An Apolipoprotein(a) Peptide Delays Chylomicron Remnant Clearance and Increases Plasma Remnant Lipoproteins and Atherosclerosis In Vivo

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Objective—Humans with high expression of apolipoprotein(a) [apo(a)] and high plasma levels of lipoprotein(a) [Lp(a)] are at increased risk for atherosclerosis, but the mechanism is not known. We have previously shown that the KIV_{5-8} domain of apo(a) has unique cell-surface binding properties, and naturally occurring fragments of apo(a) encompassing this domain are thought to be atherogenic in humans. To investigate the effect of KIV_{5-8} on lipoprotein metabolism and atherosclerosis in vivo, we created several independent lines of liver-targeted KIV_{5-8} transgenic mice.

Methods and Results—The transgenic mice have plasma apo(a) peptide concentrations that are similar to Lp(a) concentrations in humans at risk for coronary artery disease. Remarkably, the transgenic mice had a 2- to 4-fold increase in cholesterol-rich remnant lipoproteins (RLPs) when fed a cholesterol-rich diet, and a 5- to 20-fold increase in atherosclerosis lesion area in the aortic root. Using an in vivo clearance study, we found only slight differences in the triglyceride and apolipoprotein B secretion rates between the 2 groups of mice, suggesting an RLP clearance defect. Using an isolated perfused mouse liver system, we showed that transgenic livers had a slower rate of RLP removal, which was retarded further when KIV_{5-8}, full-length apo(a), or Lp(a) were added to the perfusate. An apo(a) peptide that does not interact with cells, K(IV3), did not retard RLP removal, and low-density lipoprotein (LDL) had a much smaller effect than Lp(a).

Conclusions—We propose that high levels of apo(a)/Lp(a), perhaps acting via a specific cell-surface binding domain, inhibit hepatic clearance of remnants, leading to high plasma levels of RLPs and markedly enhanced atherosclerosis. We speculate that the KIV_{5-8} region of apo(a) competes with one or more receptors for remnant clearance in the liver and that this process may represent one mechanism accounting for increased atherosclerosis in humans with high secretion levels of apo(a). (Arterioscler Thromb Vasc Biol, 2005;25:0-0.)

Key Words: apolipoprotein(a) ■ atherosclerosis ■ hepatic clearance ■ lipoprotein(a) ■ remnant lipoproteins

Lipoprotein(a) [Lp(a)] is a low-density lipoprotein (LDL)-like lipoprotein in which a glycoprotein called apolipoprotein(a) [apo(a)] is attached covalently to the apolipoprotein B100 (apoB100) of LDL. Apo(a) can also be noncovalently associated with the apoB of very-low-density lipoprotein (VLDL) and remnant lipoproteins (RLPs). Apo(a) is made up of variable numbers of protein domains called kringles, designated KIV{1} through KIV{10} (see Figure I, available online http://atvb.ahajournals.org). Interest in apo(a)-containing lipoproteins arises from the finding that high plasma levels of Lp(a) are an independent risk factor for atherothrombotic coronary artery disease and stroke. Postulated mechanisms include apo(a)-mediated inhibition of plasmin activation on fibrin, stimulation of smooth muscle cell proliferation and migration, increased retention of Lp(a) in the arterial wall, and alterations in endothelial function. Although studies in mice have supported these mechanisms, none has been shown to be directly responsible for the increased risk of coronary artery or cerebrovascular disease in humans.

Previous studies in our laboratory revealed that cholesterol-loaded macrophages, which are prominent cells in atherosclerotic lesions, and other cells can bind apo(a) and native Lp(a) with high affinity. The interaction with cells is mediated by sequences within the KIV_{5,8} domain. We postulated that this interaction, which is modulated by interferon-γ, may trigger a signal transduction pathway that affects atherogenesis. Of interest, Scanu et al have shown that Lp(a) is degraded in vivo by elastases, yielding a KIV_{5,8}-containing peptide that is found in atherosclerotic...
lesions and that has been postulated to be atherogenic in this milieu. In this context, the goal of this study was to determine the effect of the KIV5-8 peptide on atherogenesis in vivo. To accomplish this goal, we created several lines of KIV5-8 transgenic mice. Importantly, the plasma level of the apo(a) peptide in these mice, unlike those in mice or rabbits expressing full-length apo(a), was similar to the elevated levels of Lp(a) in humans at risk for coronary artery disease. We found dramatic effects on plasma RLP levels and atherogenesis that may have novel implications related to Lp(a) atherogenicity and RLP clearance in humans.

