Modification of Apolipoprotein(a) Lysine Binding Site Reduces Atherosclerosis in Transgenic Mice

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Abstract

Lipoprotein(a) contributes to the development of atherosclerosis through the binding of its plasminogen-like apolipoprotein(a) component to fibrin and other plasminogen substrates. Apolipoprotein(a) contains a major lysine binding site in one of its kringle domains. Destruction of this site by mutagenesis greatly reduces the binding of apolipoprotein(a) to lysine and fibrin. Transgenic mice expressing this mutant form of apolipoprotein(a) as well as mice expressing wild-type apolipoprotein(a) have been created in an inbred mouse strain. The wild-type apolipoprotein(a) transgenic mice have a fivefold increase in the development of lipid lesions, as well as a large increase in the focal deposition of apolipoprotein(a) in the aorta, compared with the lysine binding site mutant strain and to nontransgenic littermates. The results demonstrate the key role of this lysine binding site in the pathogenic activity of apolipoprotein(a) in a murine model system. (*J. Clin. Invest.* 1997. 100:558–564.) Key words: lipoprotein • apolipoprotein • lipoprotein(a) • fibrin • mouse

Introduction

Elevated plasma levels of the lipoprotein(a) [Lp(a)][1] are a major independent risk factor for atherosclerosis and related disease states including myocardial infarction, cerebral stroke, peripheral vascular disease, and restenosis. 12 of 14 published prospective studies conclude that Lp(a) levels predict the development of cardiovascular disease (1, 2, and references therein). While Lp(a) closely resembles LDL in its content of cholesterol, phospholipid, and apolipoprotein B-100, it is distinguished by the presence of an additional glycoprotein known as apolipoprotein(a) [apo(a)], which was found to have a remarkable homology to plasminogen (3). Plasminogen is a serine protease zymogen which contains five distinct kringle domains, followed by a catalytic domain. Apo(a) consists of multiple tandem repeats of domains closely resembling plasminogen kringle 4, followed by a single kringle 5–like and inactive protease–like domain.

Several hypotheses have been proposed to account for the pathogenicity of Lp(a) (4–6). In vitro studies show that apo(a) competes for the binding of plasminogen to fibrin and other substrates and reduces the generation of active plasmin (7–14). A resulting decrease in fibrinolysis can prolong the persistence of mural thrombi which stimulate repair processes that lead to the local thickening of the vessel wall. A second effect of the inhibition of plasmin generation is the reduction in plasmin-dependent activation of TGF-β, resulting in increased proliferation and migration of vascular smooth muscle cells (15, 16). These scenarios have received support from in vivo studies in primates (17), humans (18, 19), and transgenic mice (20, 21). The human apo(a) gene has been expressed in transgenic mice, an animal species normally lacking this gene, by introduction of a human apo(a) transgene under the control of the mouse transferrin promoter. In three separate studies, apo(a) transgenic mice have been found to develop significantly more fatty streak–type lesions in their proximal aorta when maintained on a high fat diet than their control littermates (22–24). In contrast, another study failed to show a significant degree of atherosclerosis associated with the apo(a) transgene (25), suggesting that the mixed genetic background into which the transgene was inserted may influence the atherogenic phenotype.

Immunocytochemical analysis of the aortic lesions in transgenic mice demonstrated focal colocalization of apo(a) within the fatty streak lesions, as well as local decreased level of plasmin, decreased level of active TGF-β and increased markers of smooth muscle activation (21). The suppressed amount of active TGF-β was proposed to promote lesion development by altering the phenotype and function of endothelial and smooth muscle cells in the vessel wall (21, 26). Each of these pathways may depend on the ability of apo(a) to possess functional lysine/fibrin binding sites resembling its plasminogen homolog.

The nature of the lysine binding site (LBS) of plasminogen kringle 4 has been explored by x-ray crystallography, chemical modification, and mutagenesis. The site is composed of a trough lined by three aromatic residues (Trp62, Phe64, Trp72) flanked at one end by two anionic residues (Asp55, Asp57) and at the other end by two cationic residues (Lys35, Arg71) (27, 28; numbering based on plasminogen kringle 5 convention). The nature of this site allows favorable van der Waals interaction with the hydrocarbon backbone of ω-amino acid ligands such as lysine and ϵ-aminocaproic acid (EACA), while the charged groups interact electrostatically with the opposed carboxylate and ω-amino ends. Site-directed mutagenesis has confirmed the importance of these seven binding site residues, but suggested that only one of the two cationic residues (Arg71) may be sufficient for high-affinity binding (29, 30). Se-

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1. *Abbreviations used in this paper:* EACA, ϵ-aminocaproic acid; LBS, lysine binding site; Lp(a), lipoprotein(a); r-apo(a), recombinant apo(a).

