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Reconstitution of Lipoprotein(a) by Infusion of Human Low Density Lipoprotein into Transgenic Mice Expressing Human Apolipoprotein(a)*

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Lipoprotein(a) (Lp(a)) is an atherosclerosis-causing lipoprotein that circulates in human plasma as a complex of low density lipoprotein (LDL) and apolipoprotein(a) (apo(a)). It is not known whether apo(a) attaches to LDL within hepatocytes prior to secretion or in plasma subsequent to secretion. Here we describe the development of a line of mice expressing the human apo(a) transgene under the control of the murine transferrin promoter. The apo(a) was secreted into the plasma, but circulated free of lipoproteins. When human (h)-LDL was injected intravenously, the circulating apo(a) rapidly associated with the lipoproteins, as determined by nondenaturing gel electrophoresis. Human HDL and mouse LDL had no such effect. When h-VLDL was injected, there was a delayed association of apo(a) with the lipoprotein fraction which suggests that apo(a) preferentially associated with a metabolic product of VLDL. The complex of apo(a) with LDL formed both *in vivo* and *in vitro* was resistant to boiling in the presence of detergents and denaturants, but was resolved upon disulfide reduction. These studies suggest that apo(a) fails to associate with mouse lipoproteins due to structural differences between human and mouse LDL, and that Lp(a) formation can occur in plasma through the association of apo(a) with circulating LDL.

Lipoprotein(a) is a cholesterol ester-rich particle comprised of a low density lipoprotein (LDL)[1] and a highly polymorphic glycoprotein called apolipoprotein(a) (apo(a)) (1, 2). The apo(a) of Lp(a) is attached to apolipoprotein B-100 (apoB-100) by a putative disulfide linkage (3–5), although other interactions may contribute to the tight association of these two proteins (6, 7). The site of assembly of the Lp(a) particle *in vivo* is not known. Apo(a) and apoB-100 mRNA are both expressed in significant quantities only in the liver (8, 9). Metabolic studies suggest that unlike LDL, Lp(a) is not initially secreted as a triglyceride-rich lipoprotein (10). It is not known if apo(a) couples to apoB in the hepatocyte and is secreted as a pre-formed lipoprotein, or whether apo(a) joins the lipoprotein particle in the plasma. Individuals with abetalipoproteinemia are unable to secrete apoB-100 and yet have apo(a) in their plasma which circulates free of lipoproteins (11). Therefore, apo(a) can be secreted from the liver independently of apoB-100.

The physiological role of Lp(a) remains elusive. Lp(a) has an unusual species distribution and is present in the plasma of the hedgehog (12), marmoset (13), old world monkeys (8, 14), great apes (14), and humans (1), but not in other animals. To date, the only experimental animal models available to study the *in vivo* metabolism and pathophysiology of Lp(a) have been primates. In order to study the site of formation and metabolism of Lp(a) and its role in atherogenesis, we have established a transgenic mouse line expressing the human apo(a) under the control of the mouse transferrin promoter.

EXPERIMENTAL PROCEDURES

Materials—Human LDL (density (d) = 1.019–1.063 g/ml), VLDL (d < 1.006 g/ml), and HDL (d = 1.063–1.215 g/ml) were prepared as previously described (15). IgG-1A[2], an anti-human apo(a) mouse monoclonal antibody, was obtained from Dr. Gerd Utermann (Innsbruck, Austria). A polyclonal rabbit anti-human apoE antibody was obtained from Dr. Carl Weisgraber (Gladstone Institute, University of San Francisco, San Francisco, CA), and goat anti-rabbit IgG was obtained from Cappell (Durham, NC). [α-^{32}P]dCTP and [γ-^{32}P]ATP were purchased from ICN Radiochemicals (Irvine, CA). Restriction enzymes and *Escherichia coli* DNA polymerase I (Klenow fragment) were obtained from New England Biolabs (Beverly, MA). The nylon and nitrocellulose membranes were from Schleicher & Schuell (NY-TRAN™) and Amersham Corp., respectively. The minigel and transfer apparatus used for immunoblotting were both from Bio-Rad). The ϵ-aminocaproic acid and horseradish peroxidase were obtained from Sigma.

Development of Human Apo(a) Transgenic Mouse—Four human apo(a) expression constructs were made using either the mouse metallothionein I (16), mouse albumin (17), cytomegalovirus (18) or mouse transferrin (TF in plasmid name) enhancer/promoter 5′ sequences (19) linked to a human apo(a) cDNA (20). The apo(a) transgene construct (pTF1a17) was derived from the apo(a) expression vector pRK5ha17 (21). This expression vector contains the cytomegalovirus promoter/enhancer and an immunoglobulin G intron (22) fused to 25 nucleotides of the apo(a) 5′-untranslated sequence, the apo(a) secretion signal sequence, 17 tandem copies of a

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[1] The abbreviations used are: LDL, low density lipoprotein; apo(a), apolipoprotein(a); Lp(a), lipoprotein(a); apoB-100, apolipoprotein B-100; apoE, apolipoprotein E; VLDL, very low density lipoprotein; HDL, high density lipoprotein; ELISA, enzyme-linked immunoabsorbent assay; β-ME, β-mercaptoethanol; m-, mouse; h-, human.

