Transgenic Rabbits Expressing Human Apolipoprotein(a) Develop More Extensive Atherosclerotic Lesions in Response to a Cholesterol-Rich Diet

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Abstract—High lipoprotein(a) [Lp(a)] levels constitute an independent risk factor for the development of atherosclerosis. However, the relationship between Lp(a) and atherosclerosis is not fully understood. To examine the effect of Lp(a) on the development of atherosclerosis, we studied transgenic rabbits expressing human apolipoprotein(a) [apo(a)], which was assembled into Lp(a) in the plasma. Human apo(a) transgenic rabbits fed a 0.3% cholesterol diet for 16 weeks had more extensive atherosclerotic lesions than did nontransgenic rabbits, although the cholesterol levels in the plasma of both groups were similarly elevated. Compared with the lesions in control rabbits, the areas of the atherosclerotic lesions in human apo(a) transgenic rabbits were significantly increased in the aorta, the iliac artery, and the carotid artery. Furthermore, human apo(a) transgenic rabbits on a cholesterol-rich diet had a greater degree of coronary atherosclerosis than did control rabbits. Immunohistochemical analysis revealed that human apo(a) was frequently deposited in the atherosclerotic lesions of transgenic rabbits. We conclude that Lp(a) may have proatherogenic effects in the setting of a cholesterol-rich diet in transgenic rabbits. **(*Arterioscler Thromb Vasc Biol*. 2001;21:88-94.)**

Key Words: apolipoproteins ■ atherosclerosis ■ lipoproteins ■ transgenic rabbits

High plasma levels of Lp(a) are associated with atherosclerotic diseases. In many human studies, elevated levels of plasma Lp(a) have been found to be associated with an increased risk of atherosclerotic coronary heart disease, stroke, and restenosis,[1–3] although several studies did not detect such an association.[4,5] The involvement of Lp(a) in the pathogenesis of atherosclerosis has been strongly suggested by the presence of Lp(a) in human atherosclerotic lesions.[6,7]

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The Lp(a) particle closely resembles LDL in lipid composition and in the presence of apoB-100. Lp(a) is distinguished from LDL by the presence of an additional protein component designated apo(a), which is complexed to apoB-100 by a disulfide linkage.[1] Some difficulties in determining the role of Lp(a) in atherosclerosis in vivo have been encountered because of the lack of appropriate experimental animals. This is because apo(a) is naturally present exclusively in Old World monkeys and humans, whereas 1 nonprimate species, the hedgehog, has independently evolved an apo(a)-like protein.[8] The development of transgenic mice expressing human apo(a) has provided an alternative means for the study of apo(a) functions.[9,10] However, unlike apo(a) in humans, in which nearly all plasma apo(a) is associated with apoB-100, the human apo(a) in the transgenic mice circulates in a free form in the plasma rather than in association with the murine LDL.[11] In spite of this, expression of human apo(a) resulted in increased aortic atherosclerosis when these apo(a) transgenic mice were fed a high-fat diet,[9,12,13] although several studies failed to demonstrate the atherogenic effect of apo(a), even when double-transgenic mice expressing human apo(a) plus apoB-100 were used.[14,15]

To study the metabolic and pathological consequences of Lp(a), we[16] and others[17] generated transgenic rabbits expressing human apo(a) and demonstrated that human apo(a) is associated with rabbit apoB to form Lp(a)-like particles in the plasma. Apart from Lp(a) formation, rabbits have some unique features of lipoprotein metabolism and rapidly develop atherosclerosis when fed a cholesterol-rich diet.[18] To determine whether Lp(a) would increase the susceptibility to atherosclerosis, we studied the response of the human apo(a) transgenic rabbits to a cholesterol diet and quantified the extent of atherosclerosis in transgenic and nontransgenic rabbits.

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TABLE 1. Plasma Lipid and Human Apo(a) Levels in Transgenic Rabbits on Chow and Cholesterol-Rich Diets for 16 Weeks

	Chow Diet				Cholesterol Diet			
	Total Cholesterol, mg/dL	Triglycerides, mg/dL	HDL-C, mg/dL	Human Apo(a), nmol/L	Total Cholesterol, mg/dL	Triglycerides, mg/dL	HDL-C, mg/dL	Human Apo(a), nmol/L
Male								
Transgenic rabbits (n=5)	40±17	37±4.3	23±6	11.88±6.7	673±80	77±31	ND	26.95±12.9
Nontransgenic rabbits (n=7)	35±14	46±11	28±9	0	761±261	69±46	ND	0
Female								
Transgenic rabbits (n=6)	62.3±23	39.2±7	40±8	11.3±4.2	1388±656*	61±14	54.7±5	29.37±11
Nontransgenic rabbits (n=8)	61.2±26	37.1±5.4	39±9	0	1033±375	42±8	52.2±9	0

Values are mean±SD. ND indicates not determined.
*$P<0.05$ vs nontransgenic control.

