Apolipoprotein[a] is not associated with apolipoprotein B in human liver

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Abstract The aim of this research was to determine whether apolipoprotein[a] (apo[a]) is linked to apolipoprotein B (apoB) in human liver. Four ELISAs were developed: *1)* a competition assay that measures apoB; *2)* a competition assay that measures apo[a]; *3)* a capture assay based on capture of apo[a] by a polyclonal antibody and detection of co-immobilized apoB using a monoclonal antibody; and *4)* a capture assay based on capture of apo[a] using a polyclonal antibody and detection of immobilized apo[a] using a monoclonal antibody. Assays *2* and *4*, therefore, measure apo[a] either free or in complex with other proteins, while assay *3* measures apo[a] associated with apoB. The levels of apo[a] ranged from 25 to 440 μg/g liver in nine individual liver samples. There was no significant difference between apo[a] levels in individual human liver samples measured using ELISA *1* or *3;* however, it was not possible to detect apo[a]/apoB using assay *3*. ApoB was present in human liver homogenates at levels ranging from 90 to 700 μg/g measured using assay *1*. These results suggest, therefore, that apo[a] is not coupled to apoB in the liver and may be secreted in the free form to bind with low density lipoprotein (LDL) in the extracellular fluid or plasma.—**Wilkinson, J., L. H. Munro, and J. A. Higgins.** Apolipoprotein[a] is not associated with apolipoprotein B in human liver. *J. Lipid Res.* 1994. **35:** 1896–1901.

Supplementary key words enzyme-linked immunoassay • lipoprotein[a] • secretion

Elevated plasma levels of lipoprotein[a] (Lp[a]) are an independent risk factor for atherosclerosis and coronary heart disease (1–7). Lp[a] in the circulation consists of low density lipoprotein (LDL)-like particles in which apoB is linked to a highly glycosylated protein apolipoprotein[a] (apo[a]) through a disulfide bond (2). Apo[a] exhibits close homology with plasminogen and contains an inactive form of the protease domain, one copy of the kringle V domain, and multiple copies of the kringle IV domain (8). Thirty four isoforms of apo[a] have now been identified with molecular masses between 400 and 900 kD (9). Plasma levels of Lp[a] show a wide range from <1 mg/dl to >500 mg/dl and there is an inverse relationship between the molecular mass and the circulating level of Lp[a] (10, 11).

A number of independent pieces of evidence have shown that in humans and primates the liver is the site of synthesis of apo[a] (2, 12–15); however, very little is known about intracellular events in the synthesis of apo[a] and its regulation. This is due in part to the lack of suitable and easily available animal models. An elegant study by White, Rainwater, and Lanford (14) of Lp[a] secretion by baboon hepatocytes has concluded that newly synthesized apo[a] is not associated with apoB in the cell but assembled into Lp[a] particles after secretion. However, the form in which apo[a] is secreted in vivo and the intracellular events in its transit through the human liver cell are unknown.

The primary aim of the present study was to determine whether apo[a] is associated with apoB in human liver. For this study, we developed four immunoassays; capture and competition ELISA to measure total apo[a], a capture ELISA to measure apo[a] in complex with apoB, and a competition ELISA to measure total apoB. The results indicate that apo[a] in human liver is not associated with apoB and therefore that apo[a] is probably not secreted as a complex with apoB in lipoprotein particles.

METHODS

Human liver samples

Liver samples were obtained with the help of Dr. P. Ingleton, Department of Pathology, University of Sheffield, and Dr. A. J. Strang, The Liver Research Laboratories, Queen Elizabeth Hospital, Birmingham. These samples were either transplant material or normal tissue removed with pathological samples during surgery. These samples were frozen immediately in liquid nitrogen and kept at −70°C until used. Liver samples were thawed and homogenized (20% w/v) in Tris-buffered saline

Abbreviations: LDL, low density lipoprotein; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline.

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(TBS) (50 mM Tris, 200 mM NaCl, pH 7.4) containing 0.4% taurocholate as a solubilizing agent, using a Potter-Elvejhem top-driven homogenizer.

Human blood

Blood samples were taken randomly from patients attending the Cardiothoracic Lipid Clinic at the Northern General Hospital, Sheffield. PMSF and EDTA were added to give final concentrations of 20 μM and 100 μM, respectively. Red cells were pelleted by centrifugation (1000 *g* for 10 min). Protease inhibitors PMSF (10 μl, 20 mM), aprotinin (100 μl, 15–30 TIU/ml), and EDTA (10 μl, 100 mM) were added to each 10 ml of plasma which was stored in the refrigerator and used within 24 h.