342-base pair sequence homologous to the kringle 4 domain of plasminogen followed by a kringle 5 and protease-like domain. The 3′-untranslated region of apo(a) was fused to an SV40 early region polyadenylation signal. To make pTF1a17, the cytomegalovirus promoter was replaced with a 3-kilobase (kb) *Eco*RI-*Bam*HI fragment of the mouse transferrin promoter (19).

A 12.8-kb *Not*I-*Hpa*I fragment from pTF1a17 was purified free of vector sequences and microinjected into fertilized C57 Bl/6xSJL F_2 hybrid mouse eggs (23). Mice that contained the transgene were identified by dot blot hybridization of DNA extracted from tail tissue. One male mouse (275-4) expressed the transgene and was bred to C57 Bl/6xSJL F_1 females to establish a line.

Enzyme-linked Immunoabsorbent (ELISA) Assay of Plasma Apo(a)—The plasma levels of apo(a) in the mice were determined using a sandwich ELISA assay (11, 24). In this assay, the capture antibody is a rabbit anti-human Lp(a) polyclonal antibody and the detecting antibody is a mouse anti-human apo(a) monoclonal antibody, IgG-IA[2] conjugated to horseradish peroxidase (25). The assay was standardized using purified human Lp(a), and the values are reported as Lp(a) total mass. Approximately one-third of Lp(a) mass is protein (apoB-100 and apo(a)) (3, 26), and the molecular mass of the recombinant apo(a) approximates that of apoB-100 (~500,000 kDa) (21). Therefore, the plasma concentration of the recombinant apo(a) was estimated to be one-sixth of the Lp(a) concentration determined by the ELISA assay.

RNA Blot Analysis of Apo(a) Expression in Transgenic Mice—Total RNA was purified from various tissues (liver, kidney, jejunum, heart, brain, testis, white fat) by guanidinium isothiocyanate-CsCl centrifugation (27). An aliquot (10 μg) was size-fractionated on a 1.5% agarose-glyoxal gel, transferred to a nylon membrane, hybridized with a ^{32}P-labeled 2.7-kb *Sma*I fragment from the pRK5ha17 expression construct (21), and washed as previously described (28). After exposure to film, the membrane was stripped and re-hybridized with a ^{32}P-labeled 3.1-kb *Eco*RI fragment from the mouse transferrin cDNA (kindly provided by Dr. G. Stanley McKnight, University of Washington) and exposed to film.

Immunoblot Analysis of Apo(a) Synthesis in Transgenic Mice—Mice were anesthetized with an intraperitoneal injection of 90 mg/kg sodium pentobarbital, and the ascending aorta was cannulated and the right atrium slit. Mice were perfused with 25 ml of ice-cold 0.9% NaCl containing bovine serum albumin (2 mg/ml). The indicated tissues were homogenized in four volumes of 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM 1,10-phenanthroline, 0.1 mM leupeptin, 1 μg/ml pepstatin, 0.5 μg/ml aprotinin, and 20 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 800 × *g* for 2 h at 4 °C. The supernatant was collected, and 80 μg of total protein (in 10 μl) were mixed with 5 μl of buffer A (15% (w\v) SDS, 8 M urea, 5 mM dithiothreitol, 62.5 mM Tris-HCl, pH 8.0) and 10 μl of loading buffer (10% (w/v) glycerol, 0.15% (w/v) bromphenol blue, 2.3% (w/v) SDS, 5% (v/v) β-mercaptoethanol (β-ME), 62.5 mM Tris-HCl, pH 6.8). After boiling for 5 min, 15 μl of sample were loaded onto a 5% SDS-polyacrylamide gel and electrophoresis was performed for 1 h at 200 V at 4 °C. The proteins were transferred to nitrocellulose at 200 V for 1 h and immunoblotted as described (24) using IgG-1A[2] conjugated to horseradish peroxidase (25). After washing, the filter was developed using the protocol and reagents of the ECL Western Blotting Detection System Kit™ (Amersham Corp.) and exposed to XAR-5 film (Kodak).

Analysis of Apo(a) Distribution in Transgenic Mice Plasma—A total of 100 μl of mouse and human plasma was adjusted to a density of 1.215 g/ml with KBr, and ultracentrifugation was performed for 11 h at 4 °C using a Beckman TLA-100 rotor at 436,000 × *g*. Aliquots (15 μl) of total plasma, the top ($d < 1.215$ g/ml) and bottom ($d > 1.215$ g/ml) fractions were added to 20 μl of buffer A and 50 μl of loading buffer. A 10-μl aliquot was boiled for 5 min and then subjected to electrophoresis on a 5% SDS-polyacrylamide gel. Five μl of mouse plasma and one μl of human plasma (~0.5 μg of Lp(a)) were adjusted to a volume of 20 μl in 0.9% NaCl. After the addition of 5 μl of sucrose dye (50% (w/v) sucrose, 0.625% (w/v) bromphenol blue, 0.2 M glycine, 25 mM Tris, pH 8.3), one-fifth of the total volume was loaded onto a 4% nondenaturing polyacrylamide gel. Electrophoresis was performed at 4 °C for 2 h at 150 V. The gel was soaked for 12 h at 22 °C in 250 ml of 10 mM dithiothreitol, 1.5 M Tris-HCl, pH 8.8. Immunoblotting was performed as described above.

