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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, $K(IV_2)_3$, 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;25:1704-1710.)**

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.[1,2] Apo(a) can also be noncovalently associated with the apoB of very-low-density lipoprotein (VLDL) and remnant lipoproteins (RLPs).[3–5] Apo(a) is made up of variable numbers of protein domains called kringles, designated KIV_1 through KIV_{10} (Figure I, available online http://atvb.ahajournals.org).[6] 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.[7,8] 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.[9–11] 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.[12–14] The interaction with cells is mediated by sequences within the KIV_{5-8} domain.[14] We postulated that this interaction, which is modulated by interferon-γ, may trigger a signal transduction pathway that affects atherogenesis.[14,15] Of interest, Scanu et al[16,17] have shown that Lp(a) is degraded in vivo by elastases, yielding a KIV_{5-8}-containing peptide that is found in atherosclerotic

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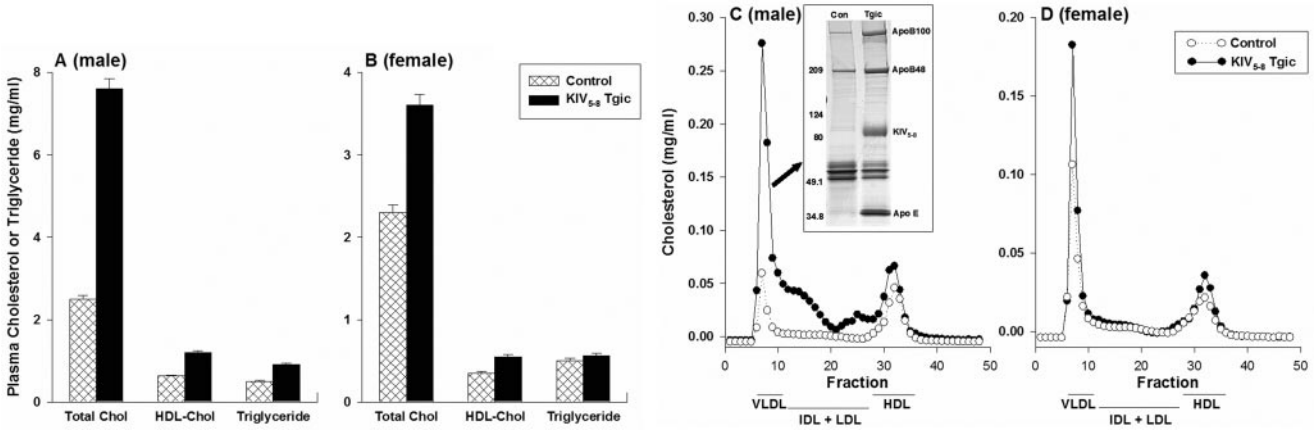

Figure 1. KIV_{5-8} transgenic mice in the FVB background have a marked increase in plasma non-HDL cholesterol. A and B, Littermate male control (n=25) and KIV_{5-8} transgenic (*Tgic*) (n=32) mice and female control (n=28) and KIV_{5-8} transgenic (n=37) mice were fed a cholesterol/cholate diet for 18 weeks and then analyzed for total plasma and HDL cholesterol and triglyceride concentrations. Control, hatched bars; transgenic, black bars. The transgenic total plasma cholesterol value and the transgenic HDL cholesterol value were statistically different from the control values for both sexes ($P<1.5\times10^{-6}$). Only the male transgenic triglyceride value was statistically different from the control value ($P=2\times10^{-9}$). C and D, The plasma cholesterol fast protein liquid chromatography (FPLC) profiles corresponding to these 4 groups of mice. Control, open circles; transgenic, closed circles. The various lipoprotein fractions are represented by lines beneath the graphs: VLDL, 6 to 11; intermediate-density lipoprotein and LDL, 12 to 27; and HDL, 28 to 35. Inset in C, SDS-polyacrylamide gel electrophoresis (4% to 20%) of FPLC fractions 6 to 11 from control and KIV_{5-8} transgenic FVB male mice. The molecular mass markers (kDa) are indicated on the left.