Methods

Rabbits

Transgenic rabbits expressing human apo(a) were produced by use of a protocol described previously.[16] The human apo(a) transgenic construct consists of the full-length human apo(a) cDNA fragment containing 17 copies of kringle 4 repeats under the control of the mouse transferrin promoter (pTfHa17) as described previously.[11] Eleven heterozygous transgenic (5 males and 6 females) and 15 nontransgenic (7 males and 8 females) littermate rabbits aged 4 to 5 months were fed a diet containing 0.3% cholesterol and 3% soybean oil by weight for 16 weeks. To monitor the response to a cholesterol-rich diet, blood was drawn from the auditory medial artery weekly for the first 4 weeks and biweekly thereafter. All animal experiments were performed with the approval of the Animal Research Committee of the University of Tsukuba.

Analysis of Plasma Lipids and Lipoproteins

Plasma total cholesterol, HDL cholesterol (HDL-C), and triglycerides were measured by use of enzymatic assays (Wako Chemicals). Plasma lipoprotein analysis was performed after rabbits were fed the cholesterol-rich diet for 8 and 16 weeks, as described.[19] The plasma levels of human apo(a) and Lp(a) formation in transgenic rabbits were studied as described.[20]

Quantification of Atherosclerotic Lesions

Rabbits were euthanized with an overdose injection of sodium pentobarbital solution. A thoracotomy and laparotomy were performed to expose the heart and the entire "arterial tree," which included both common carotid arteries, the whole aorta, and both common iliac arteries. They were removed and immediately immersed in cold PBS, and the adventitial fat was carefully removed. Arterial trees were opened longitudinally and pinned out flat on Styrofoam sheets and stained with Sudan IV after fixing in 10% neutral buffered formalin for 24 hours. To measure the atherosclerotic lesions, the entire arterial trees were photographed with Fujichrome Sensia II film, and the slides were scanned at a resolution of 1012 pixels in 24-bit full color with use of a Polascan 35 Ultra scanner (Polaroid Co). The area of sudanophilic lesions relative to the surface area of each artery was measured by use of a computerized MacSCOPE image analysis system (Mitani Corp) and expressed as a percentage of the area of the whole artery. The left coronary artery was used for the evaluation of coronary atherosclerosis, as described.[21]