Infusion of Human and Mouse Lipoproteins into Apo(a) Transgenic Mice—Human (h-)VLDL, h-LDL, and h-HDL fractions were separated from plasma pooled from five normolipidemic subjects who had no detectable plasma Lp(a) by either the ELISA assay (11, 24) or immunoblotting (24). Mouse (m-)LDL ($d = 1.019$–1.063 g/ml) was isolated from 3 ml of plasma derived from five hepatoma-bearing mice that had markedly elevated LDL levels.[2] The chemical composition of mouse LDL was comparable to that of human LDL (triglyceride to protein ratios: 0.46 *versus* 0.40; total cholesterol to protein ratios: 1.5 *versus* 1.8) (29). The lipoproteins were dialyzed against 0.15 M NaCl, and then the protein concentrations of LDL, HDL, and VLDL were determined by the method of Lowry (30). The apoB-100 content in purified VLDL was estimated to be one-third of the total protein content (31).

Three transgenic mice were anesthetized with sodium pentobarbital (90 mg/kg). Each mouse received a 100-μl intravenous bolus of lipoproteins via the external jugular vein. The amount of lipoproteins injected was calculated so that the molar ratio of plasma apo(a) to injected apoB-100 or HDL protein was 1 to 50. Blood was collected by retro-orbital puncture at various time points after the injection. For each set of experiments, 5 μl of each sample, prepared as described above, was loaded onto a 4% nondenaturing polyacrylamide gel under non-reducing conditions. After electrophoresis, the proteins were transferred to nitrocellulose and immunoblotted with IgG-1A[2] as described.

At each time point after the *in vivo* infusion of h-VLDL, total lipoproteins were separated from 100 μl of mice plasma by ultracentrifugation at $d = 1.215$ g/ml for 11 h at 436,000 × *g* in a Beckman TLA-100 rotor; 20-μl aliquots of the lipoprotein fractions were then mixed with 20 μl of buffer A and 50 μl of loading buffer. The samples were boiled for 5 min, and 10 μl were loaded onto a 12% SDS-polyacrylamide gel. An immunoblot was performed using a rabbit anti-human apoE immune serum as described (32).

In Vitro Incubation of Human and Mouse Lipoproteins with Apo(a) Transgenic Mice Plasma—Twenty μl of transgenic mouse plasma was incubated at 37 °C in a total volume of 40 μl with the same purified lipoproteins that were used in the *in vivo* experiments. The final molar ratio of apo(a) to apoB-100 (and apo(a) to total proteins in the case of HDL) was 1 to 50. The incubations were performed for the same time intervals as the *in vivo* experiments. A single aliquot of mouse plasma was incubated in 0.15 M NaCl for 6 h at 37 °C as a negative control. Immediately after the incubation, 15 μl of 0.15 M NaCl and 5 μl of sucrose dye were added to a 5-μl aliquot from each time point and 10 μl were subjected to electrophoresis on a 4% nondenaturing polyacrylamide gel, followed by immunoblot analysis using IgG-1A[2].

Incubation of h-LDL with Plasma from Apo(a) Transgenic mice in the Presence of ε-Aminocaproic Acid—Transgenic mouse plasma (20 μl) was incubated with h-LDL for 30 min in the absence or presence of 5, 20, 50, 100, 200, and 500 mM ε-aminocaproic acid. After incubation, the samples were size-fractionated on 4% nondenaturing polyacrylamide gels and immunoblotting was performed as previously described. To determine if ε-aminocaproic acid could disrupt the apo(a)·LDL complex formed *in vitro* or *in vivo*, 20 μl of post-injection mouse plasma containing the apo(a)·LDL complex was adjusted to a final concentration of 50, 200, 500 mM ε-aminocaproic acid in 0.15 M NaCl and incubated for 15 min at 22 °C. Aliquots (10 μl) were combined with 10 μl of 0.15 M NaCl and 5 μl of sucrose dye. Six μl were loaded onto a 4% nondenaturing polyacrylamide gel and immunoblotted with IgG-IA[2].

Stability of the Apo(a)·LDL Complex in Presence of Denaturant and Chaotropic Agents—A 5-μl aliquot of the mouse plasma containing the apo(a)·LDL complex obtained *in vivo* and *in vitro* was added to 50 μl of sample buffer containing 15% (w/v) SDS, 6 M urea, 0.2 M Tris-HCl, pH 6.8, in presence or absence of 30% (v/v) ethylene glycol. The samples were boiled for 5 min, and 5 μl were loaded onto a 5% SDS-polyacrylamide gel and blotted with the IgG-IA[2]. Duplicate samples were prepared adding 5% (v/v) β-ME prior to electrophoresis.

RESULTS

A total of 1762 eggs were microinjected with the apo(a) fusion genes and transferred into pseudopregnant females. Among 181 offspring, 61 contained the transgene as determined by dot hybridization of DNA obtained from tail homogenates. A sensitive ELISA immunoassay was used to measure plasma levels of Lp(a) in these mice (11, 24). The assay was standardized using purified human Lp(a) and the values are expressed as Lp(a) total mass. Of the 61 transgenic mice, only one of 21 pTF1a17 mice expressed the apo(a) transgene and had a plasma Lp(a) concentration of 9 mg/dl,

[2] R. E. Hammer, unpublished observations.

which corresponds to an apo(a) mass of ~1.5 mg/dl. This founder mouse, which contained the apo(a) transgene under the control of the transferrin promoter (Fig. 1), was mated, and a line was established. The heterozygous progeny of this founder had plasma Lp(a) levels that ranged from 6 to 15 mg/dl with a mean of 8.5 mg/dl.