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 KIV_{5-8} peptide on atherogenesis in vivo. To accomplish this goal, we created several lines of KIV_{5-8} 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.[18–20] 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 KIV_{5-8} Transgenic Mice

Starting at 8 weeks of age, KIV_{5-8} 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 KIV_{5-8} peptide in the transgenic mice was ≈140 nmol/L, 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, particularly 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 KIV_{5-8} 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 KIV_{5-8} 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

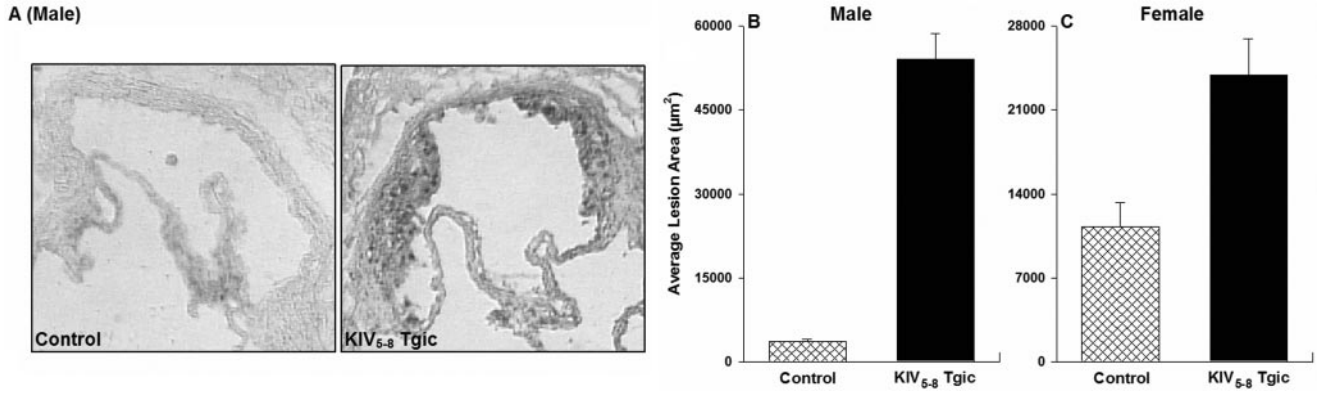

Figure 2. KIV_{5-8} transgenic mice in the FVB background have a marked increase in aortic root atherosclerosis. A, Oil Red O-stained aortic root sections from the control and transgenic (*Tgic*) male mice described. B and C, Average areas of aortic root lesions from the male and female control (hatched bars) and transgenic (black bars) mice described in Figure 1. The transgenic mice had statistically larger lesions than the controls ($P=1\times10^{-8}$ and 7×10^{-4} for males and females, respectively). The square root-transformed values for male control and transgenic lesion sizes were 59.2±3.3 and 226.3±10.9 μm, respectively. The square root-transformed values for the female control and transgenic lesion sizes were 97.3±9.0 and 145.8±9.4 μm, respectively.

with control mice: 8896±1080 versus 3949±661 μm^2 (male) and 18 168±1770 versus 10 438±1876 μm^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 above 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 above, 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.[14] Therefore, we determined whether $K(IV_2)_3$ transgenic mice had similar or different characteristics compared with KIV_{5-8}