Lipoprotein fractions

LDL (d 1.019–1.063 g/ml) and Lp[a]-enriched fractions (d 1.063–1.165 g/ml) were prepared from human plasma by differential centrifugation (16). The Lp[a] fraction was separated into apo[a] and LDL minus apo[a] by layering reduced Lp[a] beneath a 30–7.5% KBr gradient and centrifugation for 24 h at 70,000 *g* max (17). The gradients were removed in 1-ml fractions and the load layer (apo[a]) and floating fractions (apoB) were used to test the specificity of the apo[a] and apoB antibodies by immunoblotting as described previously (16).

Antibodies

The following primary antibodies were used in these studies: *i,* affinity-purified monospecific polyclonal anti-Lp[a] (sheep) for phenotyping (Immuno Ltd.); and *ii,* monospecific affinity-purified polyclonal antibody to Lp[a] (Immuno Ltd.). These anti-Lp[a] antibodies are polyclonal and recognize epitopes throughout the length of the apo[a] molecule. Consistent with the details supplied by Immuno Ltd., we found that neither Lp[a] antibody crossreacts with plasminogen or albumin using ELISA, dot blotting, and immunoblotting after SDS-PAGE. *iii,* A mouse anti-human apo[a] monoclonal antibody (clone M1A2 reported by Boerhingher, the supplier, to recognize the multiple kringle IV of apo[a] and demonstrate no crossreactivity with plasminogen); and *iv,* monoclonal antibodies against human apoB, MAC 129 and MAC 131 hybridoma supernatants, were a gift from Dr. Ermanno Gherardi. These monoclonal antibodies recognize epitopes in the N-terminal 48% of apoB (18). Secondary antibodies were donkey anti-sheep IgG coupled to alkaline phosphatase (Sigma) for primary polyclonal antibodies and goat anti-rat and anti-mouse IgG coupled to alkaline phosphatase (Sigma) for primary monoclonal antibodies.

Competition ELISA for apo[a]

One hundred and fifty μl of Lp[a] standard (3.5 μg Lp[a] ml in sodium bicarbonate buffer, 0.1 M, pH 9.6) was placed in 96-well plates (Nunc). The plates were sealed in plastic bags and left overnight at room temperature. The wells were emptied and washed four times with TBS containing 0.5% (w/v) Tween 20 and 0.5% RIA grade bovine serum albumin (BSA) (wash buffer) followed by blocking with TBS containing 3% BSA (blocking buffer) added to each well for at least 2 h. The wells were emptied and 100 μl of primary antibody (anti-Lp[a]) diluted up to 1–20000 in TBS containing 1% BSA containing 0.4% taurocholate (incubation buffer) was added together with 50 μl of the same buffer, Lp[a] standard, or unknown Lp[a] (plasma or solubilized liver homogenate) diluted with incubation buffer. The plates were shaken for about an hour and left overnight at 4°C. The wells were emptied, washed four times, and 150 μl secondary antibody coupled to alkaline phosphatase (diluted 1–1000 in incubation buffer) was added. The plates were left at room temperature with shaking for 4 h. The wells were emptied and washed four times with wash buffer followed by 0.1 M glycine buffer, pH 10.4, containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$. Bound alkaline phosphatase was then measured by addition of *p*-nitro-phenylphosphate (Sigma). The color development was read at 405 nm using a Anthos HT11 plate reader.

Antibody dilution curves were prepared using a range of primary antibody concentrations. Dilutions of primary antibody that gave 50–75% inhibition of the maximum binding to the immobilized Lp[a] were used in subsequent assays of standards or unknown samples.

Competition curves were prepared using a range of concentrations of Lp[a] standard (0–20 mg/dl) and were always performed on each ELISA plate. Lp[a] was always assayed in a range of diluted unknown samples to ensure that values fell on the linear part of the curve. Each dilution was determined in duplicate or triplicate.

Competition ELISA for apoB

This was performed as described previously for determination of rabbit apoB except that human LDL-apoB was used as standard and MAC 131 or 129 was used as antibody (19).