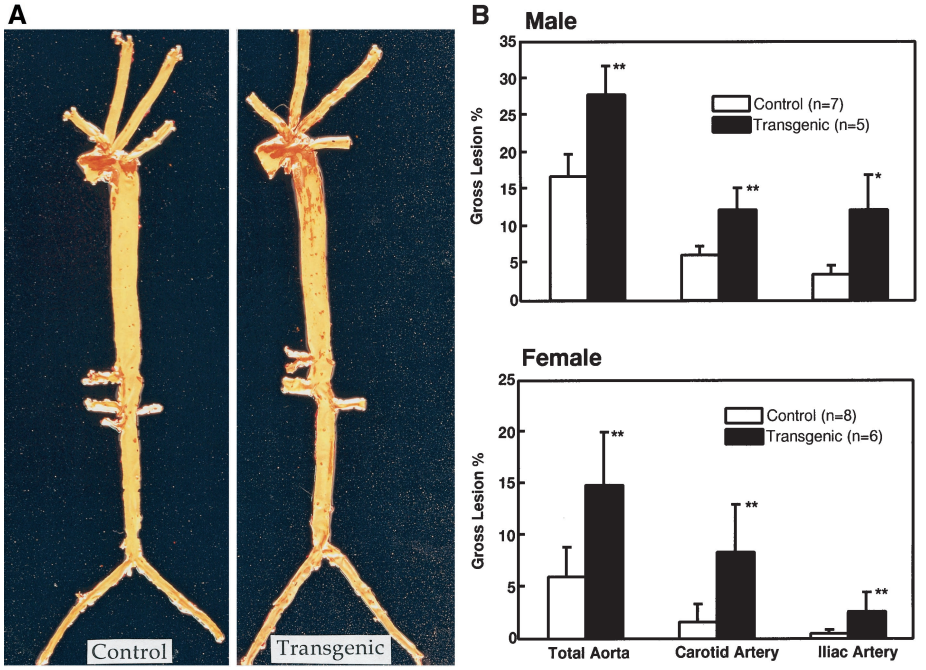

Figure 1. Atherosclerotic lesions in human apo(a) transgenic rabbits. A, Representative photographs of pinned out arterial trees from a male transgenic rabbit (right) and a nontransgenic control rabbit (left). B, Quantitative analysis of the lesion areas in each artery covered by Sudan IV staining. **$P<0.05$ and *$P=0.05$ for transgenic rabbits vs nontransgenic control rabbits.