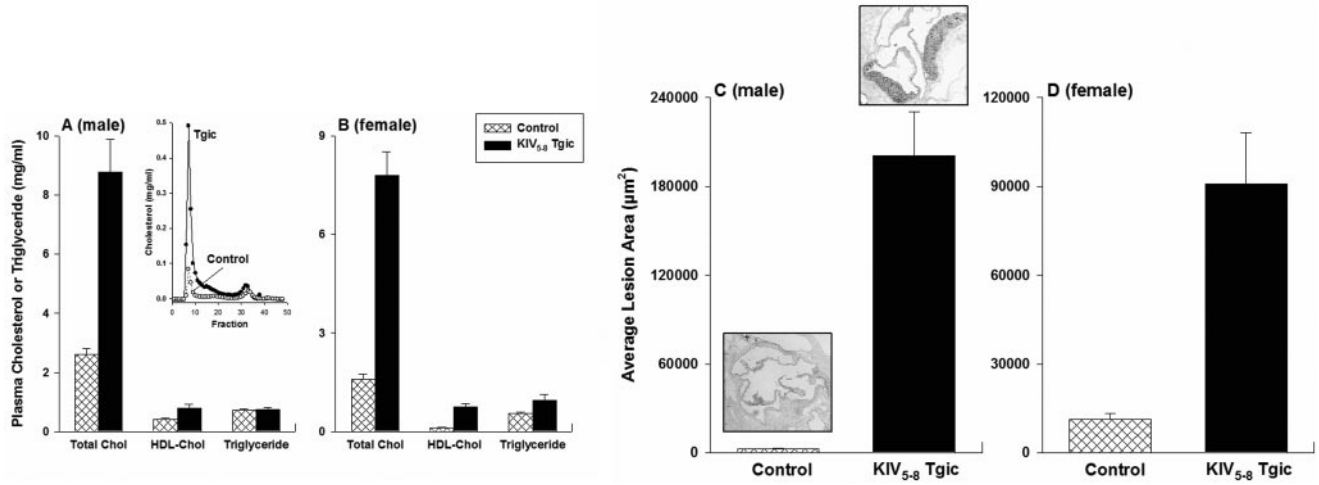

Figure 3. KIV_{5-8} transgenic mice in the C57BL/6J background have a marked increase in plasma non-HDL cholesterol and aortic root atherosclerosis. A and B, Littermate male control (n=10) and KIV_{5-8} transgenic (*Tgic*) (n=11) mice and female control (n=11) and KIV_{5-8} transgenic (n=10) mice were fed a cholesterol/cholate diet for 11 weeks and then analyzed for total plasma and HDL cholesterol and triglyceride concentrations. Control, hatched bars; transgenic, black bars. The transgenic total plasma cholesterol value and the transgenic HDL cholesterol value were statistically different from the control values for both sexes ($P<0.01$). Only the female transgenic triglyceride value was statistically different from the control value ($P=0.007$). Inset in A, The plasma cholesterol FPLC profiles corresponding to male control and transgenic mice. Control, open circles; transgenic, closed circles. C and D, Average areas of aortic root lesions from male and female control (hatched bars) and transgenic (black bars) mice. The transgenic mice had statistically larger lesions than the controls ($P=2\times10^{-4}$ and 7×10^{-5} for males and females, respectively). The square root-transformed values for male control and transgenic lesion sizes were 43.2±8.13 and 437.8±32.0 μm, respectively. The square root-transformed values for the female control and transgenic lesion sizes were 100.7±10.6 and 288.5±27.5 μm, respectively. Insets in C, Oil Red O-stained sections of aortic root sections from the control and transgenic male mice.

transgenic mice. Importantly, the plasma level of the $K(IV_2)_3$ peptide in these mice (245 nmol/L) was even higher than that of the KIV_{5-8} peptide in the KIV_{5-8} transgenic mice. The plasma level of this peptide was not influenced by diet. As shown in Figure IIA (available online http://atvb.ahajournals.org), $K(IV_2)_3$ transgenic and control mice on the FVB background had similar total cholesterol, HDL cholesterol, and triglyceride levels in the plasma. Moreover, the total cholesterol levels in both transgenic and control mice were the same before and after diet. Moreover, lesion areas in the aortic root were relatively small and similar between $K(IV_2)_3$ transgenic and control mice (Figure IIB). These data demonstrate specificity of the KIV_{5-8} domain in influencing plasma VLDL/RLP cholesterol and aortic root lesion area.

Triglyceride, ApoB-100, and ApoB-48 Secretion Rate Are Not Altered in KIV_{5-8} 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 KIV_{5-8} 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 [^{35}S] 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 that the increase in VLDL/RLP in the transgenic mice was primarily caused by a decrease in clearance of these particles.