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quence analysis of human apo(a) cDNA revealed that only the most carboxy-terminal of its 37 kringle 4–like domains retains the seven residues of the plasminogen kringle 4 LBS, with only a single conservative substitution (Lys35 to Arg; 3). [This kringle is denoted K4-37 in some publications, following the numbering of McLean et al. (3), while other publications refer to it as K4 type 10.] It was suggested that this kringle would account for most of the lysine affinity of apo(a) (3, 31). Subsequent analyses of in vitro and naturally occurring mutations support this prediction (32–36).

To evaluate the function of apo(a) LBS in vivo, we replaced the two key anionic residues of this kringle (Asp 55,57) with alanine by in vitro mutagenesis, and expressed the recombinant apo(a) [r-apo(a)] protein in cultured mammalian cells. The mutated apo(a) retains only 30% of the lysine binding activity of the wild-type construct (37). We now report on the effects of this mutated apo(a) on atherosclerosis and apo(a) deposition in the vessel wall of transgenic mice created in the inbred FVB strain.

Methods

Expression plasmids. As we previously described (37), wild-type and LBS defective forms of apo(a) cDNA were constructed to encode eight kringle 4–like domains plus the single kringle 5–like and protease-like domains of human apo(a) (3). For the LBS form, the asp55 and asp57 residues of the LBS of kringle 4-37 were replaced by alanine residues. The wild-type [pCMha8] and LBS mutant [pCMha8lysmuta] apo(a) expression plasmids contain cytomegalovirus promoter sequences ligated to apo(a) cDNA sequences encoding the secretion signal sequence, the first two kringle 4–like repeats, a fusion kringle containing 101 bp of the third kringle 4–like repeat with 241 bp of K4-32, followed by kringles 4-33-37, kringle 5–like, and the protease-like domain.

Fibrin binding assay. Recombinant proteins were produced in cultured human embryonic kidney 293 cells (37). The conditioned serum-free supernatant was concentrated by Centriprep concentrators (Amicon Corp., Beverly, MA) to $\sim$ 50-fold. The concentrations of wild-type and mutant apo(a) were measured by ELISA using purified wild-type r-apo(a) as standard, and adjusted to the same final concentration with concentrated 293 cells conditioned serum-free supernatant. The fibrin binding assay was adapted from those of Harpel et al. with minor modification (7, 38). 96-well microtiter plates (Nunc, Inc., Naperville, IL) were coated with 5 μg of human fibrinogen (American Diagnostica Inc., Greenwich, CT) in 100 μl of 0.1 M bicarbonate buffer, pH 8.5, overnight at 4°C and blocked with 3% BSA in PBS (150 nM NaCl, 10 mM phosphate, pH 7.4) plus 0.05% Tween 20 (PBST). The fibrinogen was treated with 1 U/ml of human thrombin for 30 min at 37°C to convert it into fibrin and subsequently subjected to partial plasmin proteolysis with 10 ng of plasmin for 30 min at room temperature and later inactivated with serine protease inhibitor 0.1 mM PMSF (Sigma Chemical Co., St. Louis, MO) for 20 min at room temperature. Dilutions of concentrated wild-type and mutant r-apo(a) supernatant were then added to the treated wells and incubated overnight at 4°C with or without 0.2 M EACA followed by washing with PBST buffer. The same dilutions of 293 cells conditioned supernatant were diluted to the same extent to serve as negative control for the background of binding from the concentrated supernatant. Lp(a) polyclonal antibody [goat anti–human Lp(a)], the horseradish peroxidase–conjugated secondary antibody, and *p*-nitrophenylphosphate (Sigma) color development were used for detection as indicated previously (38). The data were the average of three experiments, each done in duplicate. Different concentrations of 293 cells conditioned supernatant give similar absorbance readings that were approximately the same as those from the negative control, indicating that the components in the serum-free media are not detectable by apo(a)-specific antibody in the in vitro fibrin binding assay.