Methods

A detailed Methods section is available online at http://atvb.ahajournals.org.

Results

VLDL/Remnant Lipoprotein Cholesterol and Atherosclerosis Are Markedly Increased in KIV5-8 Transgenic Mice

Starting at 8 weeks of age, KIV5-8 transgenic and control littermate mice on the FVB background were fed a cholesterol-enriched, cholate-containing diet, and 18 weeks later the mice were analyzed for total and high-density lipoprotein (HDL) plasma cholesterol, plasma triglyceride, plasma lipoprotein profile, and atherosclerotic lesion size in the aortic root. At 8 weeks of age, just before dietary intervention, the plasma concentration of the KIV5-8 peptide in the transgenic mice was ~140 nmol, and this value did not change after cholesterol/cholate feeding. The average weights of the transgenic and control mice were almost identical (30.3±0.43 grams, and 30.3±0.35 grams for males, and 27.8±0.43 grams, and 27.3±0.47 grams for females, respectively). Before dietary intervention, the total plasma cholesterol was ~1.3-fold higher in transgenic mice than in control mice. As shown in Figure 1A and 1B, the total plasma cholesterol was increased ~3-fold in male transgenic mice and 1.5-fold in female transgenic mice after 18 weeks on the diet. Thus, ingestion of the cholesterol/cholate diet exacerbated this difference in cholesterol levels. Plasma HDL was relatively low and elevated ~2-fold in the male transgenics. Plasma triglyceride was also relatively low and increased ~1.8-fold in male transgenic mice. Similar results were obtained for an independent line of transgenic and control mice on the FVB background (data not shown).

The lipoprotein profiles in Figure 1C and 1D show that the rapidly eluting cholesterol-rich peak was much higher in transgenic mice, particular in male transgenic mice. There was a minor difference in the size of the slowly eluting HDL-peak, and the intermediate-eluting intermediate-density lipoprotein/LDL peak was modestly increased in male transgenic mice. The rapidly eluting fractions from male control and transgenic mice were subjected to SDS-polyacrylamide gel electrophoresis (Figure 1C, inset). ApoB100 and apolipoprotein B48 (apoB48) were increased in the fraction from transgenic mice, suggesting that the rapidly eluting peak contained both VLDL and remnant lipoproteins (RLPs). Moreover, the fraction from the transgenic mice contained the KIV5-8 peptide as well as markedly increased apolipoprotein E (apoE), which could be caused by longer resident time in the plasma. These data suggest that apoE is not displaced by KIV5-8 from the RLPs. Aortic root lesion area was markedly greater in male transgenic versus control mice (Figure 2A and 2B) and modestly increased in female transgenic versus control mice (Figure 2C). In a second line of transgenic mice on the FVB background, atherosclerosis was also increased compared
with control mice: $8896 \pm 1080$ versus $3949 \pm 661 \mu m^2$ (male) and $18168 \pm 1770$ versus $10438 \pm 1876 \mu m^2$ (female). Thus, the expression of the KIV$_{5-8}$ transgene is associated with increases in VLDL/RLP cholesterol and aortic root lesion area.

To examine the effect of the KIV$_{5-8}$ transgene in another genetic background, we performed a similar study to that described in mice on the C57BL/6J background. At 8 weeks of age, the total plasma cholesterol was 2-fold higher in transgenic mice than in control mice. As shown in Figure 3A and 3B, the C57BL/6J transgenic mice of both sexes had marked increases in total plasma cholesterol after dietary intervention. As stated, HDL levels were relatively low and moderately increased, particularly in the female mice. Plasma triglyceride was similar in male transgenic and control mice and modestly elevated in female transgenic mice. The lipoprotein profile showed a marked increase in the rapidly eluting peak (male profile shown in inset to Figure 3A). Finally, the aortic root lesion area was massively increased in transgenic versus control mice (Figure 3C and 3D).

