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Research Article

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Lipoprotein(a) Vascular Accumulation in Mice

In Vivo Analysis of the Role of Lysine Binding Sites Using Recombinant Adenovirus

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Abstract

Although the mechanism by which lipoprotein(a) [Lp(a)] contributes to vascular disease remains unclear, consequences of its binding to the vessel surface are commonly cited in postulated atherogenic pathways. Because of the presence of plasminogen-like lysine binding sites (LBS) in apo(a), fibrin binding has been proposed to play an important role in Lp(a)'s vascular accumulation. Indeed, LBS are known to facilitate Lp(a) fibrin binding *in vitro*. To examine the importance of apo(a) LBS in Lp(a) vascular accumulation *in vivo*, we generated three different apo(a) cDNAs: (a) mini apo(a), based on wild-type human apo(a); (b) mini apo(a) containing a naturally occurring LBS defect associated with a point mutation in kringle 4-10; and (c) human-rhesus monkey chimeric mini apo(a), which contains the same LBS defect in the context of several additional changes. Recombinant adenovirus vectors were constructed with the various apo(a) cDNAs and injected into human apoB transgenic mice. At the viral dosage used in these experiments, all three forms of apo(a) were found exclusively within the lipoprotein fractions, and peak Lp(a) plasma levels were nearly identical (~45 mg/dl). *In vitro* analysis of Lp(a) isolated from the various groups of mice confirmed that putative LBS defective apo(a) yielded Lp(a) unable to bind lysine-Sepharose. Quantitation of *in vivo* Lp(a) vascular accumulation in mice treated with the various adenovirus vectors revealed significantly less accumulation of both types of LBS defective Lp(a), relative to wild-type Lp(a). These results indicate a correlation between lysine binding properties of Lp(a) and vascular accumulation, supporting the postulated role of apo(a) LBS in this potentially atherogenic characteristic of Lp(a). (*J. Clin. Invest.* 1997. 100:1493–1500.) Key words: lipoprotein(a), analysis • atherosclerosis, physiopathology • gene transfer • transgenic mice

Introduction

Lipoprotein(a) [Lp(a)]¹ is a macromolecular complex found in human plasma that is structurally distinct from low density lipoproteins (LDL) by addition of a single protein, apolipoprotein(a) [apo(a)]. This hydrophilic glycoprotein is associated with the apolipoprotein B (apoB) moiety of LDL through a disulfide bond joining the carboxyl-terminal region of both molecules (1, 2). Numerous prospective and retrospective studies have correlated the presence of high (> 30 mg/dl) Lp(a) plasma levels with the incidence of atherosclerosis, although a smaller, but not insignificant, number of clinical studies have found no evidence of such a correlation (for review see references 3 and 4). These discrepancies may be explained in part by the considerable heterogeneity of Lp(a) composition within the human population primarily attributable to polymorphism within the apo(a) gene. However, without a clear understanding of the mechanism by which Lp(a) contributes to vascular disease, it is not possible to identify apo(a) alleles that may contribute to atherogenic risk.

A characteristic of Lp(a) central to nearly all of the hypotheses accounting for its atherogenic properties is its propensity for accumulation in the vessel wall. There is very little controversy regarding this aspect of Lp(a), given the extensive evidence from direct observation and *in vitro* demonstrations of its affinity for vessel wall components. Immunochemical analyses of atherosclerotic lesions in human arterial samples and bypass grafts have demonstrated marked accumulation of Lp(a), correlating with plasma levels of the donor (5–7). In the absence of atherosclerotic lesions, high-level deposition of Lp(a) has been observed in areas of vascular inflammation (8). Several studies in which animal models of atherosclerosis were infused with human-derived Lp(a) have yielded similar findings. For example, binding analysis of Lp(a) and LDL infused into c57BL/6 mice demonstrated enhanced deposition of Lp(a) on arterial surfaces relative to LDL, especially in areas prone to lesion development (9). Preferential accumulation of Lp(a) at sites of vessel injury and lipid accumulation has also been demonstrated in rabbits (10).

There are numerous potential mechanisms for accumulation of Lp(a) in the vessel wall that can be attributed to both the well-documented cellular interactions of its lipid moiety (LDL) or through binding of its unique glycoprotein constituent to vascular cells and matrix components. In light of several important findings concerning the properties of apo(a), however, it is the latter interaction that has come to be the focus of much investigation into Lp(a) pathophysiology. In this regard, the most important new insight came from the cloning and sequencing of the apo(a) gene, which revealed a high degree of homology between apo(a) and plasminogen (PMG) (11), a protease-containing multiple high affinity lysine/fibrin binding

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1. Abbreviations used in this paper: B-Gal, B-Galactosidase; e-ACA, ε-aminocaproic acid; huB tg mice, mice expressing the human apo B transgene; LBS, lysine binding site; Lp(a), lipoprotein(a); Lp(a)WT, Lp(a) containing wild-type mini apo(a); PMG, plasminogen.

sites (LBS). Most notably, both apo(a) and PMG contain triple-loop structural motifs known as kringles. PMG contains five unique krigle domains, while apo(a) contains 11 distinct krigle types. All but one of these krigle types are highly homologous to PMG krigle 4 [referred to as apo(a) kringles 4-1 through 4-10; see Fig. 1], and the final krigle domain resembles PMG krigle 5. Apo(a) also contains a protease domain similar to that of PMG, although it does not exhibit significant proteolytic activity and cannot be activated since the zymogen cleavage site is nonfunctional (11). Based on these features of apo(a), it has been postulated that Lp(a) may function as a pro-atherogenic molecule by competitive inhibition of PMG binding and activation on fibrin surfaces, disrupting important regulatory processes dependent on plasmin activity. Consistent with this, a number of *in vitro* studies have demonstrated that Lp(a) does compete with PMG for binding to fibrin, and reduces the generation of active plasmin (12–14). Potentially atherogenic consequences of reduced plasmin activity include a reduction in clot lysis and plasmin-dependent activation of TGF- β , a cytokine involved in the maintenance of normal phenotype and function of endothelial and smooth muscle cells in the vessel wall. Studies carried out using apo(a) transgenic mice have given support to these predictions, demonstrating increased formation of fatty streak lesions (15), reduced levels of plasmin and TGF- β (16, 17), and diminished clot lysis (18).

