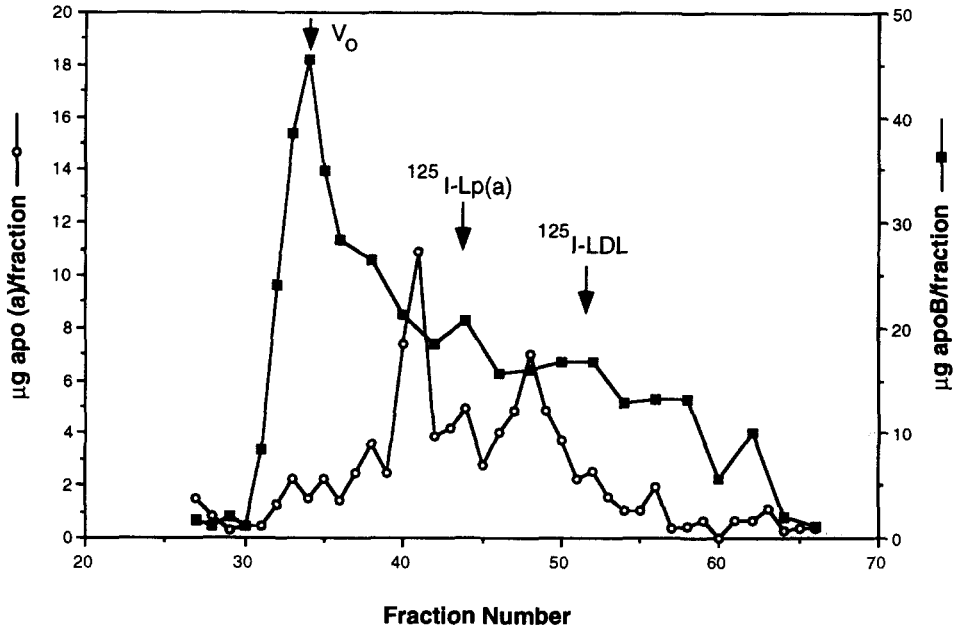

Fig. 7. Apo[a] and apoB elution profiles of a GuHCl plaque extract subjected to gel exclusion chromatography. After extraction of plaques with PBS, the residual tissue was re-extracted with 6 M GuHCl. This extract was then chromatographed on a Sephacryl 400 HR column (1.5 × 95 cm) in the presence of 6 M GuHCl and 2-ml fractions were collected and then monitored for apo[a] (○) and apoB (■) contents by appropriate RIAs as described in Materials and Methods.

on the shorter lag period of conjugated diene formation after Cu^{2+}-induced oxidation (24). Perhaps an association of Lp[a] with such connective tissue as proteoglycans renders Lp[a] more prone to oxidation than LDL as was recently reported (25). As increases in fluorescence at 360 ex/430 em of LDL is indicative of modification of apoB-100 by reactive aldehydes formed during lipid peroxidation to form Schiff-base adducts (26), the low fluorescence level relative to the conjugated dienes level would suggest that hydroperoxides formed in Lp[a] are more resistant to decomposition and subsequent formation of reactive aldehydes than hydroperoxides in A-LDL.

We cannot currently exclude the possibility that the oxidation measured in the Lp[a]-like particles extracted from lesions was due to post-mortem modifications, as insufficient lipoprotein could be isolated from the small samples obtained at surgery to assess levels of oxidation. However, when REM values were obtained by immunoblotting agarose electrophoretograms of PBS extracts for apo[a] from cases obtained at surgery, the mean value was no higher in the autopsy cases than in the surgery cases. In fact, they were slightly lower. Thus, oxidation of Lp[a]-like particles in plaques as assessed by increases in REM, which we have found to parallel increases in fluorescence in A-LDL (20), does not appear to be greater in autopsy samples than in surgical samples.

Apo[a] extracted from lesions with PBS could also be found in the void volume fraction after gel filtration of samples of a d < 1.10 g/ml ultracentrifuge fraction. As apo[a] comigrated with apoB on nondenaturing gels in which the bands were identified by appropriate immunoblots, it is likely that the void volume contains apo[a] and apoB in the same particle, probably in the form of Lp[a] self-aggregates or Lp[a] complexed to other plaque components. Lp[a] was previously shown to self-aggregate in the presence of Ca^{2+} ions (27, 28) and to interact with arterial proteoglycans (29) and with fibrin(ogen) (30, 31).

Characterization of apo[a]-containing particles in the GuHCl extract was of particular interest, as it contained roughly 80% of the immunoreactive apo[a] found in plaques (11). Evidence for the presence of Lp[a]-like particles in the GuHCl extract came from affinity chromatography studies in which particles containing both apo[a] and apoB in approximately the size range of Lp[a] could be isolated using Sepharose-anti-apoB. In addition, the elution profile of GuHCl extracts on Sephacryl 400 HR demonstrated the presence of Lp[a]-like particles that were only slightly larger than plasma Lp[a], as well as some particles slightly larger than LDL. The chemical and/or structural changes that were responsible for these modifications in size relative to plasma Lp[a] still need to be determined.

Apo[a] immunoblotting of SDS-PAGE gels under reducing conditions provides information on the apo[a] isoform pattern in plasma and tissue (9, 10). In our study we found that the isoform patterns were about the same in both plasma and the PBS extract, consistent with results reported by Rath et al. (13) on human atherosclerotic lesions, although the relative intensities of

the two were not always identical, the reason for which still needs to be explored. Likewise, plasma and GuHCl extracts showed similar bands depicting apo[a] isoforms on SDS-PAGE, suggesting the presence of the same isoforms.

In conclusion, the data from this study suggest that lipoproteins containing apo[a] are present in human atherosclerotic lesions in a variety of different forms, some resembling Lp[a] but chemically modified such as by oxidation. Other particles may represent Lp[a] complexed with themselves or with other tissue components to form aggregates. Still other particles found in GuHCl extracts that are smaller than plasma Lp[a] could represent degraded Lp[a].

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