Construction of transgenic mice. For transgenic expression, the cytomegalovirus promoter of the above expression plasmids was replaced with a 500-bp promoter fragment of the human apoAI gene (39, 40). The gene fragment was generated by PCR using primers 5′-GACACTCGAGCCCGGGAGACCTGCAAG3′ and 5′-GACAGTCGACGAAGGGCCGTGGGGGAC3′. After digestion with XhoI and SalI, this fragment was ligated into the XhoI site of the pCMha8 or pCMha8Lysmuta. A 4.8-kb XhoI plus HpaI fragment from pAIha8 or pAIha8Lysmuta was purified from vector sequences and microinjected into oocytes of inbred FVB mice using standard procedures (40). Transgenic founders were identified by analyzing tail DNA for the presence of apo(a) sequence by Southern blot hybridization.

Detection of apo(a). Plasma levels of wild-type and mutant apo(a) in the transgenic mice were initially determined by ELISA (Strategic Diagnostics, Newark, DE) using r-apo(a) as standard. Concentration was further estimated by Western blotting of mouse plasma with detection by rabbit polyclonal antiserum against r-apo(a), compared with dilutions of purified wild-type r-apo(a). The two transgenic mouse strains in this study contained $\sim$ 15 nM plasma apo(a) for the wild-type apo(a) and 30 nM plasma apo(a) for the LBS mutant apo(a).

Northern blot analysis of RNA. mRNA was isolated using poly(A) Quick mRNA kit (Promega Corp., Madison, WI) and subjected to electrophoresis on 1.5% agarose/0.6 M formaldehyde gel and transferred to nylon membrane (Micron Separations, Inc., Westboro, MA). The filter was hybridized with a ^{32}P-labeled probe derived from apo(a) cDNA (3).

Mouse breeding and diets. Two mouse lines expressing either human wild-type or mutant LBS apo(a) transgenic were maintained by mating transgene-positive offspring to FVB mice. The transgenic mice used in these studies were heterozygous for either wild-type or mutant LBS apo(a). The nontransgenic littermates served as controls. All mice were weaned at 3 wk of age and housed in a full barrier transgenic facility on a 12-h light–dark cycle with free access to food and water.

At about 10 wk of age, all mice were switched from a normal mouse chow diet (4.5% fat, rodent chow; Purina Mills, St. Louis, MO) to an atherogenic diet containing 15% total fat including 1.25% cholesterol; 0.5% sodium cholate, and 7.5% casein (1.25% cholesterol diet 384144; ICN Biomedicals, Inc., Carson, CA). Blood was collected for lipid measurements at 4 and 18 wk on the high fat diet, after which the animals were killed. The transgenic and control littermates were housed in the same cages during high fat diet treatment.

Lipid measurements. Total plasma cholesterol levels and HDL cholesterol levels were measured using an enzymatic method (Sigma). The HDL cholesterol was measured after the precipitation of LDL and VLDL fractions of the sera with phosphotungstic acid and $MgCl_2$ (PTA/$MgCl_2$). Briefly, 25 μl of plasma was mixed with 5 μl of PTA/$MgCl_2$, vortexed, and the mixture was incubated at room temperature for 5 min. The samples were centrifuged for 5 min at 3,000 rpm in a microcentrifuge. 5 μl of the supernatant was used for HDL cholesterol determination.

Vascular lesion assay. The aortic sectioning, lipid staining, and lesion scoring were performed blindly with modifications to the method described previously (22, 41). Briefly, after 18 wk on the atherogenic diet, animals were killed, and the heart and attached aorta were collected in 10% PBS by gross-cutting the bottom half of the heart off under a dissecting microscope. The heart was embedded in OCT embedding medium (Miles Inc., Kankakee, IL) and immediately frozen on dry ice. 10-μm sections separated by 50 μm each were prepared. The first and most proximal section of the aorta was taken 80 μm distal to where the aorta becomes rounded and the aortic valves are distinct. For oil red O staining, sections were collected on gelatin-coated slides which were fixed in formaldehyde by placing slides in a dish with a formaldehyde-soaked gauze pad and covered overnight at 4°C before staining with oil red O and hematoxylin and

counterstaining with light green (L-5382; Sigma). The area of oil red O staining lesion was measured at a magnification of 100 by a calibrated eyepiece with square micron units. The mean lesion per section per animal was calculated for each individual and group of animals. The significance levels were determined by Mann-Whitney U statistical analysis (42). For apo(a) immunostaining, 10-μm sections were collected on poly-L-lysine (Sigma) coated slides and immediately fixed in ice-cold acetone for 90 s, and kept at −20°C until used.