Based on the presence of multiple PMG krigle 4-like domains in apo(a), the PMG inhibition hypothesis assumes that one or more of these have retained a functional LBS. PMG krigle 4 contains one of the two high affinity LBS found in PMG, both of which have been extensively characterized at the structural level. Based on the crystal structure of PMG krigle 4, seven amino acid residues contribute to the LBS (19). Comparison of these seven critical residues of the PMG krigle 4 LBS to those present in each of the 10 unique krigle 4 homologues of human apo(a) revealed that krigle 4-10 retains a nearly identical site, with only a single conservative substitution (Lys35 to Arg35). The importance of the krigle 4-10 LBS has received considerable support from the identification of a lysine binding defective form of human Lp(a) that had a nonconservative Trp to Arg (W > R) substitution in apo(a) occurring at residue 72 of krigle 4-10 (20). This same substitution is also present in lysine binding defective rhesus monkey apo(a) (21, 22). Combined with the structural evidence, these findings in human apo(a) variants and rhesus apo(a) strongly implicate the krigle 4-10 LBS in the binding of Lp(a) to lysine-Sepharose. The binding of Lp(a) to fibrin, however, may not be entirely mediated by the krigle 4-10 LBS, as demonstrated in the recent study of Klezovitch et al. (23). In this study, Lp(a) and apo(a) containing the krigle 4-10 LBS defect bound to fibrin with affinity similar to that of wild-type Lp(a), suggesting that this interaction is dependent on additional binding sites within apo(a). The location of additional LBS(s) remains to be firmly established, although several lines of evidence suggest they may reside within kringles 4-5 through 4-8 (24–29). LBS(s) within these kringles are thought to play a role in assembly of the Lp(a) particle by promoting noncovalent interactions with apoB-100, but they appear to be masked within the Lp(a) particle, unavailable for interaction with lysine-Sepharose (25, 28).

In this study, we have evaluated the importance of Lp(a)'s lysine interaction in determining its vascular accumulation. This interaction was explored by expressing wild-type apo(a) and lysine binding-defective forms of apo(a) in mice using ad-

enovirus mediated gene transfer. Unlike most previous studies of apo(a) in transgenic mice, apo(a) was expressed in human apoB transgenic mice in order to study the properties of the *in vivo* assembled Lp(a). We examined *in vitro* lysine interactions of the various forms of Lp(a), and measured corresponding *in vivo* Lp(a) vascular accumulation. The results of this study suggest that Lp(a)'s propensity for vascular accumulation is closely related to its lysine binding properties, binding that is conferred to a large degree by the krigle 4-10 LBS.

Methods

Plasmids and oligonucleotides. Plasmid pCMha8 contains apo(a) cDNA encoding the secretion signal sequence, kringles 4-1 and 4-2, a fusion krigle combining the first 101 bp of the second krigle 4-2 repeat (4-2₂) with 241 bp of krigle 4-5 (aka 4-32; numbering based on reference 11), kringles 4-6 through 4-10, krigle 5-like domain, and the protease domain. This miniaturized apo(a) cDNA [mini apo(a)] provided the wild-type comparison in this study, and the lysine binding-defective cDNA constructs described below were based on this design. Plasmid pLAL.RSV contains the left terminus of adenovirus type 5 (0–1.3 map units) followed by the RSV promoter, a polycloning site, and the bovine growth hormone terminator.

Oligonucleotides (GENSET Corp., La Jolla, CA) used for PCR, sequencing, and reverse transcription: aK37F - 5' GTAATGGCCAGAGTTATCGAGG 3', aK37R - 5' ACTCTTTCCCATTTCCCAAAC 3', AMG10-3 - 5' CCCAGCATCCGCCCGCAGTACTGC 3', RhaF2 - 5' CCATGGCTGTTTCTGAACAAGCAC 3', RhaR2 - 5' GTGCTTGTTACAGAAACAGCCATGG 3', RhaF3 - 5' GAAACCCCCGGGGTACAGGAGTGCTACTACCAT 3', RhaRT - 5' CCACTTGAGGCTTCCACAATCAAATGAAGAGG 3', RAD-1 - 5' TCACTTAGTGAGCTCGTCGAG 3'.

Site-directed mutagenesis. Mutations were introduced into the apo(a) cDNA using a recombinant PCR technique. A portion of the apo(a) cDNA (Alu I–Avr II) was subcloned into pLAL.RSV, and a short PCR product was generated between the mutagenic primer AMG 10-3 and RAD-1. (PCR reaction contained 40 ng of the target DNA, 0.1 nmol each primer, 0.2-mM dNTP, 0.5-U Taq polymerase, 1.25-U pfu polymerase in manufacturer-supplied buffer for pfu polymerase [Boehringer Mannheim Biochemicals, Indianapolis, IN]; cycling: 94°C for 30 s, 52°C for 30 s, followed by 72°C for 90 s—30 cycles). This PCR product was gel-purified and used as a megaprimer in a second PCR reaction containing pCMha8 as target DNA (no RAD-1 sequence) with primers 37F and RAD-1 (conditions as above, but with 5 ng of megaprimer and 58°C annealing temperature). This product containing the mutation was then reinserted into the apo(a) cDNA using restriction sites MscI and AvrII. The DNA sequence of the mutation, as well as the surrounding PCR-generated region, was confirmed by sequencing.