To determine the tissue distribution of apo(a) expression, RNA blot analysis was performed using an apo(a)-specific probe (Fig. 2, *panel A*). Apo(a) mRNA was detectable in all tissues analyzed. When the same blot was stripped and hybridized with a mouse transferrin probe, expression of mouse transferrin was limited to the liver, brain, testis (visible with longer exposure), and white fat. Previously, this 3.0-kb fragment of transferrin 5′-flanking DNA has been shown to faithfully direct expression of the human growth hormone gene (19) and the human low density lipoprotein receptor gene in transgenic mice (33). The loss of the normal hierarchy of transferrin expression in this line of mice may be due to positional effects.

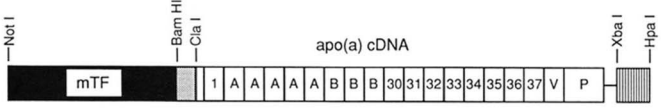

FIG. 1. **Human apo(a) expression construct.** The expression vector (pTF1a17) contains the mouse transferrin enhancer/promoter (*black box*), an immunoglobulin intron (*stippled box*), and the transcription termination signal from SV40 (*striped box*). The recombinant apo(a) cDNA includes 25 nucleotides of the 5′-untranslated region, signal sequence, five copies of the common kringle 4-like domain (the so-called "A" repeat), three copies of the "B" repeat (which differ from the "A" repeat by 3 base pairs), all the unique kringle 4-like domains (kringles 1 and 30–37), kringle 5, the protease-like domain, and part of the 3′-untranslated region.

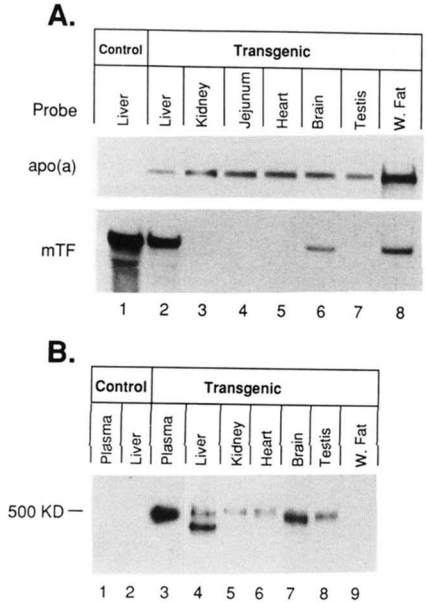

FIG. 2. **Tissue distribution of apo(a) mRNA and protein expression.** *Panel A*, 10 μg of total RNA were extracted from various tissues as described (27) and size-fractionated on a 1.5% agarose glyoxal gel. The RNA was transferred to a nylon membrane, hybridized with an apo(a)-specific probe, a ^{32}P-labeled 2.7-kb *Sma*I fragment from pRK5ha17 (> 10^8 cpm/μg) (21), then stripped and rehybridized with a ^{32}P-labeled 3.1-kb *Eco*RI mouse transferrin cDNA probe. *Panel B*, 48 μg of total protein from tissue homogenate were loaded onto a 5% SDS-polyacrylamide gel under reducing conditions, transferred to a nitrocellulose membrane and blotted with 20 μg/ml of a human apo(a)-specific monoclonal antibody, IgG-1A^2, conjugated to horseradish peroxidase (25). The membrane was exposed to XAR-5 film for 3 min.

The distribution of apo(a) protein expression was assessed by immunoblot analysis of solubilized tissue samples using an apo(a)-specific monoclonal antibody, IgG-1A^2, and the results are shown in Fig. 2 (*panel B*). Immunoreactive material was identified in all tissues examined (visible with longer exposure). A single immunoreactive band with an apparent mass of ~500 kDa was observed in the plasma of the transgenic mice (*lane 3*). In contrast, there were two proteins visible in the tissue samples; the larger protein (~500 kDa) was of identical size to that seen in plasma, whereas the smaller protein (~300 kDa) was most prominent in the liver and not present in the plasma. The same apo(a) cDNA has been expressed in a human kidney cell line (293 cells), and two proteins of the same size were identified (21). Pulse-chase experiments performed in the transfected 293 cells indicated that the 300-kDa protein was a precursor of the mature, 500-kDa form (21), with the size difference presumably due to glycosylation. To determine the contribution of each tissue to apo(a) expression, densitometry was performed and the relative amount of tissue-specific expression was determined after correction for tissue weight. The liver was the major site of apo(a) synthesis and produced 15 times more apo(a) protein than the brain and 40 times more than kidney.