The KIV$_{5-8}$ domain of apo(a) was originally chosen because this domain, but not the KIV$_2$ domain, was found to mediate the interaction of apo(a) with cells. Therefore, we determined whether K(IV$_2$)$_3$ transgenic mice had similar or different characteristics compared with KIV$_{5-8}$
Triglyceride, ApoB-100, and ApoB-48 Secretion Rate Are Not Altered in KIV5,8 Transgenic Mice

To determine whether the increase in plasma VLDL/RLP concentration in the transgenic mice was caused by enhanced secretion of triglyceride and apoB-100 and apoB-48, we conducted an in vivo study in male KIV5,8 transgenic and control mice on the FVB background after 18 weeks on the cholesterol/cholate diet. The mice were fasted for 4 hours and then injected intravenously with a solution containing Triton WR-1339 to block triglyceride clearance and [35S] methionine to measure apolipoprotein secretion. As shown in Figure IIIA (available online http://atvb.ahajournals.org), the baseline triglyceride level was ~3-fold higher in transgenic mice compared with control mice. However, the slopes of the 2 curves over time, which reflects secretion rate, were very similar. Moreover, there were no significant differences in apoB-100 or apoB-48 secretion rate between transgenic and control mice (Figure IIIB). These data suggest specificity of the KIV5,8 domain in influencing plasma VLDL/RLP cholesterol and aortic root lesion area.

Infusion of KIV5,8, Apo(a), or Lipoprotein(a) Decreases Chylomicron Remnant Clearance in Perfused Mouse Livers

To directly examine chylomicron remnant (CR) clearance, livers from control or KIV5,8 transgenic mice were perfused with 125I-labeled rat CRs in the absence or presence of apo(a) peptides or lipoproteins. As shown in Figure 4, both KIV5,8 peptide and full-length apo(a) significantly decreased the percent removal of CRs per pass through the livers of nontransgenic mice. Next, we compared the ability of freshly isolated control versus KIV5,8 transgenic livers to clear CRs in the absence of peptide coinfusion (Figure 5A). CR clearance by transgenic liver was significantly less than that by nontransgenic liver, most likely because KIV5,8 peptide perfuses the transgenic liver in vivo. We then assessed whether CR clearance by the transgenic liver could be lowered further by coinfusion with KIV5,8 peptide or, as a negative control, K(IV2)3 peptide. As shown in Figure 5B, CR clearance was further decreased by KIV5,8 peptide but not by K(IV2)3 peptide. Finally, we assessed blockage of CR clearance in transgenic liver by apo(a), lipoprotein(a), and LDL (Figure 6). Both apo(a) and lipoprotein(a) were as effective or more effective in blocking CR clearance, whereas LDL was a much less effective inhibitor of CR clearance.