Cloning rhesus apo(a) kringles 4-5 through 4-10. RNA was extracted from rhesus monkey liver (California Regional Primate Center, Davis, CA) using RNAsat-60 (Tel-Test B, Friendswood, TX), subjected to DNase treatment, then reverse transcribed (3- μ g RNA incubated with 15-ng RhaRT in a volume of 12 μ l at 65°C for 10 min, then supplemented with Superscript II/buffer (Gibco BRL, Gaithersburg, MD) and incubated for 1 h at 42°C). The first strand cDNA was then treated with RNase, extracted with phenol/chloroform, and purified by passage through Chromospin-100 columns (Clontech, Palo Alto, CA). This product was then subjected to PCR with RhaRT and RhaF3 to amplify the segment of rhesus apo(a) cDNA between krigle 4-5 and krigle 4-10. 5 μ l of the RT product was subjected to PCR as above (cycling: 94°C for 30 s, 60°C for 30 s, followed by 72°C for 120 s—30 cycles).

pLAL.RSV-RHa was assembled by insertion of a HindIII–EarI fragment of rhesus apo(a) PCR product into analogous sites present in mini apo(a) cDNA, creating the same krigle 4-2/4-5 fusion present in mini apo(a).

Adenovirus expression constructs. Ad.RSV-mini apo(a), a recombinant adenoviral vector for expression of mini apo(a) was constructed by subcloning the mini apo(a) cDNA (from pCMha8) into vector pLAL.RSV by ligation of a 3.3-kb XhoI–KpnI fragment and a 0.4-kb KpnI–PstI fragment to produce pLAL.RSVmini apo(a). This plasmid was linearized at a unique SpeI site immediately after the terminator sequence and ligated to XbaI restricted adenovirus DNA (Ad5Pacl, a variant of adenovirus type 5 having both E1 and E3 region deletions [30], kindly provided by Dr. F. Graham, McMaster University, Hamilton, Ontario, Canada). Ligation products were transfected into 293 cells using Lipofectamine (Gibco BRL), and the resulting plaques were screened for apo(a) sequences using PCR. Ad.RSVapo(a)4-10m and Ad.RSVapo(a)RH were produced from their respective pLAL.RSV constructs by the same procedure. Large scale preparations of plaque-purified Ad.RSVmini apo(a) were produced in 293 cells and titered as described previously (31).

Mouse procedures and virus administration. All animal experimental protocols were submitted and received approval from the Lawrence Berkeley Laboratory Animal Welfare Committee. Human apoB transgenic mice have been described previously (32). The founder line used for these studies expresses high levels of human apoB with an average plasma level of 72 mg/dl. All mice were fed Purina mouse chow (#5001). Under these conditions, the mice do not develop Oil Red O–staining lesions by 16 wk of age (33). Female apoB transgenic mice (12–16 wk of age) were injected with apo(a) adenovirus titers ranging from 1×10^8 to 8×10^8 plaque forming units per gram. Viral suspensions (in 137-mM NaCl, 10-mM Tris [pH 7.5], 1-mM MgCl₂, and 10% glycerol) were injected through the tail vein in a total volume no greater than 0.15 ml.

Lp(a) measurement and lipoprotein isolation. Blood was collected from mice by tail venipuncture, plasma was separated by centrifugation and supplemented with Complete protease inhibitor cocktail (Boehringer Mannheim Biochemicals). Immunoblotting was performed as described previously (31), using a polyclonal goat anti-Lp(a) antiserum which lacks cross-reactivity with PMG (Biodesign International, Kennebunk, ME) diluted 1:4000. Antibody binding was detected using alkaline phosphatase conjugated anti-goat IgG (Sigma Chemical Co., St. Louis, MO) diluted 1:5000, followed by BCIP/NBT (Bio-Rad Laboratories, Hercules, CA).

For Lp(a) and apo(a) determinations, plasma samples were subjected to density gradient ultracentrifugation as described (32), to obtain the total lipoprotein fractions ($d < 1.21$ g/ml) and lipid-free protein fraction. Lp(a) was measured in mouse plasma and lipoprotein fractions by a sandwich ELISA assay. Purified IgG prepared from goat polyclonal antiserum to human apo(a) (International Immunology Corp., Murrieta, CA) was bound to the microtiter plate well as a capture antibody. A horseradish peroxidase conjugate of the same antibody was used for detection of apo(a) after addition of the chromogenic substrate *o*-Phenylene diamine. Standardization of the method was based on in-house human lipoprotein calibrators independently measured using a commercially available standardized Lp(a) ELISA kit (Strategic Diagnostics, Newark, DE). Standard curves were calculated with appropriate blanks and controls using Logit-Log data transformation. All reported Lp(a) concentrations were calculated from triplicate analysis.

Lysine-Sepharose chromatography. Lipoprotein fractions ($d < 1.21$) derived from 100 μ l of plasma from adenovirus-treated mice were dialyzed against Buffer A (0.15-M NaCl, 10-mM phosphate, 0.01% EDTA [pH 7.4]), then applied to a 2.5-ml lysine-Sepharose FPLC column (Sigma Chemical Co.). Fractions (50 μ l) were collected immediately after the void volume. After two column volumes of Buffer A, Buffer A containing 0.1-M ϵ -aminocaproic acid (ϵ -ACA, Sigma Chemical Co.) was applied, and a second set of fractions was collected. Fractions were assayed for the presence of Lp(a) by reducing SDS-PAGE and immunoblotting.

Preparation of aortic sections, immunostaining, and quantitation. Groups of 10 female apoB transgenic mice, 10–14 wk of age, were injected with Ad.RSVmini apo(a), Ad.RSVapo(a)4-10m, or Ad.RSV-