Infusion of KIV_{5-8}, Apo(a), or Lipoprotein(a) Decreases Chylomicron Remnant Clearance in Perfused Mouse Livers

To directly examine chylomicron remnant (CR) clearance, livers from control or KIV_{5-8} transgenic mice were perfused with ^{125}I-labeled rat CRs in the absence or presence of apo(a) peptides or lipoproteins. As shown in Figure 4, both KIV_{5-8} 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 KIV_{5-8} 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 KIV_{5-8} peptide perfuses the transgenic liver in vivo. We then assessed whether CR clearance by the transgenic liver could be lowered further by coinfusion with KIV_{5-8} peptide or, as a negative control, $K(IV_2)_3$ peptide. As shown in Figure 5B, CR clearance was further decreased by KIV_{5-8} peptide but not by $K(IV_2)_3$ 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.

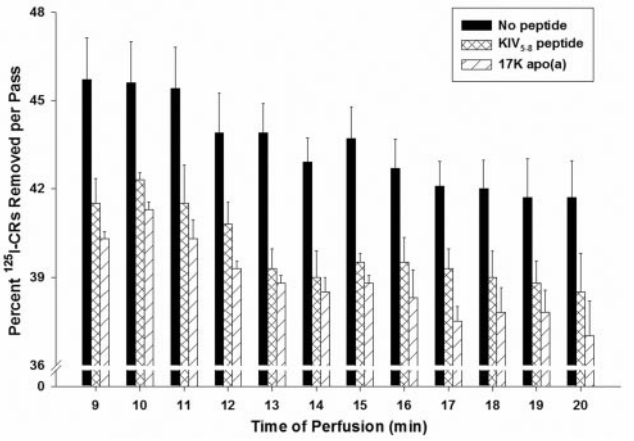

Figure 4. KIV_{5-8} peptide and 17K apolipoprotein(a) partially inhibit the clearance of chylomicron remnants in perfused FVB mouse liver. Shown is the percent removal of ^{125}I-labeled rat chylomicron remnants (CRs) removed per pass at 9 to 20 minutes after the start of the perfusion. The perfusions consisted of ^{125}I-CRs alone (black bars), ^{125}I-CRs plus KIV_{5-8} peptide (cross-hatched bars), and ^{125}I-CRs plus 17K apo(a) (diagonal-hatched bars). The amounts of apo(a) protein added were equimolar to the apolipoprotein E on the CRs. There were statistical differences between ^{125}I-CRs alone and ^{125}I-CRs plus KIV_{5-8} peptide ($P \leq 0.04$) and between ^{125}I-CRs alone and ^{125}I-CRs plus 17K apo(a) ($P \leq 0.02$). There were 4 to 7 mice in each group.

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) KIV_{5-8} peptide that lacked the domain (KIV_9) necessary for covalent attachment to apoB-100, and the mice lacked human apoB-100 that is also necessary for this interaction.[21] However, most of the peptide was associated with apoB-containing plasma lipoproteins, likely by noncovalent interactions, as demonstrated by immunoblots of the FPLC (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/L, which is within the fourth quintile of Lp(a) levels in humans (134 to 279 nmol/L).[20] In contrast, full-length apo(a) transgenic mice and rabbits have very low levels of apo(a), eg, 15 nmol/L and 11 nmol/L, respectively,[18,19] which correspond to the lowest quintile of human Lp(a) levels (0 to 30 nmol/L). 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[22] described a