Discussion

A discussion of the potential relevance of these findings to humans requires first an analysis of the model used in this study. Although human plasma contains full-length apo(a) covalently attached primarily to LDL, the mice here expressed a truncated apo(a) KIV5,8 peptide that lacked the domain (KIV5) necessary for covalent attachment to apoB-100, and the mice lacked human apoB-100 that is also necessary for this interaction. However, most of the peptide was associated with apoB-containing plasma lipoproteins, likely by noncovalent interactions, as demonstrated by immunoblots of the fast protein liquid fractions (Figure 1 and data not shown). Moreover, the fundamental finding of the study, namely the effect of the peptide on RLP clearance, was shown to occur with both full-length apo(a) and human Lp(a) in the perfused liver system. The major advance of this model over previous transgenic apo(a) models is that the plasma level of the apo(a) peptide in these mice was ~140 nmol, which is within the fourth quintile of Lp(a) levels in humans (134 to 279 nmol). In contrast, full-length apo(a) transgenic mice and rabbits have very low levels of apo(a), eg, 15 nmol and 11 nmol, respectively, which correspond to the lowest quintile of human Lp(a) levels (0 to 30 nmol). Thus, our mouse model provided a unique opportunity to observe the effects of an apo(a) peptide at levels similar to Lp(a) levels in humans at high risk for coronary artery disease. During the revision of this manuscript, Schneider et al described a
mouse with very high levels of Lp(a) in the plasma. Interestingly, the plasma VLDL particles of this mouse had increased triglyceride and cholesterol levels. Although these increases were less than those described here, the mice were fed a chow diet instead of the remnant-promoting cholesteryl-cholate diet used here. The authors speculated that VLDL particles with bound apo(a) may undergo slower proteolytic processing and thus accumulate in the plasma, which is consistent with our model. Finally, Scanni et al.\(^1\) have published a number of studies showing that free apo(a) peptides are found in human urine and are likely generated in the plasma by elastases acting on intact Lp(a). Most intriguingly, one of these peptides, termed “F2,” contains the KIV\(_{5-8}\) domain, and it is this peptide that is found in lesions and thought to be atherogenic.\(^1\)\(^,\)\(^1\)\(^6\)\(^,\)\(^2\)\(^3\)

A survey of the literature revealed no studies that were specifically designed to examine the potential relationship between Lp(a) levels and plasma remnant lipoprotein in humans. Apo(a) has been found on human apoE-containing triglyceride-rich lipoproteins and RLPs, which may represent up to 20% of apo(a)-containing particles.\(^1\)\(^,\)\(^2\)\(^,\)\(^3\)\(^\)\(^5\) Hoppichler et al.\(^2\)\(^4\) found that normolipidemic patients with coronary artery disease had a 2- to 4-fold higher percentage of apo(a) in the triglyceride-rich lipoprotein fraction compared with normolipidemic healthy subjects. Moreover, Song et al.\(^2\)\(^5\) showed an association between polymorphisms of triglyceride-rich lipoprotein receptors, including the VLDL receptor and LRP, and plasma levels of Lp(a), and suggested that these receptors may mediate the uptake of Lp(a) in humans. Argraves et al.\(^2\)\(^6\) showed that the VLDL receptor plays a role in the cellular uptake of Lp(a) both in cultured cells and in vivo, and Marz et al.\(^2\)\(^7\) reported that high-molecular-weight forms of Lp(a) can interact with LRP. Finally, Rader et al.\(^2\)\(^8\) observed that a patient deficient in apoE had elevated Lp(a) levels and relatively slow catabolism of a buoyant subpopulation of Lp(a). Thus, it is possible that certain subpopulations of Lp(a) compete for the uptake of remnant lipoproteins in humans. In addition, a potential relationship between apo(a)-containing lipoproteins and high levels of remnant lipoproteins might be missed if Lp(a) is quantified solely by isolating particles in the LDL density range.

The increase in cholesterol-rich VLDL/RLPs in the KIV\(_{5-8}\) transgenic mice undoubtedly contributes to the dramatic increase in atherosclerosis, because these types of lipoproteins are known to be atherogenic.\(^1\)\(^9\)\(^,\)\(^1\)\(^6\)\(^,\)\(^2\)\(^3\) However, it is possible that the peptide has an additional atherogenic effect at the level of the arterial wall. We attempted to obtain evidence for this mechanism by comparing subpopulations of control and transgenic mice whose plasma non-HDL cholesterol levels are overlapping. The only model in our study for which this analysis was possible was female FVB mice. There were 7 control mice and 13 transgenic mice in the non-HDL cholesterol range between 2.15 and 3.05 mg/mL. The average non-HDL cholesterol values were 2.49 ± 0.11 and 2.61 ± 0.07 mg/mL, respectively (P = 1.8; not significant). The average lesions areas in \(\mu\)m\(^2\) were 10 516 ± 1853 in the control mice and 18 785 ± 3178 in the transgenic mice, which represents a 1.8-fold statistically significant difference (P = 0.04).