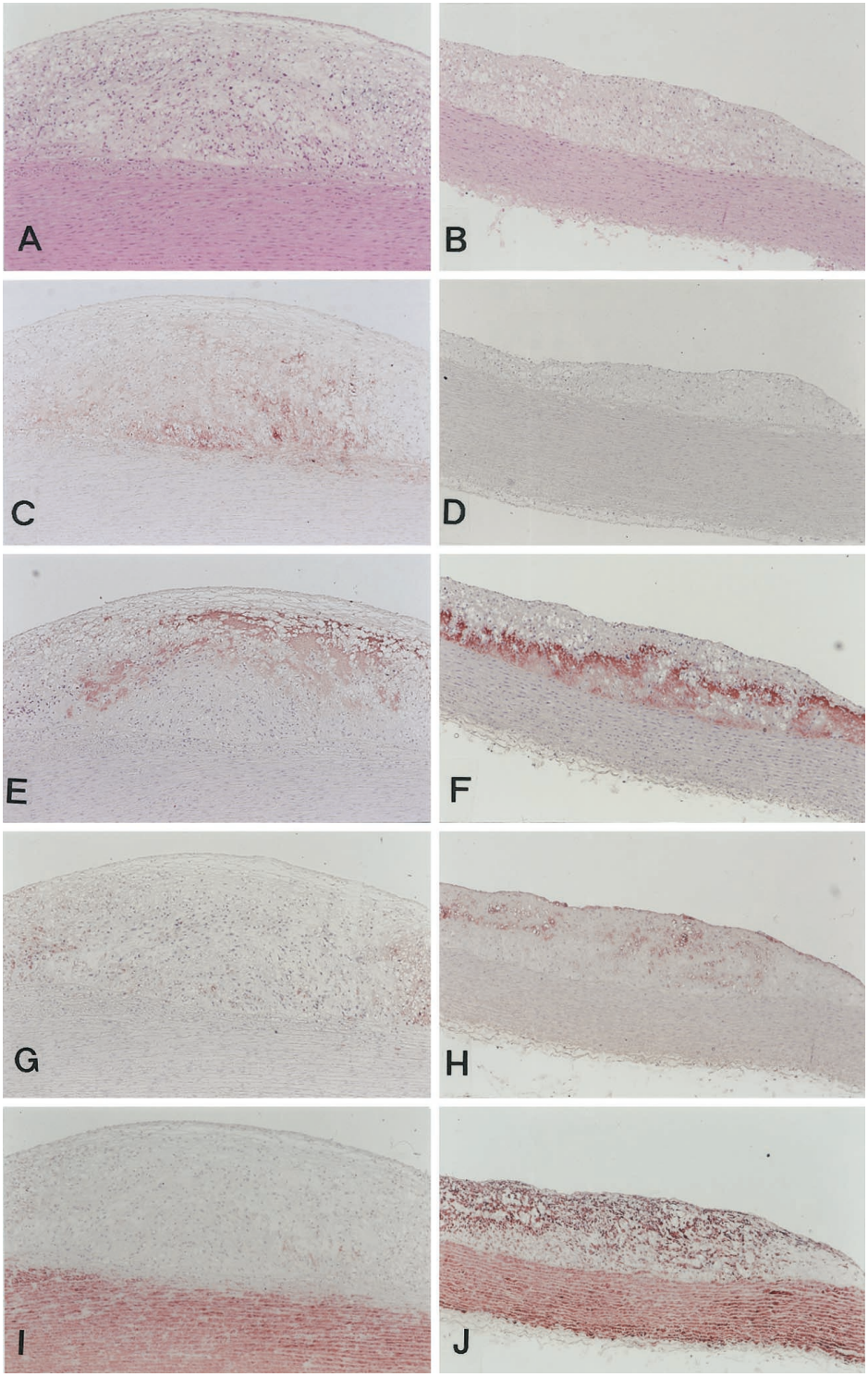

Figure 2. Histological and immunohistochemical analysis of aortic atherosclerosis in transgenic and nontransgenic rabbits. Serial sections (5 μm thick) were taken at the thoracic aorta at the same position for each aorta and stained with hematoxylin-eosin (A and B), anti-human apo(a) (C and D), anti-apoB (E and F), anti-macrophage (G and H), anti–smooth muscle α-actin (I and J), anti-vimentin antibodies (K and L), anti-human apo(a) (M), anti-macrophage (N), and anti–smooth muscle α-actin antibodies (O). Magnification was as follows: panels A to J, $\times$50; panels K and L, $\times$100; and panels M to O, $\times$200. Note that mature SMCs in the media are clearly stained with anti–α-actin mAb. In foam cell–rich areas, there is apo(a) deposition in the extracellular matrix, but it is not associated with macrophages or SMCs (M to O).

Histology and Immunohistochemical Staining

For qualitative characterization of the lesions, segments of the aorta and coronary artery from selected animals were cut into cross sections, embedded in paraffin, and stained with hematoxylin-eosin and elastica–van Gieson. To study cellular components and lipoprotein deposits in the lesions, immunohistochemical staining with anti-rabbit macrophage monoclonal antibody (mAb, RAM-11), anti–smooth muscle α-actin HHF-35 mAb (Enzo Biochemicals), anti-vimentin mAb (Enzo), anti-apoB polyclonal serum (Rockland Inc), and anti-apo(a) mAb was