apo(a)RH (3×10^8 pfu/g). Plasma Lp(a) concentration was determined on day 7, and mice were killed on day 12. Two additional mice were injected with the same titer of a control virus, AD.RSV B-Gal and killed 7 days after injection. The entire heart was removed, rinsed in PBS, then frozen in OTC mounting compound. A segment of the proximal aorta was cut into 8- μ m sections for quantitative fluorescence staining procedure described previously (16). Briefly, after aortic sections were fixed in ice-cold acetone, they were incubated with Tris-buffered saline (TBS), 3% BSA for 30 min, followed by sheep anti-human polyclonal Lp(a) antibody [Immunoscientific; antiserum shown to lack cross-reactivity with plasminogen or the apoB component of Lp(a)] diluted 1:200 in TBS, 3% BSA, followed by rabbit anti-sheep IgG-FITC (Jackson ImmunoResearch Labs, Inc., West Grove, PA) diluted 1:80 in TBS, 3% BSA. All incubations were performed at room temperature. Two pairs of serial sections, separated by 350 μ m, were examined from each mouse. Imaging and quantitation were performed according to Mosedale et al. (34). Three separate fields of the vessel wall, selected randomly in phase-contrast image, were captured from each section for immunofluorescence quantitation. Sections adjacent to each pair were treated in similar fashion with omission of the primary antibody to determine background staining. This background intensity was subtracted from the mean of each section to give specific fluorescence intensity. In addition, to evaluate the frequency of vascular cell transduction by adenovirus vectors under these experimental conditions, sections of control mice injected with AD.RSV B-Gal (B-Galactosidase) were examined for B-Gal immunostaining using mouse anti-B-Gal FITC (Jackson ImmunoResearch Labs, Inc.).

Statistical difference between mean Lp(a) accumulation and mean areas of focal accumulation were calculated using the Mann-Whitney U test for nonparametric analysis.

Results

Apo(a) expression and Lp(a) formation in apoB transgenic mice injected with recombinant apo(a) adenoviruses. The packaging requirements of the adenovirus system used in this study necessitated the use of a miniaturized version of apo(a) [mini apo(a), Fig. 1]. Based on several lines of evidence from studies using molecular modeling, mutagenesis and proteolytically-derived apo(a) fragments, the deletion of NH₂-terminal kringle domains from apo(a) should not significantly impact properties of mini apo(a) relevant to this study (24–28). The two putative lysine binding defective apo(a) cDNAs were analogous to mini apo(a) in their construction, containing structural modifications reported to be associated with diminished lysine-Sepharose binding properties (20, 22). Apo(a)4-10m contains a single W–R substitution at position 72 of kringle 4-10, and apo(a)RH is a chimeric human–rhesus apo(a) with rhesus monkey apo(a) kringles 4-5 through 4-10 substituted for homologous human apo(a) kringles (Fig. 1). As in rhesus apo(a), apo(a)RH lacks a kringle 5 homologue, although this is predicted to have little impact on lysine binding. To determine if sequence differences in the three forms of apo(a) would lead to significant differences in immunoreactivity, quantitative immunoblotting was carried out using three different Lp(a) antisera to detect the three different forms of apo(a). At two fixed concentrations of Lp(a) preparations (determined by reducing SDS-PAGE and silver staining), there was no more than 5% difference in immunoreactivity between the three forms. Compared to Lp(a) isolated from transgenic mice containing the 17 kringle form of apo(a), the miniaturized forms of apo(a) exhibited \sim 50% lower immunoreactivity (data not shown).

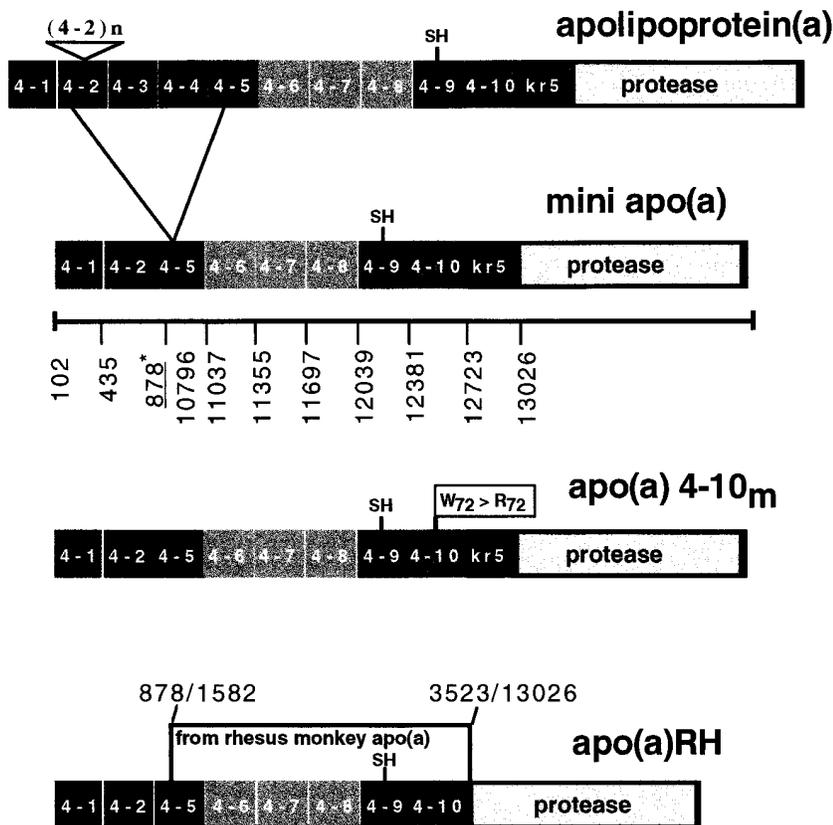


Figure 1. Schematic representation of the three apo(a) cDNAs encoded by adenovirus vectors. The structures of mini apo(a), apo(a)4-10m, and apo(a)RH are aligned with a generalized apo(a) structure with 10 unique kringle 4-like domains (designated 4-1 through 4-10; kringle 4-2 variably repeated), one kringle 5-like domain, and a protease domain. Deletion of kringles 4-3 and 4-4 and fusion of kringles 4-2₂ and 4-5 are indicated by lines between apo(a) and mini apo(a). Nucleotide numbering (according to reference 11) is given at kringle repeat junctions and at the point of fusion in kringle 4-5. In apo(a)4-10m, a single codon was altered in kringle 4-10 to reproduce the W₇₂>R₇₂ substitution. Apo(a)RH has kringles 4-5 through 4-10 derived from rhesus monkey apo(a) and lacks a kringle 5-like domain. Numbering (rhesus according to reference 21) is given at fusion points; fusion of kringle 4-2₂ and rhesus kringle 4-5 is identical to that in mini apo(a).