Sandwich-capture ELISA

ELISA plates were prepared by pipetting 100 μl of 1:100 dilution of anti-human Lp[a] in 0.1 M bicarbonate buffer, pH 9.6, into each well. The plates were sealed in plastic bags and left overnight at room temperature. The wells were emptied and washed four times with wash buffer followed by blocking buffer for at least 2 h. A range of concentrations of Lp[a] standards (0–40 mg/dl) or plasma or solubilized liver homogenate in 100 μl incubation buffer were placed in the wells. The plates were left overnight at 4°C. The wells were emptied and washed four times. Bound apoB was determined by addition of a monoclonal antibody against apoB (100 μl, overnight). Alternatively,

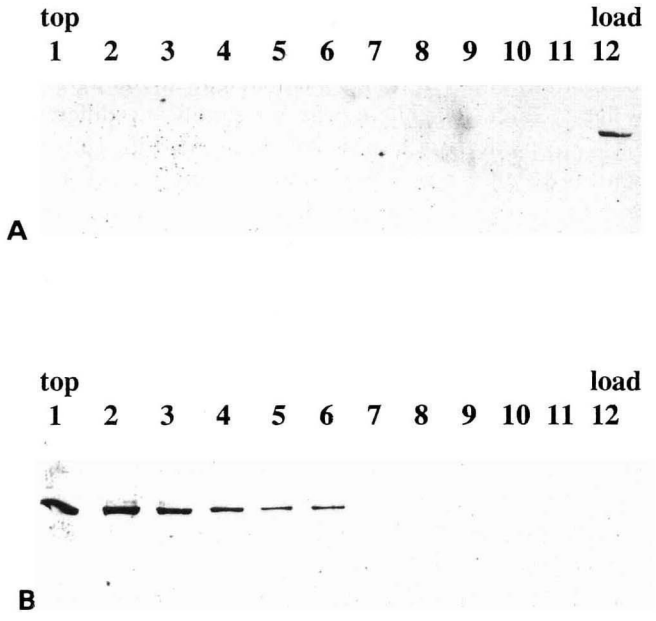

Fig. 1. Distribution of immunologically detectable apo[a] and apoB in reduced Lp[a]. An Lp[a]-enriched fraction was prepared from human plasma, reduced with dithiothreitol, and separated on a 7.5–30% KBr gradient as described in Methods. Twelve 1.0-ml samples were taken from the top of the gradient including the load layer. Parallel samples were separated by SDS-PAGE and immunoblotted with anti-apo[a] (A) or anti-apoB (B). In the figure illustrated, the monospecific affinity-purified polyclonal antibody to Lp[a] and MAC 131 against apoB were used. However, similar results were obtained with each of the anti-apo[a] antibodies and each of the anti-apoB antibodies.

bound apo[a] was determined by addition of a monoclonal antibody against apo[a]. The second antibody bound was determined using anti-rat IgG or anti-mouse IgG coupled to alkaline phosphatase as described above. A series of preliminary experiments was performed to determine the optimum amount of immobilized polyclonal anti-Lp[a] that binds to the plate and binds the highest concentration of Lp[a] investigated and to determine the dilution of MAC 128 or MAC 131 or anti-apo[a] required to be in excess of the apoB immobilized. A standard curve for Lp[a] was included on all ELISA plates and Lp[a] was always assayed in a range of dilutions of serum to ensure that values were on the linear part of the curve. Each dilution was assayed in duplicate or triplicate.

RESULTS

Determination of Lp[a] in human plasma samples

The specificities of each of the anti-apo[a] and anti-apoB antibodies were tested by immunoblotting of the fractions prepared by reduction of Lp[a]. In parallel blots, only the floating fractions were detected by anti-apoB and only the load layer by anti-apo[a] (**Fig. 1**).

The ELISA methods were extremely sensitive for assay of apo[a] or apoB/apo[a] (**Fig. 2**). The lower limit of the competition assay was 0.02 μg/ml of Lp[a] standard and that of the capture assays was 0.04 μg/ml of Lp[a] standard. The Lp[a] standard used was that provided by