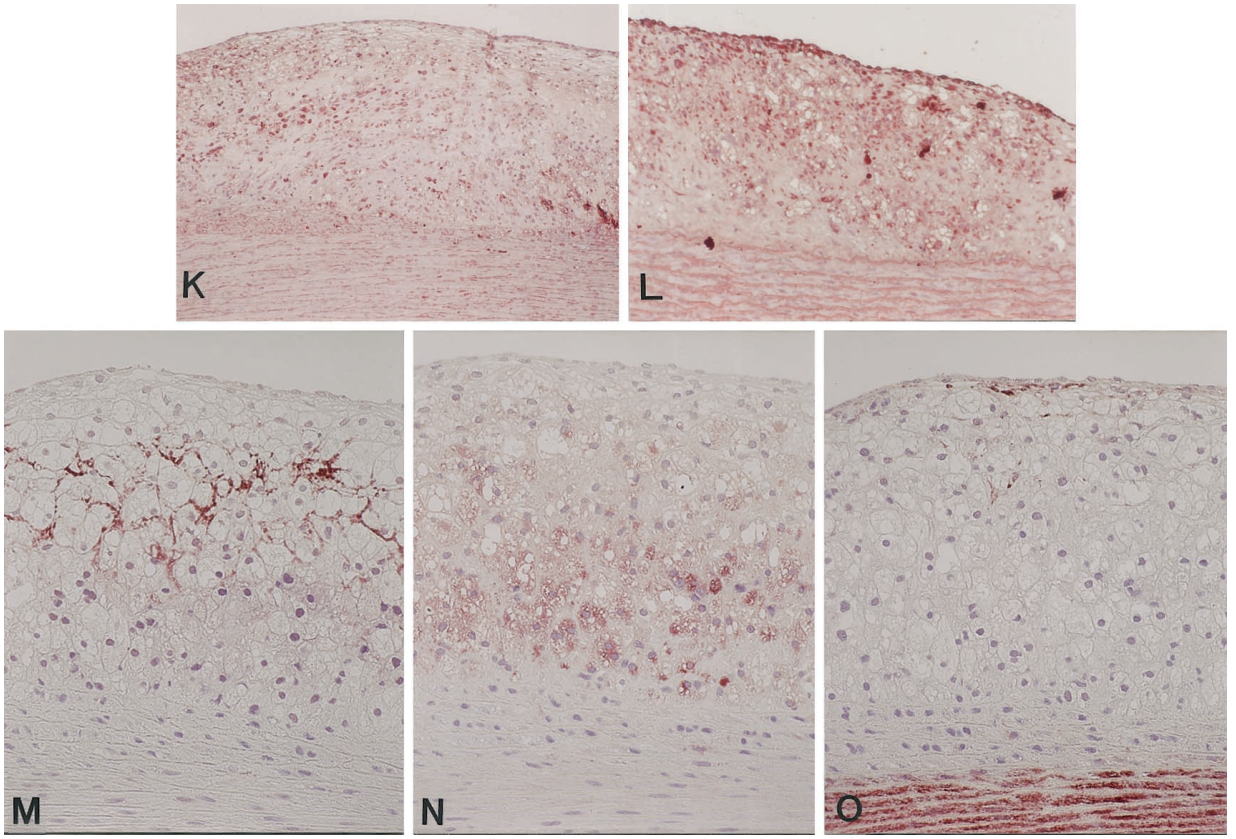

Figure 2 (cont).

performed by use of a Histofine Sab-Po(M) kit (Nichirei Co) according to the manufacturer's instructions.

Statistical Analysis

Plasma levels of cholesterol and triglycerides were compared in the groups of rabbits by using the Student *t* test, and the lesion areas were compared by using the Mann-Whitney *U* test for nonparametric analysis. In all cases, statistical significance was set at $P<0.05$.

Results

Plasma Lipids and Lipoprotein Profiles

The mean levels of human apo(a) in transgenic rabbits on a standard chow diet were 11.88±6.7 nmol/L in males and 11.3±4.2 nmol/L in females. Expression of human apo(a) in transgenic rabbits did not result in a significant change in the plasma total cholesterol, triglycerides, or HDL-C levels (Table 1). Human apo(a) transgene was expressed in the liver but not in the aorta, spleen, or bone marrow (please see Figure IA, published online at http://atvb.ahajournals.org). Examination of the aorta, coronary artery, and cerebral artery in transgenic rabbits up to the age of 2 years failed to reveal any atherosclerotic lesions (data not shown).

Transgenic rabbits and nontransgenic rabbits on a cholesterol-rich diet for 16 weeks developed marked hypercholesterolemia: total cholesterol levels of plasma were remarkably increased compared with those of chow-fed rabbits (Table 1). In general, female rabbits had higher cholesterol levels than did their male counterparts. Male transgenic and nontransgenic rabbits had similarly elevated levels of plasma total cholesterol at the end of the experiment; however, female transgenic rabbits had cholesterol levels as much as 1.3-fold higher than the levels in nontransgenic female rabbits. Triglyceride concentrations in male transgenic and nontransgenic rabbits on a cholesterol-rich diet were slightly increased. In males and females, human apo(a) levels in transgenic rabbits were increased by 2.3-fold in males and 2.6-fold in females after cholesterol feeding compared with the levels in transgenic rabbits on a chow diet (Table 1). To assess whether apo(a) was associated with apoB, plasma isolated from chow- and cholesterol-fed transgenic rabbits was studied by electrophoresis on a nondenaturing polyacrylamide gel, followed by immunoblotting. As shown in Figure IB (published online at http://atvb.ahajournals.org), almost all plasma human apo(a) in cholesterol-fed transgenic rabbits was associated with rabbit apoB to form the Lp(a) complex. Under nonreducing conditions, the apo(a) molecules existed as 2 groups of bands: high molecular weight (HMW) bands and low molecular weight bands, which can be identified as 2 kinds of apo(a) isoforms on reduction, as previously reported.[16] Initially, we postulated that these HMW apo(a) bands are covalently bound Lp(a)-like particles because they were sensitive to a reducing agent. However, when the same immunoblot membranes were reprobed with anti-apoB antibody, we found that these HMW apo(a) bands were not colocalized with apoB, suggesting that the rabbit apoB/apo(a) complex was either covalently bound but at a lower efficiency or became disassociated after sample collection (please see Figure IC, published online at http://atvb.ahajournals.org). This notion may be true because the cysteine

position in rabbit apoB was not exactly at the same site as that of human apoB (Cys-4326).[17] Density fraction analysis by ultracentrifugation showed that there was a marked increase in apoB-containing lipoproteins, including lipoproteins with density <1.006 (mainly β-VLDLs), as well as those with density 1.006 to 1.02 g/mL (IDLs) and those with density 1.02 to 1.04 (LDLs) in transgenic and nontransgenic cholesterol-fed rabbits compared with chow-fed rabbits (please see Figure II, published online at http://atvb.ahajournals.org). However, there was no difference between transgenic and nontransgenic rabbits.