To determine if apo(a) produced in human apoB transgenic (huB tg) mice treated with the various recombinant adenoviral vectors was efficiently assembled into Lp(a) particles, plasma taken from the mice 7 days after injection with a range of viral titers was fractionated into total lipoproteins ($d < 1.21$) and a nonlipid fraction. The amount of apo(a) present as Lp(a) was determined by immunoblotting of these fractions for apo(a), and by measuring Lp(a) concentration in the lipoprotein fraction. As shown in Fig. 2 A, all three forms of apo(a) were detected exclusively within the lipoprotein fraction of mice injected with 3×10^8 pfu/g, while mice treated with higher titers of virus had significant levels of apo(a) in the lipid-free fraction in addition to the lipoprotein associated apo(a). Lp(a) concentrations were remarkably similar in the lipoprotein fractions of huB tg mice injected with the various adenoviral vectors, both at 3×10^8 and 6×10^8 pfu/g (Fig. 2 B).

Corresponding plasma Lp(a) levels in mice given 3×10^8 pfu/g were identical to that of the lipoprotein fraction, but in mice given higher titers, plasma levels were much greater because of the presence of free apo(a). Irrespective of apo(a) type, the concentration of Lp(a) in the lipoprotein fractions of mice was consistently limited to ~ 60 mg/dl, suggesting saturation of available human apoB occurring with very high levels of apo(a) expression. For all subsequent experiments, mice were given an optimal dosage of 3×10^8 pfu/g to obtain plasma concentrations of Lp(a) within the range of 40–50 mg/dl, while avoiding the complicating presence of free apo(a). The time course of Lp(a) in mice injected with optimal dosage of each adenoviral vector was followed up to 21 d. Lp(a) was first detectable 3 d after injection and reached peak levels around day 7. Levels declined similarly after this peak with all three vectors, but remained detectable until 14 d after injection (data not shown).

In vitro lysine-Sepharose binding properties of plasma Lp(a). To confirm the effect of apo(a) modifications on lysine binding properties, we assessed lysine binding of the three Lp(a) forms using lysine-Sepharose affinity chromatography. In this assay, lipoprotein fractions isolated from the huB tg mice treated with the three different recombinant apo(a) adenoviruses [containing ~ 60 μ g of Lp(a)] were applied to a lysine-Sepharose column, and bound Lp(a) was eluted with e-ACA, a lysine analog. As shown in Fig. 3, a significant portion of Lp(a) containing wild-type mini apo(a) [Lp(a)WT] bound to the lysine-Sepharose column and was eluted in the presence of e-ACA. Reapplication of unbound Lp(a)WT fractions to the lysine-Sepharose column revealed additional binding material in these fractions, indicating saturation of column binding sites in the primary application. In contrast, both Lp(a)RH and Lp(a)4-10m eluted from the column immediately in primary as well as secondary applications of the unbound fractions. No apo(a) immunoreactivity was detected in the e-ACA-eluted fractions. These results are consistent with previous studies documenting lysine-Sepharose binding of rhesus monkey Lp(a) (22), and human Lp(a), with and without the W₇₂>R₇₂ substitution (20).

In vivo analysis of Lp(a) vascular accumulation. To determine the effects of altered lysine binding properties on vascular accumulation of the different forms of Lp(a), accumulation was measured in huB tg mice injected with the various apo(a) recombinant adenoviruses. Mice were injected with 3×10^8 pfu/g, a titer which yielded similar 7 day plasma levels of Lp(a) with each of the three recombinant apo(a) adenoviruses. Average Lp(a) plasma levels in the three groups of mice were 42.4 ± 2.8 [Lp(a)WT], 39.8 ± 3.2 [Lp(a)4-10m], and 48.1 ± 2.5 [Lp(a)RH]. On day 12, the aortas from the three groups of