Apo(a) deposition. Essentially as described (21, 43), sections adjacent to those used for oil red O staining were incubated with TBS, 3% BSA for 30 min, followed by sheep anti–human Lp(a) antibody diluted 1:200 in TBS, 3% BSA overnight, followed by rabbit anti–sheep IgG-FITC diluted 1:80 in TBS, 3% BSA for 4 h. All reactions were performed at room temperature. Two images of the aortic wall, covering ~ 75% of the entire vessel, were randomly selected under phase contrast (×20 lens). After selecting the field, the illumination source was changed to capture and quantitate the fluorescence intensity. One to four sections were used for each animal, and slides were labeled in a blinded fashion. Fluorescent staining was detected and analyzed by a laser confocal microscope system (MultiProbe 2010; Molecular Dynamics, Inc., Sunnyvale, CA) together with NIH Image software. The gain of the photomultiplier was set so that the fluorescent intensity of the unlabeled vessel wall was 2–5% of the full scale. A region containing the vessel media was selected with a light pen and the mean gray level of the selected pixels measured. The average intensity of vessel sections without primary antibody was subtracted from this to determine the average intensity of fluorescence in arbitrary units. The area (pixels) in which the intensity is greater than the mean intensity plus one standard deviation was used to calculate the focal deposition of apo(a) in the vessel wall.

Results

Binding of r-apo(a) to fibrin. Major physiological substrates of the LBS of plasminogen kringles 1 and 4 are fibrin and cell surface proteins containing carboxy-terminal lysines (27, 44). This association results in the juxtaposition of plasminogen and plasminogen activators on two-dimensional substrates, greatly accelerating the conversion of the zymogen to active plasmin, as well as protecting it from subsequent deactivation by antiplasmins. To assess the contribution of the major LBS in K4-37 to fibrin binding activity of apo(a), wild-type and mutant r-apo(a) were tested for their binding activity to plasmin-modified fibrin. As shown in Fig. 1, the cultured medium containing wild-type or LBS mutant r-apo(a) was concentrated and assayed for binding to plasmin-treated fibrin surfaces, in the presence or absence of 0.2 M EACA. Specific binding was calculated by subtracting the binding observed in the presence of EACA from the binding observed in its absence. By Scatchard analysis of the data, the K_d for wild-type apo(a) was ~ 6 nM, and 11 nM for LBS mutant apo(a). The mutant apo(a) has an ~ 60% reduction in specific binding to the fibrin surface.

Generation of transgenic mice. To test the role of lysine/fibrin binding in the pathogenic activities of apo(a), transgenic mice strains were created with the above apo(a) constructs that contained wild-type or LBS mutations in kringle 4-37. Mice normally lack the apo(a) gene. Transgenic animals were created and maintained in the FVB genetic background, which is only mildly susceptible to diet-induced atherosclerosis. Expression was driven by a 500-bp promoter-containing region of the human apo A-I gene, previously shown to drive high level hepatic expression of adjacent transgenic coding regions (39, 40). Heterozygous mice and nontransgenic littermates were used in the following studies. The LBS mutant apo(a) heterozygotes produced ~ 30 nM of plasma apo(a), while the wild-type apo(a) mice contained 15 nM plasma apo(a). To determine the tissue specificity of apo(a) expression, Northern blot analysis was performed using a human apo(a) cDNA probe. The expected message size of ~ 4.5 kb was detected in liver and heart RNA but not from mRNA isolated from intestine or lung (data not shown). As expected, expression of the apo(a) transgene had no significant effect on the plasma total and HDL cholesterol as well as triglyceride levels both on the chow and high fat diets (Table I).