Figure 5. Chylomicron remnant clearance is decreased in perfused liver from KIV\(_{5-8}\) transgenic mice compared with liver from control mice. A, \(^{125}\)I-CRs were perfused through control (black bars) or KIV\(_{5-8}\) transgenic (Tgic; red bar) liver. There was a significant difference in clearance between the groups (P < 0.05). B, KIV\(_{5-8}\) transgenic liver was perfused with \(^{125}\)I-CRs alone (black bars), \(^{125}\)I-CRs plus KIV\(_{5-8}\) peptide (red bars), and \(^{125}\)I-CRs plus KIV\(_{5-8}\) peptide (green bars). Coinfusion with KIV\(_{5-8}\) peptide delayed the clearance significantly (P < 0.03). There were 3 to 7 mice in each group.

Figure 6. Lp(a) is a better competitor of chylomicron remnant clearance than LDL in perfused livers from KIV\(_{5-8}\) transgenic mice. The perfusions consisted of \(^{125}\)I-CRs alone (black bars), \(^{125}\)I-CRs plus KIV\(_{5-8}\) peptide (red bars), \(^{125}\)I-CRs plus 17K apo(a) (green bars), \(^{125}\)I-CRs plus Lp(a) (yellow bars), and \(^{125}\)I-CRs plus LDL (turquoise bars). The KIV\(_{5-8}\), 17K apo(a), and Lp(a) coinfusion groups were statistically different from the \(^{125}\)I-CR alone group (P < 0.03). The LDL group was different from the \(^{125}\)I-CR alone group only at 11 and 13 to 16 minutes (P < 0.05). There were 4 to 9 mice in each group.
(data not shown). Moreover, as mentioned, human lesions contain an apo(a) peptide that encompasses the KIV5,8 sequence.17 Potential atherogenic mechanisms would be different from those involving the ability of apo(a) to inhibit plasminogen activation on fibrin or to increase smooth muscle cell proliferation, because sequences within KIV5,8 have not been implicated in these processes.9,10 However, the KIV5,8 region of apo(a) mediates a very high-affinity interaction of apo(a) and Lp(a) with cells, and this interaction is regulated by interferon-γ.12–15 The high-affinity and regulatable nature of the KIV5,8-mediated interaction of apo(a) with cells has led us to postulate that the interaction triggers one or more signal transduction pathways.14 However, the identity of the putative pathways and how they may promote atherosclerosis remains to be explored.

The data in this study support a model in which the KIV5,8 peptide, but not the K(IV3) peptide, delays clearance of VLDL/RLPs in vivo and in a perfused liver model. What might be the mechanism? Chylomicron remnant clearance is thought to involve apoE-dependent binding of these particles to heparan sulfate proteoglycans in the space of Disse followed by internalization by specific hepatic receptors, notably the LDL receptor and LRP.33,34 As mentioned, there is evidence that buoyant forms of Lp(a) can interact with LRP,27 and so it is possible that apo(a) and the KIV5,8 peptide competitively inhibit remnant clearance by LRP or possibly by other receptors or proteoglycans. This inhibition of binding to LRP may be caused by either the peptide itself or lipoprotein-associated peptide. We were unable to distinguish between these 2 possibilities, because we do not know how to prevent the interaction of the peptide with remnant lipoproteins.

In summary, we have created a novel mouse model that expresses levels of an apo(a) peptide that approach the concentrations of Lp(a) in subpopulations of humans at increased risk for coronary artery disease. These mice have increased levels of highly atherogenic, cholesterol-rich VLDL/RLP-type lipoproteins, which are likely caused by a defect in particle clearance. The marked increase in atherosclerosis in these mice is undoubtedly related to the elevated levels of these lipoproteins, but there may also be direct atherogenic effects of the peptide. Although critical questions regarding relevance to human pathophysiology and mechanisms remain, these findings may eventually provide new insight into the processes of remnant lipoprotein clearance and/or Lp(a) atherogenicity. Moreover, these results suggest the need to evaluate whether humans with high levels of Lp(a) have abnormalities in postprandial lipidaemia and higher circulating levels of RLPs.

