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Low density lipoprotein receptor-negative mice expressing human apolipoprotein B-100 develop complex atherosclerotic lesions on a chow diet: No accentuation by apolipoprotein(a)

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ABSTRACT **We have generated mice with markedly elevated plasma levels of human low density lipoprotein (LDL) and reduced plasma levels of high density lipoprotein. These mice have no functional LDL receptors [*LDLR*$^{-/-}$] and express a human apolipoprotein B-100 (apoB) transgene [*Tg(apoB*$^{+/+}$*)*] with or without an apo(a) transgene [*Tg(apoa*$^{+/-}$*)*]. Twenty animals (10 males and 10 females) of each of the following four genotypes were maintained on a chow diet: (*i*) *LDLR*$^{-/-}$, (*ii*) *LDLR*$^{-/-}$; *Tg(apoa*$^{+/-}$*)*, (*iii*) *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)*, and (*iv*)*LDLR*$^{-/-}$; *Tg(apoB*$^{+/+}$*);Tg(apo*$^{+/-}$*)*. The mice were killed at 6 mo, and the percent area of the aortic intimal surface that stained positive for neutral lipid was quantified. Mean percent areas of lipid staining were not significantly different between the *LDLR*$^{-/-}$ and *LDLR*$^{-/-}$;*Tg(apoa*$^{+/-}$*)* mice (1.0 ± 0.2% vs. 1.4 ± 0.3%). However, the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice had ≈15-fold greater mean lesion area than the *LDLR*$^{-/-}$ mice. No significant difference was found in percent lesion area in the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice whether or not they expressed apo(a) [18.5 ± 2.5%, without lipoprotein(a), Lp(a), vs. 16.0 ± 1.7%, with Lp(a)]. Histochemical analyses of the sections from the proximal aorta of *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice revealed large, complex, lipid-laden atherosclerotic lesions that stained intensely with human apoB-100 antibodies. In mice expressing Lp(a), large amounts of apo(a) protein colocalized with apoB-100 in the lesions. We conclude that *LDLR*$^{-/-}$; *Tg(apoB*$^{+/+}$*)* mice exhibit accelerated atherosclerosis on a chow diet and thus provide an excellent animal model in which to study atherosclerosis. We found no evidence that apo(a) increased atherosclerosis in this animal model.**

Atherosclerosis is a complex, multifactorial process whose analysis has been greatly facilitated by the development of genetically modified mice. ApoE-deficient mice *(apoE*$^{-/-}$*)* are currently the most widely utilized animal model for the study of atherosclerosis (1, 2). *ApoE*$^{-/-}$ mice maintained on a low fat, mouse-chow diet have dramatically elevated plasma levels of cholesterol, and they develop extensive atherosclerotic lesions widely distributed throughout the aorta (1–5). The effect of other genes on the development of atherosclerosis has been examined by crossing the *apoE*$^{-/-}$ mice with other genetically manipulated animals (6–8).

In wild-type mice, ≈90% of plasma cholesterol circulates in high density lipoproteins (HDL). In *ApoE*$^{-/-}$ mice, the cholesterol is predominantly in the very low density lipoproteins (VLDL) and in the intermediate density lipoprotein fractions (IDL) (1, 2). The most common lipoprotein pattern in humans with coronary artery disease consists of elevated plasma levels of low density lipoprotein cholesterol (LDL-C) and decreased HDL-C, with or without increases in plasma IDL and VLDL. The strong association between plasma LDL-C and coronary artery disease in humans is reflected in the clinical outcome of subjects with familial hypercholesterolemia (9). Familial hypercholesterolemia is an autosomal dominant disorder caused by mutations in the LDL receptor (LDLR) gene, which encodes a cell surface receptor that binds and internalizes plasma LDL. Individuals heterozygous for a mutation at the LDLR locus have 2- to 3-fold elevated levels of LDL-C and a striking increase in the incidence of premature coronary artery disease (9). Familial hypercholesterolemia homozygotes [*LDLR*$^{-/-}$] have 6- to 10-fold elevations in LDL and develop diffuse atherosclerotic lesions in childhood.

Plasma levels of LDL are increased ≈2- to 3-fold in mice homozygous for an inactivated LDLR gene [*LDLR*$^{-/-}$] (10), but they do not develop significant atherosclerotic lesions unless they consume a high fat, high cholesterol diet (11). The relative resistance of mice to LDLR deficiency has been attributed to the production of apoB-48, in addition to apoB-100 in the liver. Whereas human liver produces only apoB-100, mice produce both apoB-100 and its truncated variant, apoB-48. Lipoprotein containing apoB-48 can be cleared from the plasma by other receptors in addition to the LDLR, and hence mouse lipoproteins do not rise as high as human lipoproteins when LDL receptors are deficient. Mice have been generated that produce human apoB-100 in the liver (12, 13). These animals show a modest increase in LDL cholesterol but again develop atherosclerosis only after ingestion of a high cholesterol, high fat diet (14, 15). The atherogenic diet required to produce vascular lesions in the *LDLR*$^{-/-}$ and apoB-100 transgenic mice significantly alters the plasma lipid profile and may affect the vessel wall independently of plasma lipid elevations (16). These features have limited the usefulness of the *LDLR*$^{-/-}$ and apoB-100 transgenic mice for atherosclerosis studies.