Lesion Analysis

Expression of human apo(a) in transgenic rabbits fed a cholesterol-rich diet for 16 weeks led to a significant increase of atherosclerotic lesions in the male and female groups. In male transgenic rabbits, expression of human apo(a) led to a 1.65-fold increase in the sudanophilic area in the aorta and a 2-fold increase in the carotid artery (Figures 1A and 1B). The atherosclerotic lesions in the iliac artery of transgenic rabbits also tended to be more extensive than those of control rabbits, but the difference was not significant ($P=0.05$, Figure 1B). In females, transgenic and nontransgenic rabbits developed less atherosclerotic lesions than did their male counterparts even though their cholesterol levels were higher than the levels in males. Like male transgenic rabbits, female transgenic rabbits showed significantly increased lesion areas in all arterial trees (2.4-fold increase in the aorta, 5.3-fold increase in the carotid artery, and 6-fold increase in the iliac artery) compared with the areas in nontransgenic rabbits (Figure 1B).

Representative histological lesions from transgenic and nontransgenic rabbit aortas are shown in Figure 2A and 2B. Human apo(a) was frequently seen in the center of the fatty fibrous lesions of transgenic rabbits but not in those of nontransgenic rabbits (Figure 2C and 2D). When the serial sections were stained with apoB antibody, we found that apo(a) was partly superimposed with apoB staining; overall, there was a 30% to ≈50% overlap of apo(a) and apoB staining(Figure 2E and 2F). To investigate the cellular components around apo(a)-containing areas, we stained these lesions with the use of specific antibodies against either macrophages or smooth muscle cells (SMCs). In apo(a)-containing areas, there were few macrophages (Figure 2G), and the majority of the cells were spindle- or stellate-shaped, resembling SMCs, but were not stained by mAb against smooth muscle α-actin, a marker for fully differentiated SMCs (Figure 2I), raising the possibility that these cells were SMCs that were either undifferentiated or dedifferentiated in phenotype. To examine this possibility, we stained the lesions with anti-vimentin mAb and showed that all these SMC-like cells, as in control rabbits, were positive for vimentin (Figure 2K and 2L). We also attempted to study the lesions enriched in macrophage-derived foam cells and found that in these lesions, as in SMC-rich lesions, apo(a) was associated with the extracellular matrix rather than foam cell cytoplasm (Figure 2M to 2O).

Coronary Atherosclerosis

Typical coronary atherosclerotic lesions from male transgenic and nontransgenic rabbits are shown in Figure 3A and 3B. Clearly, coronary atherosclerosis in transgenic rabbits was more extensive than that in control rabbits, with a 2-fold increase in coronary lumen stenosis and a 1.8-fold increase in intimal thickness (Table 2). Like the lesions in the aorta, coronary atherosclerotic lesions showed apo(a) deposition associated with apoB (Figure 3 C and 3D). Furthermore, cells located in apo(a)-containing areas were almost invariably α-actin and RAM-11 negative (Figure 3E and 3F) but positive for vimentin (not shown). We also investigated cerebral arteries in cholesterol-fed transgenic rabbits, but we did not find any atherosclerotic lesions.

TABLE 2. Coronary Atherosclerosis in Transgenic and Nontransgenic Rabbits on a Cholesterol Diet for 16 Weeks

	Stenosis, %	Maximal Intimal Thickness, mm
Transgenic rabbits (n=5)	42.0±14.9*	0.45±0.14*
Nontransgenic rabbits (n=7)	19.4±7.1	0.25±0.05

Values are mean±SD. Coronary atherosclerotic lesions were studied in male rabbits. The left main trunk stenosis and maximal intimal thickness were measured by the MacSCOPE image analysis system as described in Methods.
*$P<0.05$ vs nontransgenic control.

Discussion

In the present study, we characterized the plasma lipid, human apo(a), and lipoprotein levels in human apo(a) transgenic rabbits fed chow and cholesterol-rich diets and examined the susceptibility of these rabbits to the formation of atherosclerotic lesions. The human apo(a) transgenic rabbits used in the present study expressed 11.88 nmol/L of human apo(a) in plasma [≈3.6 mg/dL on the basis of Lp(a) molecular weight ≈3 000 000], which is equivalent to relatively low levels in humans.[1] Despite the formation of Lp(a) particles in the rabbits, which do not normally express apo(a), transgenic rabbits that were fed a chow diet for up to 2 years did not develop atherosclerotic lesions. This is consistent with the notion that a low level of Lp(a) per se is not atherogenic. On a cholesterol-rich diet, however, the transgenic rabbits, males and females, developed more extensive atherosclerotic lesions in the aorta and other arteries than did control rabbits, which is in agreement with the results from studies of human apo(a) transgenic mice.[9,12,13] Especially noteworthy in the present study was the significant increase of lesions in muscular arteries, ie, carotid, iliac, and coronary arteries in transgenic rabbits compared with control rabbits.