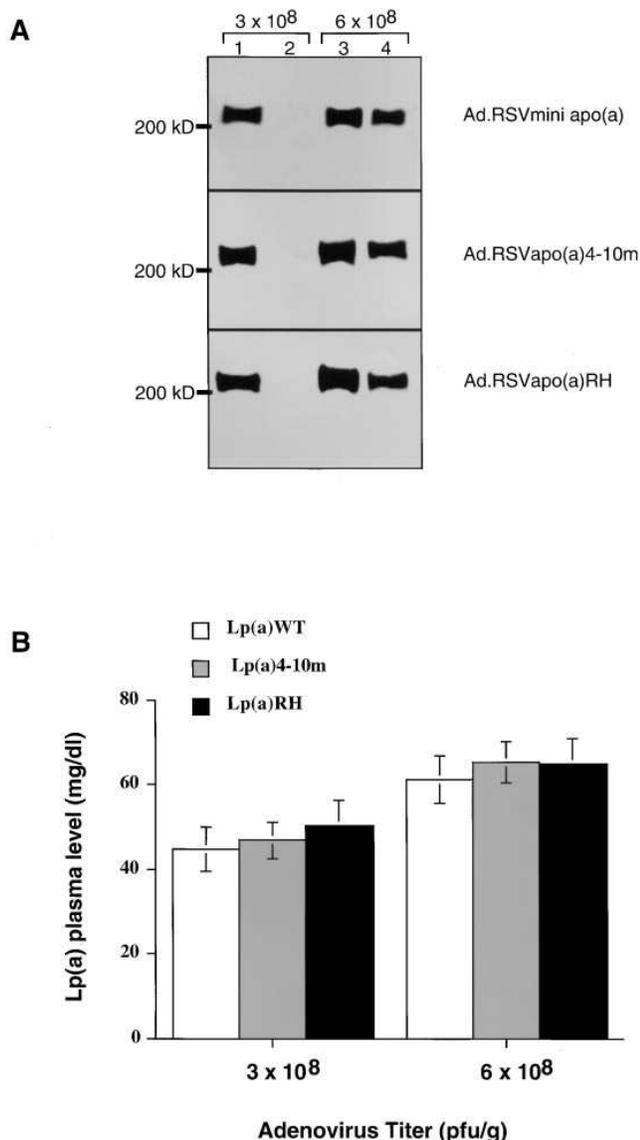


Figure 2. Expression of apo(a) and assembly of Lp(a) in huBtg mice treated with Ad.RSVmini apo(a), Ad.RSVmini apo(a)4-10m, and Ad.RSVmini apo(a)RH. (A) Mice were injected with 3×10^8 pfu/g or 6×10^8 pfu/g of indicated adenovirus vector. Lipoprotein (lanes 1 and 3) and nonlipoprotein fractions (lanes 2 and 4) from 7-day plasma samples were subjected to reducing SDS-PAGE and immunoblotting with Lp(a) antiserum. (B) Lp(a) was measured in the lipoprotein fractions ($d < 1.21$) by ELISA. Values represent the mean of concentrations in four mice \pm SEM.

mice were sectioned and examined for apo(a) immunostaining. A representative image for each of the groups is shown in Fig. 4 A, along with one image from a control mouse injected with Ad.RSV B-Gal. Using a quantitative imaging technique (16, 34), apo(a) immunofluorescence data was evaluated in two ways: (a) Integrated intensity above threshold level was normalized to the total area analyzed in the image to obtain an average fluorescence over the entire vessel wall. An average background measurement of fluorescence in adjacent sections stained with secondary antibody only was subtracted from this value to give the average specific fluorescence. (b) Focal accu-

mulation was assessed by measuring the area within a given image where scaled intensities exceeded a fixed value. The images shown in Fig. 4 A, excluding the control, are all examples of focal accumulation, where apo(a) immunofluorescence is very intense in some regions of the vessel wall and quite faint in other areas. Pixel intensity histograms corresponding to each image are given in Fig. 4 B, demonstrating how intensities in all images were scaled to a constant range of arbitrary units from 0 to 255 (detector gain setting remained constant throughout the experiment). The presence of focal accumulation in the images was predictably associated with pixels having intensities above 200 U. Thus, this value was used as the nominal threshold for determining focal accumulation area. The histogram of control mouse 214 shown in Fig. 4 B is representative of background staining obtained in the huB tg mice. These sections also served as an important control to determine the occurrence of vascular cell transduction by adenovirus vectors under the experimental conditions used in this study. 7 days after injection, immunostaining of these aortic sections for B-Gal detected no cells transduced by the control viral vector AD.RSV B-Gal, while liver sections obtained from these animals showed greater than 50% of hepatocytes were positive for B-Gal staining. This result with the control vector indicates a very low probability that the apo(a) immunoreactivity observed in these experiments was due to synthesis from viral-transduced vascular tissue.

The vascular accumulation data is presented into two ways in order to illustrate the nature of Lp(a) immunofluorescence (Fig. 5). There are multiple components to the binding, the sum of which is reflected in the mean accumulation values. High intensity focal deposition, which contributes a variable fraction of the total binding depending on the presence or ab-

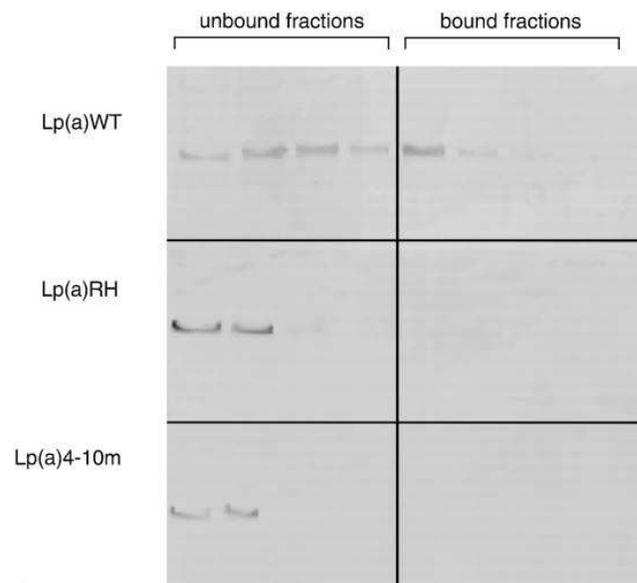
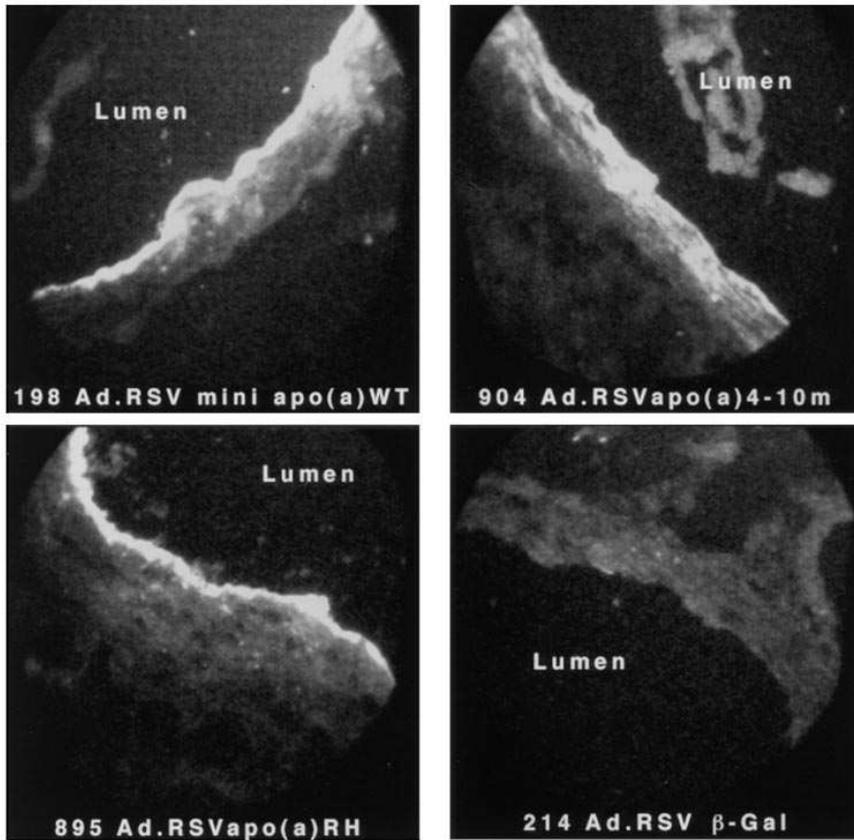