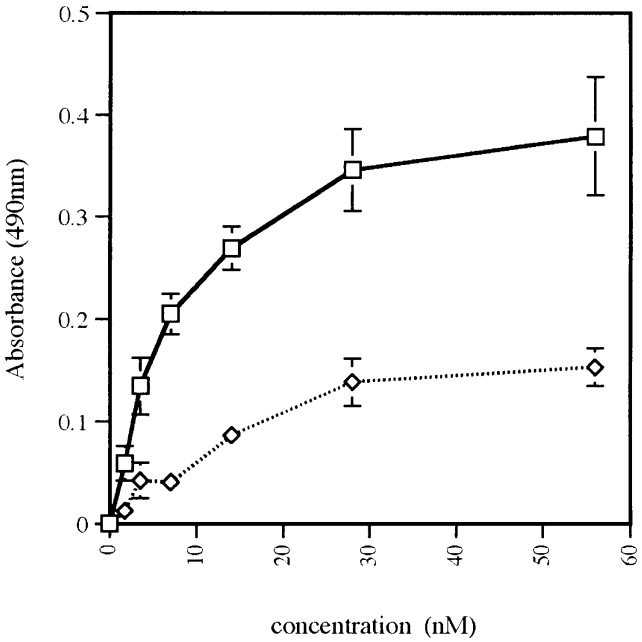

Figure 1. Disruption of the LBS of apo(a) reduces its binding to fibrin in vitro. Both wild-type and LBS mutant apo(a) constructs were expressed in 293 cells from which the serum-free supernatant was concentrated. Wild-type and LBS mutant r-apo(a) proteins were incubated with immobilized plasmin-treated fibrin in the presence or absence of 0.2 M EACA, as described in Methods. Specific binding of wild-type (*open boxes*) and mutant apo(a) (*open diamonds*) was calculated by subtracting the binding in the presence of EACA from total binding of wild-type in each case.

Vascular lesions. After the mice were fed the atherogenic diet for 18 wk, the area of lesions that stained with oil red O in the proximal aorta were quantified in 24 wild-type apo(a) transgenic mice (5 male, 19 female), 18 mutant apo(a) mice (6 male, 12 female), and 20 nontransgenic littermate controls (5 male, 15 female). The area of oil red O staining lesions for the three study groups is presented in Table II. The mice expressing the wild-type apo(a) transgene had mean lesion area 4.8-fold greater than that of mice expressing mutant apo(a) or than that of the nontransgenic control littermates. The mean lipid staining lesion area per mouse was 11,539 μm^2 for transgenic wild-type apo(a), 2,412 μm^2 for transgenic mutant apo(a), and 2,386 μm^2 for the control littermates. The mean lipid staining lesion area in the wild-type apo(a) was significantly greater than both the LBS mutant apo(a) group and the nontransgenic group by Mann-Whitney U test analysis with

Table I. Cholesterol Concentration

	WT apo(a) chow diet	WT apo(a) high fat diet	LBS mut apo(a) chow diet	LBS mut high fat diet	Nontransgenic chow diet	Nontransgenic high fat diet
Female mice	$n = 7$	$n = 7$	$n = 7$	$n = 6$	$n = 10$	$n = 5$
Total cholesterol	115.7±37.3	252.3±16.7	117.9±32.2	211.3±80.7	127.7±37.7	242.8±16.7
HDL cholesterol	79.2±22.1	79.1±38.6	94.6±29.0	72.2±26.3	79.4±18.1	95.3±28.1
Non-HDL cholesterol	33.6±20.2	173.2±106	23.2±11.1	167.4±95.1	47.6±20.4	147.4±25.7
Male mice	$n = 8$	$n = 4$	$n = 5$	$n = 7$	$n = 10$	$n = 5$
Total cholesterol	103.9±34.9	237.1±8.2	124.5±60.4	237.7±32.2	158.9±17.0	249.7±15.8
HDL cholesterol	90.2±36.6	97.9±5.6	99.6±52.4	86.7±12.3	117.1±23.6	105.4±17.0
Non-HDL cholesterol	28.5±13.9	139.2±7.1	25.2±15.5	151±47.8	41.8±40.7	144.3±18.5

The plasma cholesterol levels were determined for each three groups of mice on the low fat chow diet and after 18 wk of exposure to the high fat diet. Values in milligrams per deciliter are given for total, HDL, and non-HDL (VLDL plus LDL) fractions±SEM.

$P < 0.01$. Among the 24 wild-type apo(a) transgenic animals examined, only one animal had very low lesion score (mean lesion area = 234 μm^2). Only 1 of the 18 LBS mutant apo(a) transgenic mice had a lesion area > 10,000 μm^2, while 8 of 24 wild-type apo(a) mice exceeded this level. The difference between wild-type apo(a) and LBS mutant groups was significant despite the fact that the plasma apo(a) level in the mutant strain was nearly twofold higher than that in the wild-type strain. When each gender was considered separately, the difference in lesion area between wild-type apo(a) versus LBS mutant groups was significant for females at $P < 0.05$ and for males at $P < 0.01$. Hence these data show that expression of apo(a) leads to a significant increase in the development of fatty streak–type lesions in the aorta of transgenic mice, compared with both nontransgenic littermates, and to mice expressing even higher levels of apo(a) with the LBS of kringle 4-37 disabled by mutagenesis.