Acknowledgments

This work was supported by National Institutes of Health grants HL61313 to I.T. and HL62583 to L.S.H. and Canadian Institutes of Health Research grant MOP 11271 to M.L.K. We thank Dr Santicia Marcovina (University of Washington, Seattle, Wash) for supplying Lp(a) and Dr Patrick Tso (University of Cincinnati) for supplying rat chylomicron remnants for the binding studies.

References


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Arterioscler Thromb Vasc Biol. published online May 19, 2005;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Methods

Mice

The KIV<sub>5,8</sub> cDNA construct<sup>1</sup> with SalI ends was inserted into the Xhol polylinker site of pLIV7, which contains the hepatic control regions of human apolipoprotein E3.<sup>2</sup> The insert-containing vector was injected into oocytes from either FVB or C57BL/6J mice. In the FVB background, two independent lines of transgenic mice were established. The data presented are from a line in which the KIV<sub>5,8</sub> transgene was inserted into the X chromosome. The other line of FVB transgenic mice, in which the transgene was not X-linked, yielded similar plasma lipoprotein and atherosclerosis results, as mentioned briefly in the Results section (see below). We confirmed by Southern blotting that for each of the chosen founder lines there was a single transgene integration site (data not shown). The presence of the KIV<sub>5,8</sub> transgene was determined by PCR using the primers KIV<sub>6DEL</sub> (5'-TTGTTCAGAAACAGCCG-3') and KIV<sub>SIG</sub> (5'-TCTGAAATCAGCAGCACC-3'). The PCR conditions were 30 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min. A band of 660 bp corresponded to the region between the end of the signal sequence and the end of KIV6.

For the K(IV<sub>2</sub>)<sub>3</sub> transgene, a single copy of KIV<sub>2</sub> was cloned into pRK5 (KIV<sub>2</sub>-pRK5) and digested with BamHI.<sup>3</sup> Next, sequences corresponding to two KIV<sub>2</sub> repeats, obtained by partial digestion of the apo(a) cDNA clone λa6<sup>4</sup> with BamHI, were inserted into KIV<sub>2</sub>-pRK5. This intermediate construct [K(IV<sub>2</sub>)<sub>3</sub>-pRK5] was then digested with SalI to release the signal sequence and kringle-containing insert. The insert was cloned into the Xhol site of the vector
pLIV7, and the resulting construct, designated K(IV₂₃)-pLIV, was used to create a transgenic mouse line in the FVB background. PCR analysis was used to detect the transgene using the primers KIVSIG (above) and KIV₂ (5'-GTTCGGAAGGAGCCTCTAGGC-3') under identical conditions to those described above. A band of approximately 350 bp corresponded to the size of a single kringle. Beginning at 8 weeks of age and continuing for 11 or 18 weeks, the mice were fed a diet containing 1.25% cholesterol, 7.5% cocoa butter, 7.5% casein, and 0.5% sodium cholate (TD88051 from Harlan Teklad, Madison, WI).⁵ All mice were maintained in a 12h light/dark cycle (light cycle 7:00 a.m.-7:00 p.m.) and handled in accordance with institutional guidelines.