In the lesions of transgenic rabbits, apo(a) deposition was frequently found, but apo(a) was not completely colocalized with apoB. This suggests that apo(a) might be disassociated from Lp(a) once deposited in the intima. Lp(a) can enter the arterial intima by a mechanism similar to that of LDL[22] and Lp(a) binds to fibrin as well as to arterial wall glycosaminoglycans with a higher affinity than LDL.[23,24] This binding was found to be associated with a lysine-binding site of apo(a).[13]

In the present study, we attempted to investigate the relationship between Lp(a) and foam cells in the lesions. We found that apo(a) was predominantly associated with the extracellular matrix and was not intracellularly associated with foam cells (Figure 2M and 2N), suggesting that apo(a) may not play a role in foam cell formation.

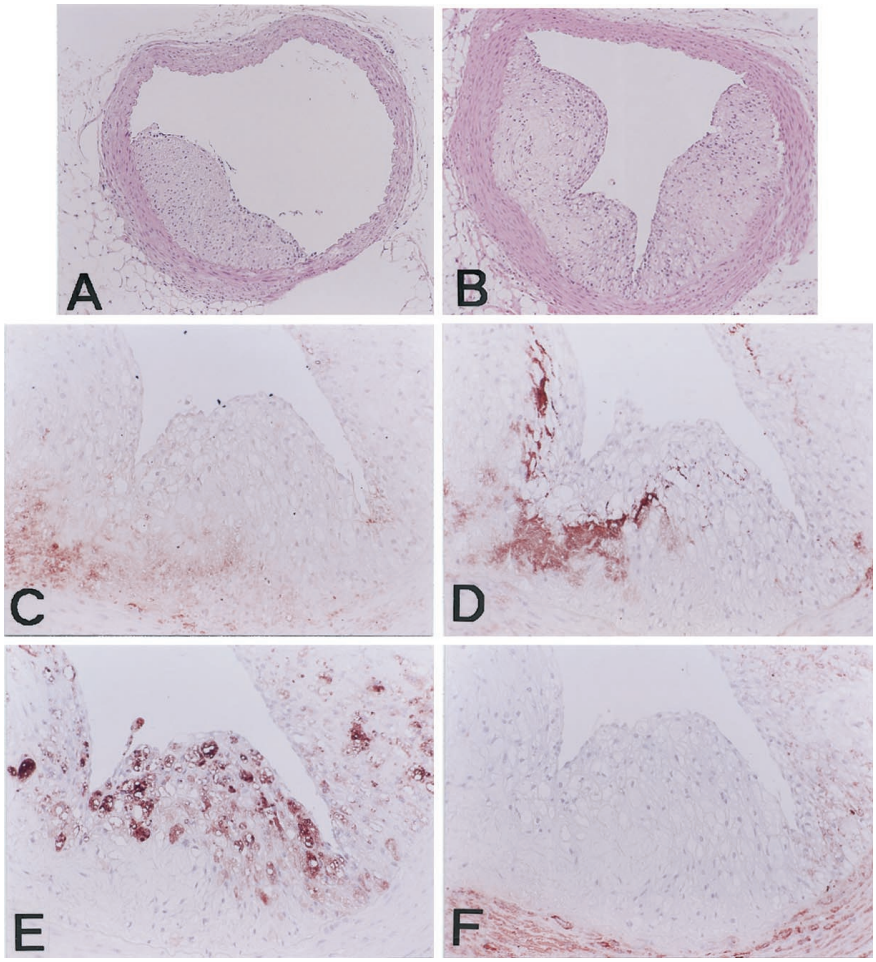

Figure 3. Coronary artery micrographs. A and B, Coronary arteries with elevated fibrofatty plaque lesions from nontransgenic (A) and transgenic (B) rabbits fed a cholesterol diet for 16 weeks (hematoxylin-eosin staining). Transgenic rabbit coronary lesions were more extensive than those of control rabbits, as quantified in Table 2. C to F, Serial sections of transgenic rabbit coronary arteries stained with anti-human apo(a) (C), anti-apoB (D), anti-rabbit macrophage (E), and anti-rabbit smooth muscle α-actin (F) antibodies. Original magnification $\times$200. Apo(a) deposits are present in the center of the lesion core (C) and are partially associated with apoB (D). However, these areas are not directly associated with macrophages (E) or α-actin–positive SMCs (F). Note that intimal cells are partially positive for smooth muscle α-actin on the right side, where there is no apo(a) deposition.