Apo(a) deposition in the aorta. To elucidate the relationship between lesion development and apo(a) accumulation in vessel walls, adjacent sections of those used for lesion quantification were assayed for apo(a) immunofluorescent staining (Fig. 2). The area per section was determined for which the fluorescent intensity was at least one standard deviation above the mean fluorescence for the entire section. Apo(a) focal deposition in wild-type apo(a) transgenic mice is 7.9-fold greater than that in LBS mutant mice, although limited apo(a) focal staining could be observed in mutant apo(a) transgenic mice. The mean area of focal apo(a) staining is 1,355 pixels per section per mouse for wild-type apo(a) transgenic mice and 170 pixels per section per mouse for mutant apo(a) mice. In the wild-type apo(a) mice, apo(a) focal staining was always colocalized with oil red O staining for lipid, while in LBS mutant apo(a) mice, limited apo(a) staining was found where no lipid lesions were detected in adjacent sections. These results are consistent with our previous observations that focal apo(a) deposition coincides with vessel wall lesion development (21, 22, 26), and further suggests that K4-37 LBS of apo(a) is involved in this effect. This is despite the fact that the average apo(a) immunofluorescence, measured for the entire tissue sample, is not significantly different between wild-type apo(a) and LBS mutant mice [14.9 and 19.3, respectively, whereas the apo(a) expression level in the LBS mutant mice is roughly twofold higher than in the wild-type apo(a) mice]. It appears that both forms of apo(a) enter the subendothelial space, whereas the lysine binding function is necessary for eventual focal high-level concentration and lesion development. This is

Table II. Lipid and apo(a) in Vessel Walls

	WT apo(a)	LBS mut apo(a)	Nontransgenic
All mice			
Lesion area (μm^2/mouse)	11539±3103 ($n = 24$)	2412±978 ($n = 18$)	2386±448 ($n = 20$)
Focal apo(a) area (pixels/section)	1355±440 ($n = 5$)	170±48 ($n = 8$)	0±0 ($n = 5$)
Mean apo(a) intensity (arbitrary units)	14.9±8 ($n = 5$)	19.3±4 ($n = 8$)	4.8±3 ($n = 5$)
Male mice			
Lesion area (μm^2/mouse)	15192±4102 ($n = 5$)	945±275 ($n = 6$)	296±127 ($n = 5$)
Female mice			
Lesion area (μm^2/mouse)	10507±3685 ($n = 19$)	3182±1428 ($n = 12$)	2931±488 ($n = 15$)

Transgenic and nontransgenic littermates were fed a high fat diet for 18 wk before killing and inspection of the aorta as described in Methods. The area of oil red O staining per mouse is presented as the mean±SEM for each group of mice: wild-type apo(a) transgenic mice; LBS mutant apo(a) transgenic mice; and nontransgenic mice. Mice are of the inbred FVB genetic background. The differences between wild-type apo(a) and LBS mutant, and wild-type apo(a) versus nontransgenic groups are significant by Mann-Whitney U test at $P < 0.01$. The mean area of focal apo(a) deposition in the aorta for each group of mice represents the number of pixels per section in which the apo(a) immunofluorescent intensity is greater than the mean intensity plus one standard deviation. The area of apo(a) staining between wild-type apo(a) transgenic and LBS mutant apo(a) transgenic mice is significantly different by Mann-Whitney U test at $P < 0.01$.