Figure 3. Binding of Lp(a)WT, Lp(a)4-10m, and Lp(a)RH to lysine-Sepharose. Lipoprotein fractions containing the indicated type of Lp(a) were subjected to lysine-Sepharose chromatography as described in Methods. Column fractions were immunoblotted with Lp(a) antiserum. Lanes 1–4 (unbound fractions) contain fractions collected immediately after the void volume. Lanes 5–9 (bound fractions) contain the first five fractions collected after application of 0.1-M e-ACA.

A



B

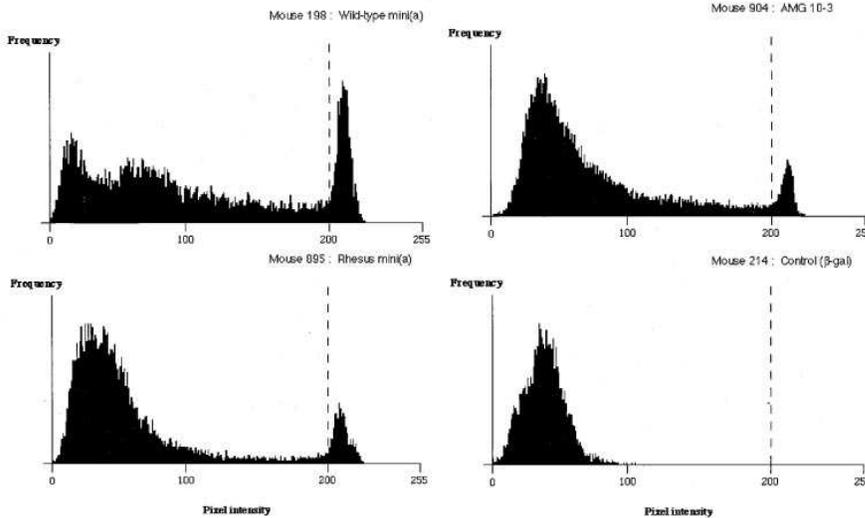


Figure 4. Immunofluorescence imaging of Lp(a) accumulation in the vessel wall. (A) 12 d after injection of adenovirus vectors, aortic sections from huB tg mice were examined for apo(a) immunoreactivity as described in Methods. Images displaying a broad range of fluorescent staining intensity are shown for each experimental group along with one image from a control group treated with adenovirus-encoding B-Gal. Control group sections, prepared 7 d after injection, were also examined for B-Gal immunoreactivity to detect possible adenovirus transduction of vascular cells. No B-Gal specific staining was detected (not shown). (B) Pixel intensity histograms are given for each of the representative images in A; the x-axis represents intensities scaled from 0–255 arbitrary units.

sence of these focal regions, is reported separately since it appears to represent a specific component of binding. Fig. 5 A shows a comparison of mean fluorescence data for the three groups of mice. Compared to Lp(a)WT, average intensity representing accumulation of Lp(a)4-10m and Lp(a)RH was reduced significantly throughout the vessel sections. Lp(a)RH accumulation was reduced by 56% ($P = 0.005$), Lp(a)4-10m by 37% ($P = 0.04$). Focal accumulation values were averaged for all images analyzed in each group of mice (Fig. 5 B), demonstrating that the frequency and severity of Lp(a) focal deposition was dramatically reduced in mice containing Lp(a)RH (72% decrease compared to Lp(a)WT; $P = 0.0001$), while in

mice with Lp(a)4-10m, focal accumulation was 40% less than Lp(a)WT ($P = 0.07$). Because of the marginal significance of the later comparison, focal accumulation of Lp(a)4-10m and Lp(a)WT was re-evaluated in a separate independent experiment. In this comparison, also shown in Fig. 5 B [Lp(a)4-10m(2) versus Lp(a)WT(2)], focal accumulation of Lp(a)4-10m was 70% reduced relative to Lp(a)WT ($P = 0.04$).

Discussion

The modifications made to apo(a) in this study comprise changes present in the two described examples of naturally oc-