To determine whether the apo(a) in the mouse plasma was incorporated into a lipoprotein particle, total lipoproteins were isolated by ultracentrifugal flotation at a density of 1.215 g/ml. Aliquots of total plasma and the top ($d < 1.215$ g/ml) and bottom ($d > 1.215$ g/ml) fractions were subjected to electrophoresis on a 5% SDS-polyacrylamide gel under reducing conditions, and immunoblotting was performed using the apo(a)-specific antibody (Fig. 3, *top panel*). In human plasma, all of the immunoreactive material was found in the top fraction (*i.e.* associated with lipoproteins), whereas in the mouse plasma all the immunoreactive material was in the bottom fraction. These results suggest that the apo(a) in the transgenic mice circulates free in plasma.

To ensure that the high salt buffers and/or ultracentrifugation procedure used to isolate the lipoproteins had not disrupted the association of apo(a) with mouse lipoproteins, aliquots of total mouse plasma and the top and bottom fractions were subjected to electrophoresis on a 4% nondenaturing polyacrylamide gel (Fig. 3, *bottom panel*). The apo(a) immu-

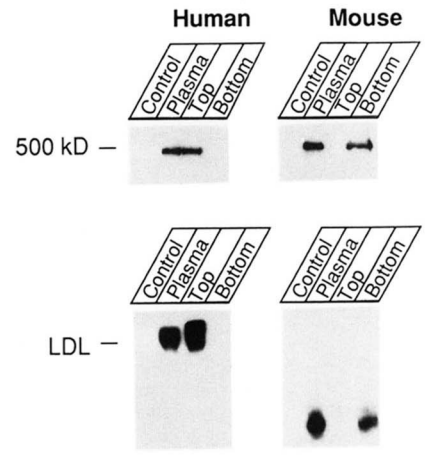

FIG. 3. **Apo(a) distribution in transgenic mouse plasma.** A total of 100 μl of plasma from humans and from transgenic mice were subjected to ultracentrifugation at a density of 1.215 g/ml. Aliquots (2 μl) of total plasma, the top fractions, and the bottom fractions were electrophoresed on a 5% SDS-polyacrylamide gel (*top panel*) or a 4% nondenaturing gel (*bottom panel*). After electrophoretic transfer, the proteins were immunoblotted using 20 μg/ml IgG-1A^2 conjugated to horseradish peroxidase as described under "Experimental Procedures." The membrane was exposed to XAR-5 film for 1 min.

noreactive material in human plasma migrated to a position that was identical to the position of LDL. In mouse plasma, the apo(a) migrated further into the gel, indicating that it was not associated with LDL. The immunoreactive material in the bottom fraction had an electrophoretic mobility identical to that of the apo(a) in mouse total plasma. Based on these studies, it was concluded that the bulk of the apo(a) protein is not tightly associated with lipoproteins in the plasma of transgenic mice.

To determine whether the apo(a) synthesized by the mouse was capable of binding to LDL, human (h-)LDL was injected into the external jugular vein of three transgenic mice and blood was collected by retro-orbital puncture at various times after the injection. The plasma was loaded onto a 4% nondenaturing polyacrylamide gel, and immunoblotting was performed with IgG-1A[2] (Fig. 4*A*). Within 1 min after the infusion of h-LDL, all of the apo(a)-reactive material shifted to a slower migrating form, reflecting the association of the free apo(a) with the infused lipoproteins. After 3 and 6 h, traces of free apo(a) were again detected (*lanes 5* and *6*). Previous metabolic studies have shown that h-LDL has a half-life of only 5 h in mice (34). Therefore, at the 3–6-h time points, much of the injected h-LDL has presumably been metabolized. When h-HDL (Fig. 4*C*) was injected there was no rapid change in the electrophoretic mobility of the apo(a)-immunoreactive material. After 3–6 h, a small portion of apo(a) showed a shift upward in its mobility. The presence of this slower migrating band at the later time points was reproducible, but the reason for its appearance is not known. When the same experiment was performed with h-VLDL, the apo(a) associated with the lipoproteins, but in a delayed fashion when compared with LDL (Fig. 5*A*).

The human lipoprotein fractions used in the *in vivo* experiments were also incubated with transgenic mouse plasma *in vitro* at 37 °C and then examined on nondenaturing gels (Fig. 4, *panels B* and *D*, and Fig. 5*B*). When mouse plasma was incubated with purified human LDL or HDL, the results were similar to those obtained *in vivo*, *i.e.* apo(a) associated with h-LDL, but not h-HDL. However, when the experiment was performed with h-VLDL, less apo(a) associated with VLDL *in vitro* (Fig. 5*B*) than had been observed *in vivo* (Fig. 5*A*). In the *in vitro* experiment (Fig. 5*B*), only 10% of the apo(a) was associated with lipoproteins after a 6-h incubation, in contrast to the *in vivo* experiment (Fig. 5*A*) in which 90% of the apo(a) was complexed with lipoproteins. In all four *in vitro* experiments using h-VLDL, there was a relative increase in association of apo(a) with lipoproteins with time, but the proportion of apo(a) that associated was never greater than 10%. The association of apo(a) with lipoproteins at the later time points may be due to *in vitro* chemical or enzymatic modification of the VLDL which exposes apo(a) binding sites on the lipoprotein. The difference in the association of apo(a) with VLDL *in vivo* and *in vitro* suggests that apo(a) preferentially associates with a metabolic product of VLDL, rather than with VLDL itself.