Of particular note, areas containing apo(a) deposits contained numerous immature or dedifferentiated SMC-like cells, as judged by morphological characteristics; moreover, the lesions were negative for smooth muscle α-actin staining but positive for vimentin staining.[25] We speculate that Lp(a) in the lesions may directly or indirectly induce these SMCs to change phenotypes or to dedifferentiate; however, this hypothesis remains to be proven. Several previous studies have demonstrated that human Lp(a) and apo(a) stimulate the proliferation of SMCs in vitro through the inhibition of TGF-β activation.[26] It is presently unknown whether transforming growth factor-β is involved in apo(a)-mediated SMC dedifferentiation.

The mean total cholesterol level in transgenic rabbits fed a cholesterol-rich diet for 16 weeks was similar to that in nontransgenic rabbits fed the same diet; nevertheless, human apo(a) levels in transgenic rabbits fed the cholesterol-rich diet were increased by 2.3-fold in males and 2.6-fold in females compared with the levels in transgenic rabbits fed the chow diet. This was an unexpected finding but was in agreement with a similar finding in apo(a) transgenic mice fed a high-fat diet.[14] We hypothesized that increased Lp(a) levels in cholesterol-fed transgenic rabbits may be attributed to downregulation of LDL receptor activity in rabbits.[27] In contrast to humans, rabbits do not normally have apo(a) or Lp(a), so the LDL receptor may be directly or indirectly involved in the removal of Lp(a) from the plasma. We recently showed that in LDL receptor–deficient Watanabe heritable hyperlipidemic rabbits expressing human apo(a), there was a 4-fold increase in plasma Lp(a) compared with plasma Lp(a) in normal transgenic rabbits.[20] In humans, however, it seems that the LDL receptor plays only a minimal role in the catabolism of Lp(a).[28]

In view of the studies in humans, our finding that apo(a) plasma levels of $\sim$10 mg/dL in transgenic rabbits on the cholesterol-rich diet can enhance cholesterol-induced atherosclerosis may be surprising. The amount of cholesterol in β-VLDL and IDL, atherogenic lipoproteins in cholesterol-fed rabbits, does not appear to explain this enhancement, inasmuch as little difference was observed between the amounts of β-VLDL and IDL cholesterol in the transgenic and nontransgenic rabbits (please see Figure II, published online at http://atvb.ahajournals.org). Transgenic and nontransgenic rabbits fed the cholesterol-rich diet had similarly reduced levels of HDL. Therefore, it is likely that the increased amount of human apo(a) in the atherogenic lipoproteins was the principal factor underlying the significant increase in the atherosclerosis in those animals. The results of the present study do not permit us to conclude that apo(a) at this level is atherogenic in rabbits fed a chow diet but, rather, that it acts as an enhancer or accelerator of the development of atherosclerosis in the setting of cholesterol diet–induced hypercho-

lesterolemia. In humans, the relative risk of atherosclerotic disease in patients with elevated Lp(a) concentrations is significantly increased in patients who also have high levels of LDL cholesterol.[29] It should be mentioned that the major atherogenic lipoproteins in cholesterol-fed rabbits are those of β-VLDL, whereas in humans, LDLs are the main atherogenic lipoproteins. In the future, we will address this issue with the use of the recently generated Watanabe heritable hyperlipidemic transgenic rabbits, which express higher levels of human apo(a) than do normal transgenic rabbits and have elevated LDL levels, as in human familial hypercholesterolemia.[20]

In summary, we have shown that human apo(a) transgenic rabbits develop more extensive atherosclerosis in the aorta, carotid artery, and coronary artery than do normal rabbits in response to a cholesterol-rich diet. Although the exact mechanism underlying this effect remains to be elucidated, the present results suggest that Lp(a) may enhance cholesterol diet–induced atherosclerosis by modifying the SMC phenotype.

Acknowledgments

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