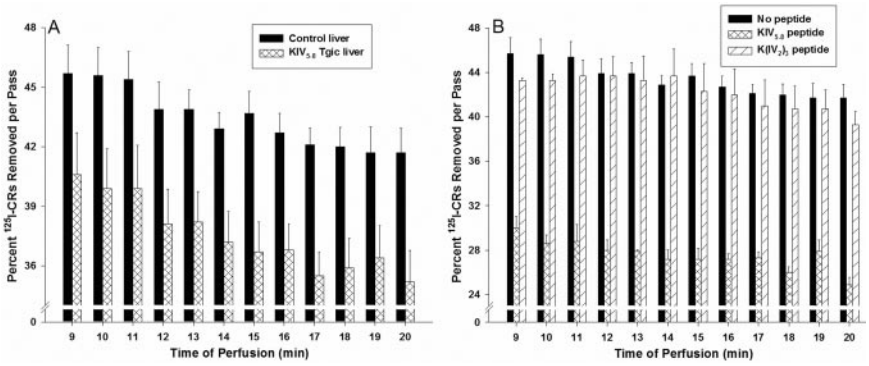

Figure 5. Chylomicron remnant clearance is decreased in perfused liver from KIV_{5-8} transgenic mice compared with liver from control mice. A, ^{125}I-CRs were perfused through control (black bars) or KIV_{5-8} transgenic (*Tgic*; cross-hatched bars) liver. There was a significant difference in clearance between the groups ($P \leq 0.025$). B, KIV_{5-8} transgenic liver was perfused with ^{125}I-CRs alone (black bars), ^{125}I-CRs plus KIV_{5-8} peptide (cross-hatched bars), and ^{125}I-CRs plus $K(IV_2)_3$ peptide (diagonal-hatched bars). Coinfusion with KIV_{5-8} peptide delayed the clearance significantly ($P \leq 0.03$). There were 3 to 7 mice in each group.

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 cholesterol/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, Scanu et al[16,23] 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.[10,16,23]

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.[3–5] Hoppichler et al[24] 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[25] 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[26] 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[27] reported that high-molecular-weight forms of Lp(a) can interact with LRP. Finally, Rader et al[28] 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.

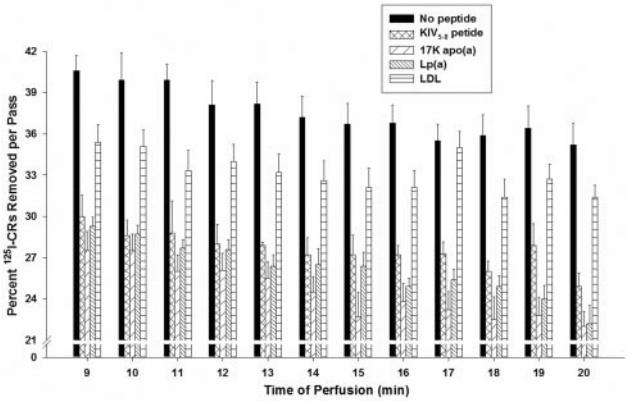

Figure 6. Lp(a) is a better competitor of chylomicron remnant clearance than LDL in perfused livers from KIV_{5-8} transgenic mice. The perfusions consisted of ^{125}I-CRs alone (black bars), ^{125}I-CRs plus KIV_{5-8} peptide (cross-hatched bars), ^{125}I-CRs plus 17K apo(a) (coarse diagonal-hatched bars), ^{125}I-CRs plus Lp(a) (fine diagonal-hatched bars), and ^{125}I-CRs plus LDL (horizontal-hatched bars). The KIV_{5-8}, 17K apo(a), and Lp(a) coinfusion groups were statistically different from the ^{125}I-CR alone group ($P \leq 0.03$). 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.

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.[29–32] 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 μm^2 were $10\,516 \pm 1853$ in the control mice and $18\,785 \pm 3178$ in the transgenic mice, which represents a 1.8-fold statistically significant difference ($P = 0.04$). Pending future studies to specifically address this issue, these data may indicate a partial effect on lesion area that is independent of plasma non-HDL cholesterol, at least in this subpopulation of mice. Of potential interest in this regard, we have shown by immunohistochemistry, using the same sheep antibody used for the Western blots, that

the peptide is present in the lesions of the transgenic mice (data not shown). Moreover, as mentioned, human lesions contain an apo(a) peptide that encompasses the KIV_{5-8} 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 KIV_{5-8} have not been implicated in these processes.[9,10] However, the KIV_{5-8} 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 KIV_{5-8}-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 KIV_{5-8} peptide, but not the $K(IV_2)_3$ 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 KIV_{5-8} 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 lipidemia and higher circulating levels of RLPs.

Acknowledgments

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