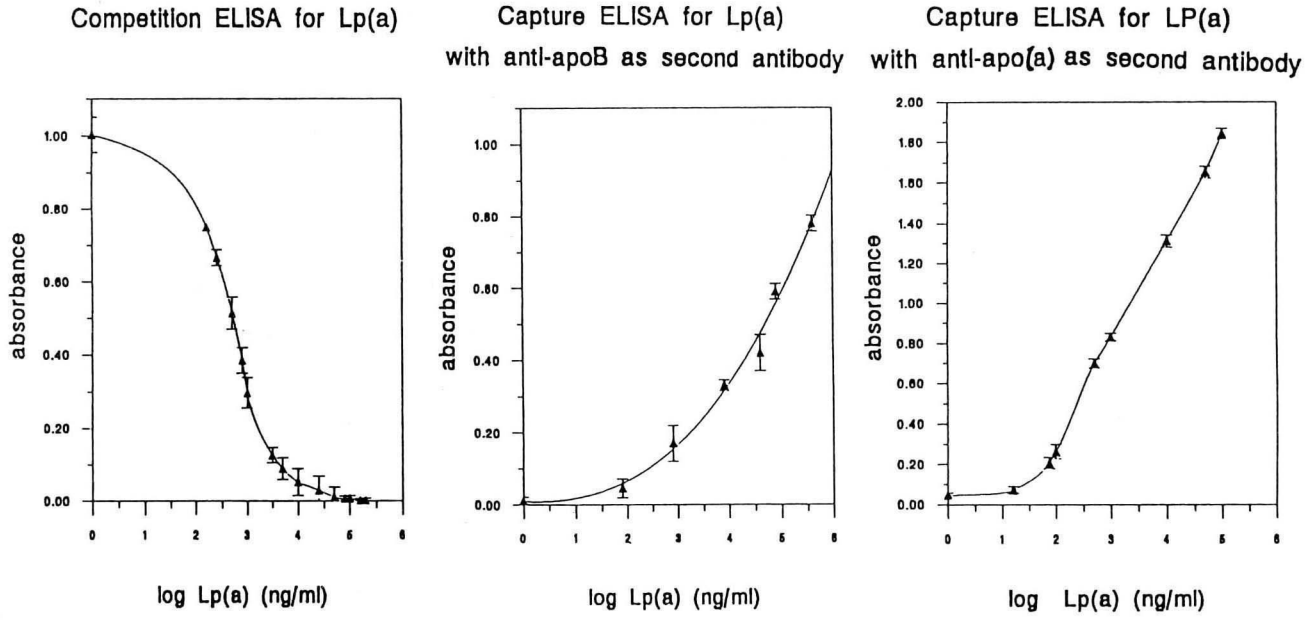

Fig. 2. Standard curves for three ELISAs. Curves were generated as described in Methods for human Lp[a] standards assayed using (a) the competition assay, (b) the capture assay using MAC 131 as second antibody, and (c) the capture assay using MIA2 as second antibody.

TABLE 1. Lp[a] in plasma determined by the capture ELISA using different second antibodies

Plasma Sample	Lp[a] mg/dl		
	MAC 129	MAC 131	Anti-Apo[a]
1	1.95	2.21	2.15
2	40.84	40.81	51.63
3	5.68	6.61	8.42
4	4.70	5.66	7.74
5	8.45	9.34	12.03

Lp[a] in human plasma was determined using the capture ELISA as described in Methods. Monoclonal antibodies MAC 131, MAC 129, and anti-apo[a] were used as the second antibody in parallel assays on the same samples. Results are the mean of three determinations. Standard deviations were less than 10%.

Immuno Ltd. and was prepared from pooled human Lp[a] samples. Assuming that 50% of the mass of the protein is apoB and 50% apo[a] this means that the competition assay and capture assays can detect 0.5 ng and 1.0 ng of apo[a] and apoB/apo[a], respectively, per assay well.

When the capture ELISA was used to determine Lp[a] with different monoclonal antibodies, MAC 131, MAC 129, or anti-apo[a], essentially similar results were obtained (**Table 1**). This observation validates the capture ELISA for plasma Lp[a], demonstrates that bound apoB is also immobilized, and that this protein can be assayed with either MAC 131 or MAC 129. It also demonstrates that the apo[a] immobilized using a polyclonal antibody can be assayed using an anti-apo[a] monoclonal antibody as second antibody.

Determination of apo[a]/Lp[a] in human liver

A range of dilutions of each human liver homogenate were assayed to ensure that all determinations fell on the linear part of the curve and that the results were parallel with the standard curve. The level of apo[a] in nine samples of human liver determined by the competition ELISA ranged from 83 μg/g wet weight to 400 μg/g wet weight (**Table 2**). There was no significant difference ($P > 0.1$) in the results obtained using either the competition assay or the capture assay with anti-apo[a] as the second antibody, although there were relatively large differences in the mean values of some samples. This is not due to sample differences as assays were performed simultaneously on the same homogenates. When either MAC 129 or MAC 131 was used in the capture ELISA, no apoB could be detected even when undiluted liver homogenates were used; however, apoB was detected in all samples of liver using the competition ELISA with either MAC 131 or MAC 129 (Table 2). The levels of apoB varied considerably between samples; however, there was no apparent relationship with the apo[a] levels in individual liver homogenates.

The lowest level of apo[a] in the samples assayed was 25 μg/g determined by the capture assay. This is equivalent to 250 ng/assay well. The lowest level of apoB coimmobilized with apo[a] that can be detected per well is 1 ng. Therefore, in this sample less that 0.25% of the apo[a] immobilized was bound apoB; in liver samples with the highest apo[a] levels it can be calculated that less than 0.001% of the apo[a] is bound to apoB.