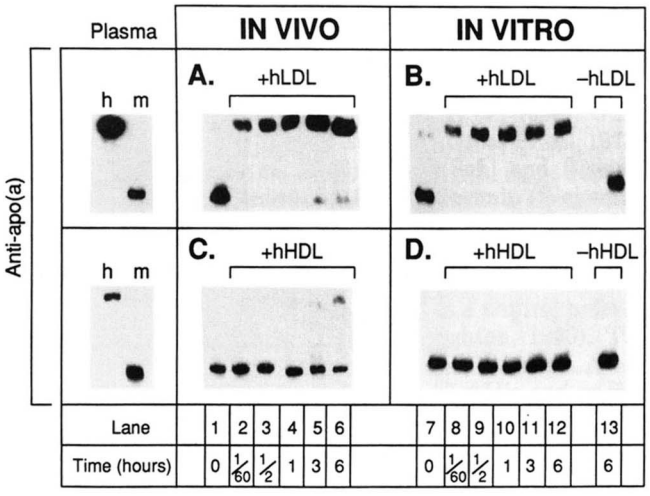

FIG. 4. ***In vivo* injection and *in vitro* incubation of human LDL and HDL in transgenic mouse plasma.** The lipoproteins were separated by ultracentrifugation and dialyzed against 0.15 M NaCl. A total of 0.65 mg of LDL or HDL (total protein) was injected into three transgenic mice and plasma was collected after 1 min, 30 min, 1 h, 3 h, and 6 h (*panels A* and *C*). Fifteen μg of the same lipoproteins were incubated at 37 °C with 20 μl of mice plasma for the indicated times (*panels B* and *D*). The samples were electrophoresed on a 4% nondenaturing gel and immunoblotted with the IgG-1A[2] as described under "Experimental Procedures." The filter was exposed to XAR-5 film for 2 min.

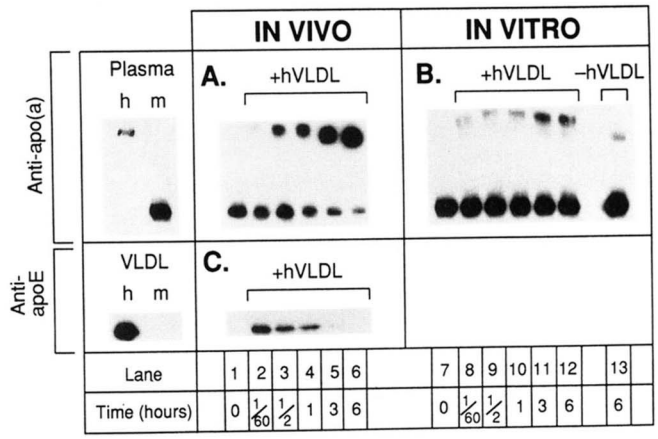

FIG. 5. ***In vivo* injection and *in vitro* incubation of human VLDL in transgenic mouse plasma.** A total of 1.95 mg of human VLDL (total protein) was injected into three transgenic mice, and plasma samples were obtained by retro-orbital puncture at the indicated times. The samples from each time point were pooled, and 5-μl aliquots were size-fractionated on a 4% nondenaturing gel and immunoblotted with IgG-1A[2] (*panel A*). A 100-μl aliquot from each time point was subjected to ultracentrifugation at a density of 1.215 g/ml, and 2 μl of the top fraction were loaded onto a 12% SDS-polyacrylamide gel. Immunoblotting was performed by incubating the filter with a rabbit anti-human apo E antiserum (1:1000 dilution) and ^{125}I-labeled goat anti-rabbit antibody (10^6 cpm/ml) (*panel C*). A total of 45 μg of human VLDL (total protein) was incubated *in vitro* with 20 μl of mouse plasma at 37 °C. The samples were loaded onto a 4% nondenaturing minigel and immunoblotted with 20 μg/ml IgG-1A[2] (*panel B*). All filters were exposed to XAR-5 film for 2 min.

VLDL secreted from the liver has multiple copies of apolipoprotein E, as well as a single copy of apoB-100. As the particle undergoes lipolysis, the apoE proteins are transferred to other lipoproteins, and the particle is converted to LDL. Therefore, the metabolism of the injected h-VLDL to h-LDL can be monitored by following the disappearance of the human apoE. At each time point after the infusion of VLDL, the lipoproteins were isolated by ultracentrifugation at a density of 1.215 g/ml and an aliquot was denatured and size-fractionated on a 12% SDS-polyacrylamide gel. An immunoblot was performed using a polyclonal rabbit anti-human apoE immune serum that does not cross-react with mouse apoE (Fig. 5*C*). The amount of human apoE in the mouse plasma declined at the same time as the amount of apo(a) associated with lipoproteins increased. This observation is consistent with the notion that large amounts of apo(a) bound to the h-VLDL lipoprotein only when it had lost its apoE and had been converted to LDL.

Approximately 70% of plasma cholesterol is transported as LDL in humans, but in mice the major cholesterol-carrying lipoprotein is HDL (35). Mice have low levels of plasma LDL

(35). To determine if the lack of association of the human apo(a) with mouse lipoproteins in the apo(a) transgenic mice was due to the low concentration of circulating mouse LDL, excess amounts of purified mouse LDL were injected (Fig. 6). When mouse LDL was infused into the transgenic animals, there was no association of apo(a) with lipoproteins (Fig. 6*A*). When mouse LDL was incubated *in vitro* with mouse plasma, only a small amount of apo(a) bound to mouse LDL (Fig. 6*B*). We conclude that the lack of association between human apo(a) and mouse lipoproteins in the transgenic mouse is due in part to structural differences between human and murine LDL.