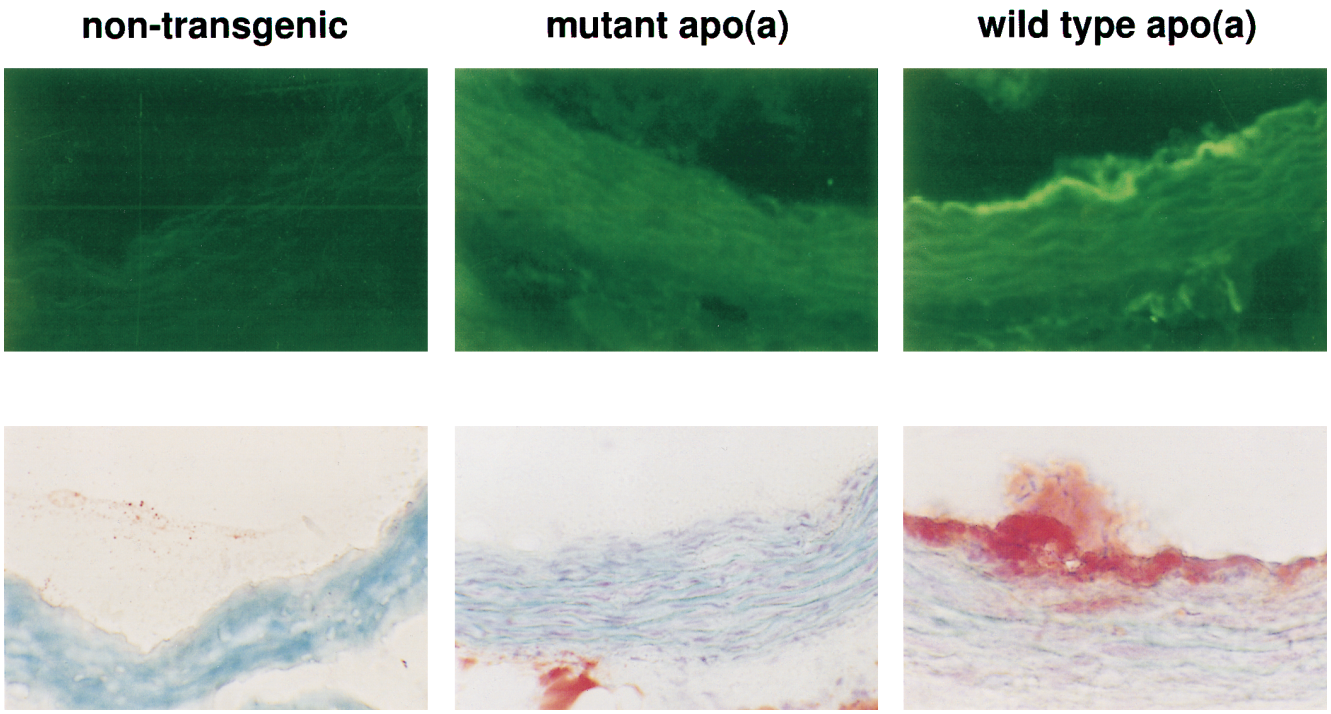

Figure 2. Oil red O and apo(a) staining in the aorta. Representative adjacent sections of aorta of the three groups of mice are shown with immunofluorescent staining for apo(a) deposition (*top*), and oil red O staining for lipid deposition (*bottom*) as described in Methods. ×400.

consistent with the proposal that decreased lysine binding ability of mutant apo(a) reduces its ability to compete with plasminogen binding to fibrin or fibrin-like substrates in vessel walls and inhibit conversion of plasminogen to active plasmin, with its atherogenic consequences.

Discussion

The kringles of plasminogen, most notably kringles 1 and 4, contain high-affinity LBS. These sites are largely responsible for the binding of plasminogen to fibrin, particularly via carboxy-terminal lysine residues that are created as fibrin becomes partially proteolyzed. This binding greatly accelerates the process of plasminogen activation, and also protects the resulting plasmin from subsequent inactivation. The binding is inhibited by lysine and analogs such as EACA, tranexamic acid, and other ω-aminocarboxylic acids (27–30). Apo(a) contains multiple, tandem copies of a domain which most closely resembles kringle 4 of plasminogen (3). Alleles of apo(a) in the human population contain a variable number of exact kringle repeats at their amino termini (ranging from ~ 10 to 30 copies) followed by an apparently constant number of kringle 4–like domains with ~ 10% variation in sequence (45, 46). Sequence analysis showed the last of these kringles (K4-37) most closely maintains the LBS of plasminogen kringle 4 (3). Expression of r-apo(a) kringle 4-37 demonstrated that it provides the major interaction of apo(a) with fibrinogen, with additional weak binding sites also present (34, 36). In vitro mutagenesis of multikringle constructs of apo(a) and expression in HepG2 cells allowed Ernst and colleagues (35) to identify two functionally distinct LBS. They concluded that the K4-37 site conferred virtually all of the lysine binding observed in a reconstituted Lp(a) lipoprotein particle, while the additional site(s) present in kringles 4-32 through K4-36 of apo(a) are masked when apo(a) is incorporated in the Lp(a) lipoprotein particle (35). In agreement with these results, we found that the LBS mutation we created resulted in a 70% loss in lysine binding activity of the isolated recombinant protein (37), as well as a significant reduction in binding to a fibrin surface in vitro (Fig. 1).