DISCUSSION

The liver is apparently the site of secretion of apo[a] in humans. The mRNA levels for apo[a] are highest in liver compared with other tissues and the apo[a] isoform in the plasma of patients who have had liver transplants is changed to that of the donor (15). The rate of secretion of apo[a] is considered to be the important factor in regulat-

TABLE 2. Determination of Lp[a] in human liver using different ELISA

Liver Sample	Capture ELISA (Anti-ApoB)	Capture ELISA (Anti-Apo[a])	Competition ELISA (Anti-Apo[a])	Competition ELISA (Anti-ApoB)
		μg/g		
1	n.d.	130 ± 11	93 ± 12	315 ± 15
2	n.d.	71 ± 9	97 ± 3	135 ± 8
3	n.d.	189 ± 19	196 ± 54	575 ± 32
4	n.d.	165 ± 20	174 ± 43	108 ± 5
5	n.d.	174 ± 15	148 ± 25	377 ± 25
6	n.d.	444 ± 51	405 ± 133	95 ± 4
7	n.d.	25 ± 5	83 ± 14	90 ± 6
8	n.d.	203 ± 45	120 ± 8	119 ± 11
9	n.d.	122 ± 8	128 ± 3	710 ± 47

Apo[a] levels in homogenates of human liver were determined as described in Methods using four ELISA: the capture ELISA with anti-apoB (MAC 131 or MAC 129) as second antibody, the capture ELISA with anti-apo[a] as second antibody, the competition ELISA with anti-apo[a], and the competition ELISA with anti-apoB (results given are for MAC 131, similar results were obtained with MAC 129). Results are the mean of at least four separate determinations ± standard deviations; n.d., not detectable.

ing Lp[a] levels (12). A major research interest of this laboratory is the mechanism of regulation of apoB secretion by hepatocytes (16, 19–21). We are therefore interested in the relationship between apo[a] and apoB synthesis and secretion. The primary point to be established is the site of coupling of apo[a] with apoB. The present study was designed to determine whether this takes place in liver. Our results clearly demonstrate that, under conditions in which apo[a] is immobilized from liver homogenates and can be measured using an anti-apo[a] monoclonal antibody, bound apoB cannot be detected, suggesting that coupling of apo[a] and apoB does not take place in the liver. One possible alternative explanation is that the epitopes for apoB are shielded in the liver. However, this does not appear to be the case as anti-apo[a], MAC 131, and MAC 129 yield similar results when used to quantify Lp[a] in plasma samples and apoB is detectable by the competition ELISA in liver samples. A further possibility is that apo[a] and apoB of Lp[a] dissociated during preparation and assay of the liver samples. However, the Lp[a] standards were treated in exactly the same way as the liver homogenates and did not dissociate. Furthermore, when Lp[a] was added to the homogenates before the assay, dissociation did not take place.

The levels of apo[a] and apoB in human liver samples vary considerably. Our previous studies of apoB levels in rat or rabbit liver (16, 19–21) showed values within the range of those found in human but with far less variation. This may reflect the fact that laboratory animals are inbred while humans exhibit considerable genetic variation. In addition, assays of rabbit and rat liver were performed on homogenates of whole liver while those of human liver were performed on homogenates of 1 gram of liver and the latter may be affected by its regional variation in the levels of apolipoproteins in the liver.

The results reported here are consistent with recent investigations from other laboratories on animal models. In baboon hepatocytes, cellular apo[a] is immunoprecipitated without bound apoB; however, secreted apo[a] in the incubation medium co-precipitates with apoB (14). Coupling of apo[a] to apoB, therefore, takes place after secretion by isolated cells. Transgenic mice transfected with the gene for human apo[a] also have free apo[a] in their plasma and when mouse LDL are transfused into these mice, the apo[a] does not associate with the lipoprotein particles (22). However, when human LDL are transfused, the apo[a] rapidly becomes bound to the LDL. The formation of apo[a]/apoB is thus dependent on some characteristic of the human apoB not shared by mouse apoB.

Our observations indicate that apo[a] is not complexed with apoB in human liver and suggest that apo[a] is secreted in a free form. As only a small proportion of apo[a] is free in human plasma (23), it would appear that apo[a] is incorporated into Lp[a] extracellularly. Although we are not aware of any other studies of apo[a]/apoB levels in human liver, our observations are consistent with the finding that patients with the genetic defect apoB-lipoproteinemia who lack circulating apoB do have low levels of apo[a] in their plasma (24).

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