**Apo(a) Peptides and Lipoproteins (Supplementary Fig. I, see below)**

The preparation and purification of KIV₅₈ and 17K apo(a) proteins was as described previously.⁴ For K(IV₂₃), conditioned medium from HEK-293 cells stably transfected with K(IV₂₃)-pLIV was passed over a lectin-Sepharose (Amersham Biosciences) column. The column was washed with HEPES-buffered saline (HBS; 20 mM HEPES pH 7.4, 150 mM NaCl) containing 1 M NaCl, and bound proteins were eluted by the addition of HBS containing 1 M NaCl and 0.5 M N-acetyl-D-glucosamine (Sigma-Aldrich). K(IV₂₃)-containing fractions were pooled, dialyzed extensively against HBS, and concentrated using polyethylene glycol (PEG) 20000 (Fluka). The final protein concentration was determined by measuring absorbance at 280 nm, with correction for Rayleigh light scattering, using a calculated extinction coefficient (E₀.1%= 1.60). Protein purity was assessed by SDS-PAGE followed by Coomassie blue staining. Lp(a), prepared as previously described⁷, was a kind gift from Dr. Santica Marcovina (University of Washington, Seattle, WA). LDL (density 1.020-1.063 g/ml) from fresh human plasma was isolated by...
to measure KIV$_{5.8}$ levels in mouse plasma, immunoglobulins were removed from plasma by incubation with protein A-Sepharose. The pre-cleared plasma was then incubated with a sheep anti-apo(a) polyclonal antibody (Ab5-33; kind gift of Hugh Hoogendorn, Affinity Biologicals Inc., Hamilton, Ontario, Canada). Immuno-complexes were then isolated by protein A-Sepharose precipitation; these samples, along with known amounts of purified KIV$_{5.8}$, were subjected to SDS-PAGE on 8% gels, followed by Western blotting using the same apo(a) antibody. The level of K(IV$_2$)$_3$ in the plasma was determined by sandwich ELISA using purified recombinant apo(a) containing three copies of KIV$_2$ as a standard. The antibody used for capture was Ab5-33 (see above); protein was detected using an anti-apo(a) monoclonal antibody.

**Plasma Lipoprotein Analysis and Aortic Root Atherosclerosis Assay**

The night before analysis, food was removed from the cages, and the mice were fasted for 16 h. The animals were then anesthetized, blood was withdrawn by cardiac puncture, and the heart was perfused with PBS. The heart and aorta were removed, perfused with PBS followed by 10% buffered formalin, embedded in OCT compound (Sakura Finetek, Torrance, CA), snap-frozen in an ethanol-dry ice bath, and stored at -70°C. Ten-micron sections were cut at -20°C using a Microm microtome cryostat HM 505E (Walldorf, Germany). Starting from the atrial leaflets, every eighth section was retained for analysis for a total of ten sections. To evaluate lesion area, the sections were stained with Oil Red O for neutral lipid and with Harris hematoxylin for nuclei. Lesion area was quantified by using a Nikon Labophot-2 microscope equipped with a Sony CCD-Iris/RGB color video camera attached to a computerized imaging system using IMAGE-
PRO PLUS 4.1 software. The mean area of intimal lipid accumulation per section from six sections was determined in a blinded fashion for individual animals.

Total plasma cholesterol and triglyceride was determined by using commercial enzymatic kits (Wako Chemicals GmbH). Plasma high-density lipoprotein (HDL) cholesterol was determined after dextran sulfate-Mg\textsuperscript{2+} precipitation of apoB-containing lipoproteins. Plasma lipoproteins were analyzed by an FPLC system consisting of two Superose 6 columns connected in series (Amersham Pharmacia). The cholesterol content of each fraction was measured by enzymatic assay. To assess the protein component of the lipoproteins, portions of six sequential FPLC fractions were pooled and concentrated 30- to 50- fold using Microcon YM10 centrifugal filter units (Millipore Corporation, Bedford, MA). Equal aliquots of each concentrated pool were loaded onto a 4-20% SDS-polyacrylamide gel (Invitrogen Corporation, Carlsbad, CA). After electrophoresis, the gel was stained with 0.05% Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO) and extensively destained to remove the excess dye.

Assay of Triglyceride and Apolipoprotein B Secretion Rate in Mice

Mice were fasted for 4 h and then injected intravenously with a solution containing 200 µCi \[^{35}\text{S} \text{methionine} \] and 500 mg/kg Triton WR-1339 (Sigma) in 0.9% NaCl. Triton WR-1339 inhibits the clearance of plasma VLDL.\textsuperscript{10} Blood samples were collected at just prior to injection (0 min) and at 60 and 120 min after injection. Aliquots of plasma were assayed for triglyceride or subjected to 4% SDS-polyacrylamide gel electrophoresis. The gels were dried and exposed to X-ray film to visualize labeled apolipoprotein B proteins. Both apo B-100 and apo B-48 bands were excised from the dried gels and counted in scintillation fluid in a liquid scintillation
counter. Protein counts were normalized by TCA-precipitable counts and the normalized values and secretion rates of apo B-100 and apo B-48 were determined as described previously.11