Two different experiments were performed to evaluate if the stability of the apo(a)·LDL complex obtained *in vivo* and *in vitro* was comparable to that of native human Lp(a). First, increasing concentrations of ϵ-aminocaproic acid were added during the *in vitro* incubation of purified h-LDL and the apo(a)-containing mouse plasma. ϵ-Aminocaproic acid is a lysine analogue that interferes with the binding of plasminogen and apo(a) to lysine residues (36). At a concentration of 100 mM, ϵ-aminocaproic acid completely inhibited the association of apo(a) with h-LDL (Fig. 7*A*). When ϵ-aminocaproic acid was added in the identical concentrations to apo(a)·LDL complexes made *in vivo*, there was no disruption in the association of apo(a) with the lipoprotein (Fig. 7*B*). An identical result was obtained when the apo(a)·LDL complex was reconstituted *in vitro* (data not shown).

Second, the stability of the reconstituted apo(a)·LDL complex was evaluated by boiling the sample for 5 min in 15% (w/v) SDS and 6 M urea in the presence or absence of the chaotropic agent, ethylene glycol. The reaction was carried out in the presence of 500 mM ϵ-aminocaproic acid to ensure that if any apo(a) dissociated from LDL, it could not reassociate with the lipoprotein prior to analysis by gel electrophoresis. The samples were loaded onto a 5% SDS-polyacrylamide gel, and immunoblotted with IgG-IA^2. The results of this analysis using human plasma, as well as LDL-apo(a) reconstituted *in vivo* or *in vitro*, are shown in Fig. 8. The apo(a) remained associated with LDL in the Lp(a) reconstituted both *in vivo* and *in vitro* (*panel A*) in the absence (*lanes 3* and *4*) or presence (*lanes 7* and *8*) of 30% (v/v) ethylene glycol. As expected, in the presence of 5% (v/v) β-ME, the apo(a) dissociated from the apoB-100 of LDL (*panel B*).

DISCUSSION

The major finding of this study is that expression of a human apo(a) transgene in mice results in synthesis and secretion of apo(a) into plasma but failure of the apo(a) to properly associate with the mouse lipoproteins. Infusion of human LDL into the transgenic mice results in a rapid association of apo(a) with lipoproteins. This association is selective since the apo(a) does not associate with h-HDL or m-LDL when these particles are infused into the mice. Therefore, h-apo(a) fails to associate with the mouse lipoproteins in the transgenic mice because of structural differences between mouse and human LDL, rather than to the low circulating levels of LDL in the mice.

Is the association of apo(a) with human LDL particles observed *in vivo* identical to that of native Lp(a)? This question cannot be answered definitively since the chemical linkage between the apo(a) and h-LDL in Lp(a) has not been

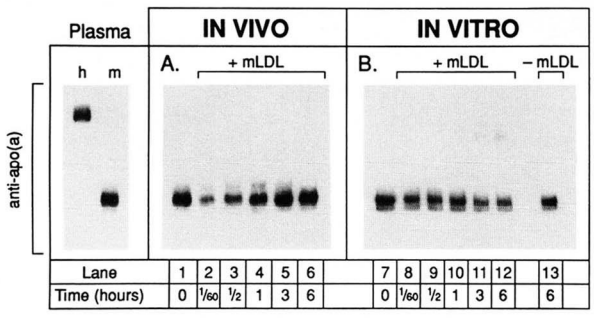

FIG. 6. ***In vivo* injection or *in vitro* incubation of mouse LDL with transgenic mouse plasma.** Mouse LDL, separated at density 1.019–1.063 g/ml, was dialyzed against 0.15 M NaCl, and 0.65 mg of total protein was infused into three transgenic mice (*panel A*) or 15 μg was incubated *in vitro* with 20 μl of transgenic mouse plasma at 37 °C (*panel B*). The samples were electrophoresed on 4% nondenaturing gels and immunoblotted using the IgG-1A^2 (20 μg/ml) as described under "Experimental Procedures."

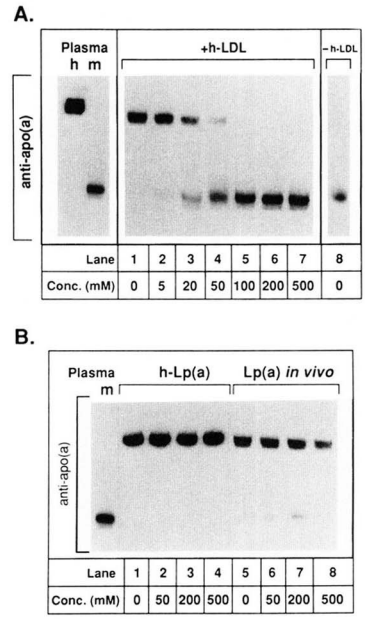

FIG. 7. **Effect of ϵ-aminocaproic acid on the association of apo(a) with h-LDL.** A total of 15 μg of purified human LDL was incubated for 30 min at 37 °C with 20 μl of transgenic mice plasma in the presence of increasing concentrations of ϵ-aminocaproic acid. The samples were electrophoresed on a 4% nondenaturing polyacrylamide gel and immunoblotted with the IgG-1A^2 (*panel A*). ϵ-Aminocaproic acid was added in different concentrations to plasma containing 2 μg of human Lp(a), or to Lp(a) reconstituted *in vivo* and *in vitro* as described under "Experimental Procedures." After 15 min at 22 °C, 0.4 μg was size-fractionated on a 4% nondenaturing polyacrylamide gel and immunoblotted using 20 μg/ml of IgG-1A^2 (*panel B*).