Scanu and colleagues initiated the search for naturally occurring lysine binding mutations in human apo(a) genes. They identified a single point mutation (trp72 to arg) in K4-37 of several individuals which nearly eliminated lysine binding, and suggested that this might represent a variant of Lp(a) which could be "benign from a cardiovascular viewpoint" (33). This group has also provided the cogent warning that one cannot presume that measurement of lysine binding of Lp(a) variants will faithfully translate to relative binding affinity of Lp(a) to fibrin itself (36, 47). Leerink and colleagues isolated lysine binding and nonbinding Lp(a) fractions from several human donors (14). Although the molecular causes of this binding difference were not determined, they reported that the lysine binding fraction of Lp(a) bound fibrin and inhibited plasminogen activation, whereas the lysine nonbinding fraction did neither (14). Since it is not yet practical to conduct human clinical trials with naturally occurring sequence variants that are of a scope to achieve statistically significant conclusions, we have used a transgenic mouse model of fatty streak development to address the in vivo effect of the LBS of apo(a) kringle 4-37. Our data reveal a significantly greater development of fatty aortic lesions in mice that are transgenic for an eight kringle construct of wild-type human apo(a) compared with mice transgenic for an apo(a) construct in which the LBS had been

destroyed by mutagenesis (asp55 to ala plus asp57 to ala). The mean lesion area per animal with the wild-type apo(a) construct is 4.8 times that of the LBS defective mutant. In fact, lesion area in LBS mutant transgenic apo(a) was only slightly higher than in nontransgenic control littermates.

The background strains of mice should be considered in the interpretation of murine model results. The animals used here were human apo(a) transgenic mice in the inbred FVB background, a strain that has mild susceptibility to diet-induced atherosclerosis. This may reduce the variability in results due to epistatic effects of genes causing resistance or susceptibility to lesion development when mixed genetic backgrounds are used. In addition, the cholesterol and cholate content of high fat mouse diets, plus the length of exposure to the atherogenic diet may also be subject to optimization for the evaluation of the effects of a particular transgenic manipulation.

It should also be noted that human apo(a) fails to form a covalently linked Lp(a) particle with mouse LDL, presumably due to sequence differences between mouse and human apo B-100 (48). In human plasma, nearly all circulating apo(a) is found in the Lp(a) particle. However, studies in human subjects infused with radiolabeled Lp(a), LDL, and apo(a) have shown that this covalent bond is exchangeable in the circulation. Knight et al. (49) and Rader et al. (50) reported that 10–25% of radiolabeled plasma Lp(a) loses its apo(a) to become LDL over the course of several days in circulation, while Bader et al. (51) reported that 80% of injected apo(a) converts to covalently linked Lp(a) after 24 h in the human bloodstream. In addition to covalent linkage, apo(a) also has a strong noncovalent interaction with LDL (52). Thus, the nature of the linkage between apo(a) and apo B in plasma, if not when bound in the vessel wall, may be more complex than once imagined. Among future studies, one can assess the role of covalent linkage of the LBS mutant apo(a) to a lipoprotein particle by crossing the transgenic apo(a) mice with transgenic strains expressing human apo B (53, 54). The results of Ernst et al. (35) that secondary LBS in apo(a) can be masked in the Lp(a) particle, led to the prediction that the kringle 4-37 LBS mutated Lp(a) in such a double transgenic mouse would possess even less residual fibrin binding and pathological activity than in our current model.

The means by which Lp(a) promotes atherosclerosis are not fully understood. Interference of plasminogen activation reduces fibrinolysis and has other pathological consequences. It has been demonstrated in tissue culture and in transgenic mice that high levels of apo(a) decrease the plasmin-dependent activation of TGF-β, resulting in a more active smooth muscle cell phenotype capable of accelerated proliferation, migration, and lipid absorption (15, 16, 21). It has been proposed that atherogenesis by apo(a) is driven by a positive feedback loop involving focal apo(a) accumulation and TGF-β inhibition (26). In this model, apo(a) begins to accumulate at sites where integrity or function of the endothelium has been compromised. As a result, concentration of active plasmin and TGF-β is locally reduced. Depression of TGF-β not only stimulates smooth muscle cells, but is associated with inflammatory damage to the endothelium and increased adhesiveness to leukocytes, which might result in further compromise of the endothelial barrier function and focal accumulation of apo(a) (26).

The results of this study are consistent with these mechanisms. Both wild-type and LBS mutant apo(a) gain access to the vessel wall. However, the consequences of the LBS mutation are marked. After 18 wk on the high fat diet, there is both a significantly greater area of oil red O staining, and of focal regions of intense apo(a) staining in the vessel wall of wild-type versus mutant apo(a) mice. Although a few cases of naturally occurring mutations in the LBS of kringle 4-37 of apo(a) have been documented to date (33), it is commonly observed that individual isolates of human Lp(a) differ in their degree of binding to lysine-Sepharose (14, 55). The results in this transgenic mouse model suggest the utility of further studies in animal models and in humans to determine whether such isoforms of the highly polymorphic Lp(a) are more benign to their human hosts.

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