**Perfusion of Isolated Mouse Livers with Chylomicron Remnants**

Rat chylomicron remnants were prepared and labeled with $^{125}$I as previously described.12-14 A single pass non-recirculating procedure was used to perfuse the livers of either control FVB or KIV$_{5,8}$ transgenic FVB mice.15 After a 5-min perfusion to remove blood from the liver, the perfusate solution containing $^{125}$I-labeled chylomicron (μg/ml) was perfused into the liver via the portal vein for 20 min remnants (1.5 at 0.5 ml/min. In certain experiments, apo(a) peptides or Lp(a) were added to the perfusate. Each protein (90 pmol) was incubated with $^{125}$I-labeled chylomicron remnants for 2 h at 37°C before perfusion, and the peptide or lipoprotein was also added to the pre-perfusion washing solution. We chose 90 pmol of apo(a) peptide because this was equimolar to the amount of apolipoprotein E that was present on the chylomicron remnant particles in the perfusate. The perfusate (exiting via the inferior vena cava) was collected at 1 min intervals and the amount of radioactivity in each sample was measured to determine the remnant uptake kinetics. To calculate the percent of $^{125}$I removal per pass the following formula was used: Percent $^{125}$I removed per pass = [$(cpm$ entering liver – $cpm$ exiting liver) / ($cpm$ entering liver)] X 100.

**Statistical Analysis**

Data are reported as means ± SE. Plasma cholesterol and aortic root lesion area were not normally distributed and were therefore analyzed by the Mann-Whitney nonparametric test. The lesional data was also square-root transformed (see figure legends) to achieve a normal
distribution.\textsuperscript{16} For the chylomicron clearance experiments, the unpaired, two-tailed $t$ test was used to compare each experimental group with the control group.

References


3. Sangrar W, Marcovina SM, Koschinsky ML. Expression and characterization of apolipoprotein(a) kringle IV types 1, 2 and 10 in mammalian cells. \textit{Protein Eng} 1994;7:723-731.


7. Bottalico LA, Keesler GA, Fless GM, Tabas I. Cholesterol loading of macrophages leads to marked enhancement of native lipoprotein(a) and apoprotein(a) internalization and degradation. \textit{J Biol Chem} 1993;268:8569-8573.


Supplementary Figure I. Schematic representation of the apo(a) variants used in this study. The numbering of the ten KIV subtypes is shown above the 17K diagram. V and P designate the KV and protease domains, respectively. Open boxes represent the KIV$_2$ repeats, and the small black box to the left of KIV$_1$ designates the signal sequence. 17K is the full-length 17-kringle recombinant apo(a). KIV$_{5-8}$ contains one copy each of the apo(a) KIV domains 5 through 8. K(IV$_2$)$_3$ contains 3 copies of the KIV$_2$ domain.
Supplementary Figure II. K(IV<sub>2</sub>)<sub>3</sub> transgenic mice in the FVB background have similar plasma lipid levels and aortic root lesion areas compared with non-transgenic mice. A, Littermate combined-sex control (n = 24) and K(IV<sub>2</sub>)<sub>3</sub> transgenic (Tgic) (n = 20) mice were fed a cholate-containing, cholesterol-enriched diet for 18 weeks and then analyzed for total plasma and HDL cholesterol and triglyceride concentrations. B, Average lesion areas from the combined-sex control (hatched bars) and transgenic (black bars) mice described above. Control, hatched bars; transgenic, black bars. The minor differences in lipids and lesion area were not statistically significant.
Supplementary Figure III. Triglyceride and apolipoprotein B secretion rates in control and KIV\textsubscript{5-8} transgenic mice are similar. Fasted mice (5-7 in each group) similar to those described in the legend to Fig. 1 were injected with Triton WR-1339 and $[^{35}\text{S}]$ methionine and blood was collected at 0, 1 and 2 hr. Plasma was analyzed for the triglyceride concentration (A) and $^{35}\text{S}$-labeled apoB-100 and apoB-48 (B). The minor differences in triglyceride and apolipoprotein secretion rates were not statistically significant.