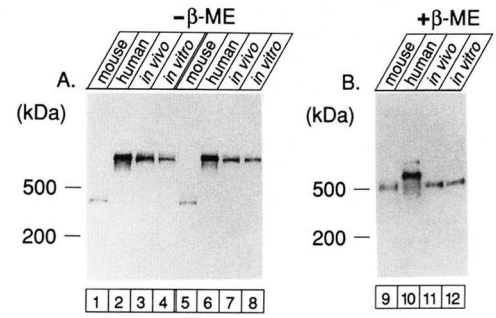

FIG. 8. **Boiling of apo(a)·LDL complex formed *in vivo* and *in vitro* in presence of SDS, urea, and ethylene glycol.** A total of 0.5 μg of human Lp(a) or apo(a)·LDL complex reconstituted *in vivo* or *in vitro* were boiled for 5 min in the presence of 15% SDS and 6 M urea with (*lanes 5–8*) or without (*lanes 1–4*) 30% (v/v) ethylene glycol (*panel A*). Identical samples were treated with the same denaturants plus 5% β-ME (*panel B*). In both experiments, 45 ng of protein were size-fractionated on 5% SDS-polyacrylamide gels and immunoblotted with IgG-1A^2 as described under "Experimental Procedures." The filters were exposed to XAR-5 film for 2 min.

fully characterized. When the Lp(a) particle is subjected to reduction using either dithiothreitol or β-ME, the apo(a) dissociates from LDL, suggesting that apo(a) is joined to apoB-100 by a disulfide linkage. However, reduction of the Lp(a) particle also disrupts the secondary structure of apo(a) by breaking the numerous disulfide bonds in the cysteine-rich kringles. Therefore, despite the fact that apo(a) dissociated from LDL upon reduction, this dissociation is not proof of a disulfide linkage between apo(a) and apoB.

We have only limited information at present as to the molecular nature of the association of apo(a) with LDL. Preliminary studies to characterize the human LDL·apo(a) complex formed *in vivo* and *in vitro* suggest that Lp(a) has been successfully reconstituted. The fact that 1) ε-aminocaproic acid can prevent the association of apo(a) with LDL, but not induce its dissociation, and 2) the reconstituted particle can withstand boiling in 6 M urea suggests that there is a covalent linkage formed between apo(a) and apoB-100.

The results of these experiments are consistent with there being a disulfide linkage between apo(a) and apoB-100. The exact residues involved in the putative disulfide bond have not been pinpointed. Only kringle 36, the penultimate kringle 4 repeat in the apo(a) gene, contains a cysteine residue that is not involved in a intra-kringle disulfide bond, so this is the presumed cysteine residue in apo(a) that binds to apoB-100 (20). The site of the cysteine residue in apoB-100 to which apo(a) binds has not been localized specifically. Unfortunately, the mouse apoB-100 gene has not been fully characterized, so the sequence differences responsible for this observed lack of association of apo(a) with mouse apoB-100 cannot be pinpointed (37).

In contrast to the rapid association of apo(a) with injected h-LDL, there was a delayed association of the apo(a) with injected VLDL. This delay was inversely related to the rate of disappearance of human apoE from the plasma. This observation suggests that apo(a) preferentially associated with a metabolic product of VLDL (*i.e.* LDL) rather than VLDL itself. In support of this hypothesis was the observation that *in vitro* incubation of mouse plasma with purified human LDL resulted in a rapid association of apo(a) with LDL, whereas with VLDL there was only a partial association of apo(a) with lipoproteins. The weaker association of apo(a) with VLDL may in part be attributed to the size of the apo(a) isoform used in these experiments. Prior studies have demonstrated that fat feeding in humans results in approximately 5% of apo(a) being associated with VLDL and chylomicrons (38). The larger apo(a) isoforms (*i.e.* >500,000 kDa) appear to preferentially associate with these triglyceride-rich particles. In the current study, a relatively small apo(a) isoform (~500,000 kDa) was used, which may contribute to the observed decreased association of apo(a) with VLDL *in vivo* and *in vitro*.

Finally, in humans, does apo(a) attach to apoB-100 in the hepatocyte or in the plasma? It has been demonstrated that disulfide bonds between other apolipoproteins can form in plasma, *e.g.* between apoE3 and apoAII (39). Our results suggest that human apo(a) may be secreted into plasma free of lipoproteins and associate with LDL in the circulation. A conclusive demonstration that the apo(a) joins LDL in plasma, rather than in the hepatocyte, awaits the development of a transgenic mouse expressing both h-apo(a) and h-apoB100 genes.

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