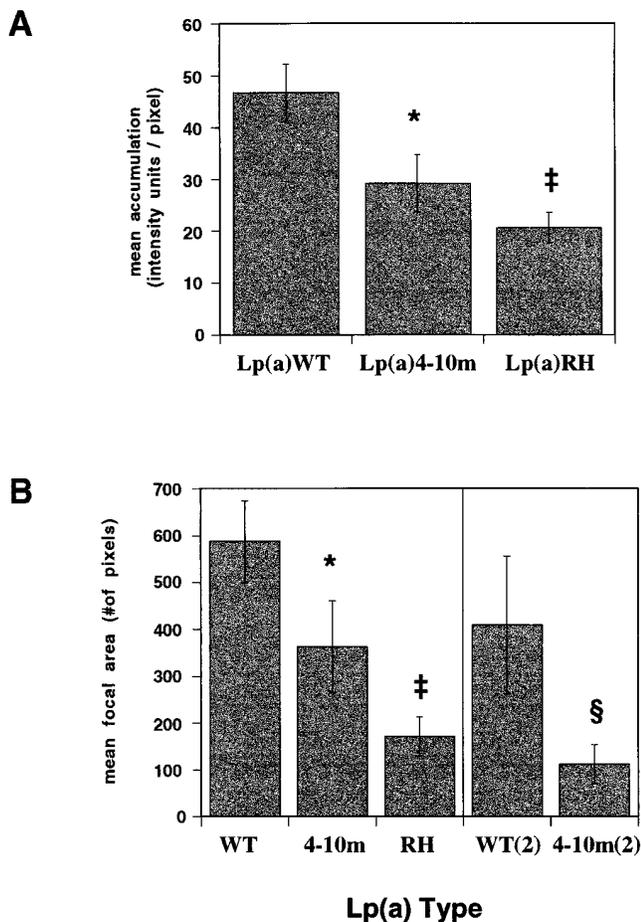


Figure 5. Quantitation of apo(a) immunofluorescent staining. Fluorescent staining was quantitated in aortic sections from mice injected with apo(a) adenovirus vectors as described previously (34). (A) Average of mean section intensity in the three groups of mice (intensity units/no. of pixels) \pm SEM. * $P = 0.04$; ‡ $P = 0.005$ (WT, $n = 9$; 4-10m, $n = 4$; RH, $n = 9$). Results are derived from a single experiment. (B) Focal staining area averaged for each group of mice (no. of pixels) \pm SEM. * $P = 0.07$; ‡ $P = 0.0001$ (WT, $n = 82$; RH, $n = 80$; 4-10m, $n = 40$; $n =$ number of images analyzed). Two values are given for the Lp(a)WT versus Lp(a)4-10m comparison since this experiment was performed twice. § $P = 0.04$ (WT(2), $n = 26$, 4-10m(2), $n = 32$).

curing lysine binding-defective forms. Compared to wild-type apo(a), apo(a)4-10m contains a single $W_{72}>R_{72}$ point mutation in kringle 4-10, while apo(a)RH contains this same substitution in the context of numerous additional amino acid differences and lacks the kringle 5-like domain present in human apo(a). Comparison of *in vitro* lysine binding properties and *in vivo* vascular accumulation of Lp(a) containing the various forms of apo(a) revealed significant correlation of these characteristics, indicative of a consequential role of apo(a) LBS in binding of Lp(a) to the vessel wall.

The use of huB tg mice was a critical aspect of this study, enabling us to study the properties of Lp(a), the predominant form apo(a) present in human plasma. We chose to focus on Lp(a) since several studies have demonstrated different properties in lipid free apo(a) versus Lp(a) (23, 25, 33). An important issue regarding apo(a) expression in the huB tg mice was that similar plasma levels of Lp(a) were achieved with the var-

ious apo(a) adenoviral vectors, and that apo(a) was present predominantly in the form of Lp(a). Injection of adenovirus vectors encoding mini apo(a), apo(a)4-10m, or apo(a)RH into huB tg mice resulted in similar dosage-expression relationships and apparently equivalent assembly efficiency of Lp(a) containing each form of apo(a). These results demonstrate that sequence differences between the various apo(a) forms had no detectable impact on Lp(a) assembly or expression of apo(a), indicating that LBS that may be required for noncovalent interaction with apoB-100 and assembly of the Lp(a) particle are functional in apo(a)RH and apo(a)4-10m.

The absence of detectable lysine-Sepharose binding with both Lp(a)4-10m and Lp(a)RH is consistent with previous characterizations of Lp(a) in rhesus monkeys and in humans. These results confirm that essential properties of these forms were reproduced in the context of mini apo(a), and that within this defined system, the $W_{72}>R_{72}$ substitution in kringle 4-10 is sufficient to disrupt lysine binding. A more extensive loss of lysine binding was observed with these modified forms of apo(a) assembled into Lp(a) particles, compared to the binding of purified apo(a) to lysine-Sepharose in this (data not shown) and other studies (25, 35). This finding is consistent with the conclusion of Ernst et al. (25) that secondary LBS located in apo(a) kringles 4-5 through 4-9 are unavailable for interaction with lysine-Sepharose when apo(a) is assembled into the Lp(a) particle.

Binding of Lp(a) to the vessel wall is known to be mediated by multiple interactions with a variety of ligands. However, loss of a highly specific interaction (i.e., the kringle 4-10 LBS with lysine containing ligands) would predictably result in significantly diminished overall binding, as was demonstrated in comparison of mean Lp(a) accumulation values in mice containing the various forms of Lp(a). Decreased vascular accumulation of Lp(a)4-10m or Lp(a)RH compared to Lp(a)WT suggests that Lp(a)'s interaction with lysine containing proteins in the vessel wall is an important determinant of its vascular accumulation, and that the kringle 4-10 LBS of apo(a) plays a significant role in this interaction. Lp(a) vascular accumulation observed in the absence of a functional kringle 4-10 LBS could arise from numerous possible sources, including the LDL portion of the particle or yet uncharacterized binding sites in apo(a). Alternatively, although the *in vitro* lysine binding data suggests that secondary LBS are masked in the Lp(a) particle, these may provide some interaction under *in vivo* conditions. Between the two modified forms of Lp(a), Lp(a)RH appears to accumulate less compared to Lp(a)4-10m, and this difference may allow identification of further binding components through analysis of structural differences between their two respective apo(a) components.

The presence of LBS in Lp(a) was associated with intense focal deposition within the vessel wall, a property that is very likely related to Lp(a)'s influence on atherogenesis. Although the dependence of Lp(a) vascular accumulation on these binding sites is consistent with their proposed role in Lp(a)'s atherogenic mechanism, an important issue in the interpretation of these results is whether short term *in vivo* accumulation can be extrapolated to long term effects related to atherogenesis. Such an evaluation of long term effects in transgenic mice expressing mini apo(a) or mini apo(a) containing a mutation which inactivated the kringle 4-10 LBS has been reported recently (36), demonstrating a significant reduction in fatty streak lesion formation and apo(a) focal accumulation in mice

containing the mutated apo(a). Under the conditions of this study, our results indicate that the structural differences in apo(a) as part of the Lp(a) particle can manifest significant differences in Lp(a) focal accumulation on a short time scale. Further, these results appear to correlate with chronic accumulation of apo(a) in longer-term studies, suggesting that the lysine binding characteristics of Lp(a), as important determinants of its vascular accumulation, may influence its eventual impact on atherosclerosis.

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