High plasma levels of lipoprotein(a) [(Lp(a)], another LDL-containing lipoprotein, also are associated with atherosclerosis in man (17), but controversy persists regarding the atherogenicity of apo(a) in mice. Apo(a), the large glycoprotein that is attached covalently to the apoB-100 of LDL in the Lp(a) particle, is not found in the plasma of most mammals, including mice. Mice expressing a human apo(a) transgene [*Tg(apoa*$^{+/-}$*)*] have apo(a) in their plasma, but it is not disulfide-linked to mouse apoB-100 (18); these mice do not develop vascular lesions on a low fat diet (19). Fat-fed apo(a) transgenic mice exhibited a significantly higher mean lesion area of fatty lesions in the aortic root than wild-type mice in some (15, 19–21), but not all (22), studies. A

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Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; IDL, intermediate density lipoproteins; LDL, low density lipoprotein; LDLR, LDL receptor; lipoprotein(a), Lp(a); VLDL, very low density lipoproteins; tg, transgene; FPLC, fast performance liquid chromatography.

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possible explanation for the conflicting results of these studies is that expression of apo(a) alone in the mouse is only marginally atherogenic. The amount of lipid staining in *Tg(apoa$^{+/-}$)* mice is modest compared with that seen in chow-fed *apoE$^{-/-}$* mice (1–5) or in fat-fed *LDLR$^{-/-}$* (11) or *Tg(apoB$^{+/+}$)* mice (14, 15).

Moreover, the relevance of the increase in aortic lipid staining in the apo(a) transgenic mice is questionable because apo(a) does not form a covalent linkage with mouse apoB-100 (18). If human apoB-100 is coexpressed with human apo(a) in the mouse, the apo(a) circulates bound to human apoB-100 as authentic Lp(a) (12, 13). The amount of aortic lipid-staining in these *Tg(apoB$^{+/-}$);Tg(apoa$^{+/-}$)* mice is not significantly higher than in mice expressing only the human apoB-100 transgene (15, 22). Perhaps Lp(a) does not induce more atherosclerosis because the level of plasma LDL is not high enough to promote lesion development. In humans, the atherogenicity of Lp(a) appears to be enhanced in individuals with elevated plasma levels of LDL (23).

In the current study, we have constructed *LDLR$^{-/-}$* mice that express a human apoB-100 transgene [*Tg(apoB$^{+/+}$)*] with or without coexpression of apo(a) [*Tg(apoa$^{+/-}$)*]. These mice have very high plasma levels of LDL and develop large atherosclerotic lesions on a low fat diet.

MATERIALS AND METHODS

Mice. *LDLR$^{-/-}$* mice were obtained from Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX) (10). These mice, which are hybrids of the 129Sv/Ev and C57BL/6 strains, were crossed with C57BL/6 X SJL hybrid mice expressing a human apoB-100 [*Tg(apoB$^{+/-}$)*] and apo(a) transgene [*Tg(apoa$^{+/-}$)*] (12). Genotypes were determined using a PCR-based assay (24), and plasmas were screened for the presence of human apoB and apo(a) as described (22). Age- and sex-matched offspring of *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* and *LDLR$^{-/-}$;Tg(apoB$^{+/+}$);Tg(apoa$^{+/-}$)* mice and of *LDLR$^{-/-}$* mice and *LDLR$^{-/-}$;Tg(apoa$^{+/-}$)* mice were used at 8 weeks of age; the offspring included 10 males and 10 females of each of the following four genotypes: *LDLR$^{-/-}$*, *LDLR$^{-/-}$; Tg(apoa$^{+/-}$)*, *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)*, and LDLR$^{-/-}$; *Tg(apoB$^{+/+}$);Tg(apoa$^{+/-}$)*. These mice were hybrids of the 129Sv/Ev, C57BL/6, and SJL strains. Ten Tg*(apoB$^{+/+}$)* mice (five males and five females) in a C57BL/6 and SJL hybrid background also were included in the study. All of the mice were weaned at 21 days and fed a cholate-free mouse-chow diet containing 6% animal fat and <0.04% cholesterol (Teklad, mouse/rat diet 7002; Harlan Teklad, Madison, WI) until they were killed at 6 mo of age. The mice were housed in a conventional, nongerm-free animal facility on 12-h dark/12-h light cycles and had free access to food and water. Venous blood was drawn by retro-orbital sinus puncture every 8 weeks from 5 to 10 animals of each genotype after the animals had fasted for 4 h.

Analysis of Plasma Lipid and Lipoproteins. Blood samples were collected into citrate-EDTA tubes. Plasma was isolated by centrifugation at 5,000 × *g* for 10 min and maintained at 4°C, or aliquoted and stored at −80°C. Plasma cholesterol and triglyceride levels were measured using enzymatic assays (Cholesterol/HP, Boehringer Mannheim and Triglyceride GPO Trinder, Sigma). Plasma lipoprotein analysis was performed using fast performance liquid chromatography (FPLC); a total of 35 fractions (1.8 ml each) was collected, and the cholesterol content was measured by fluorimetry (10). In addition, plasma was pooled from 3–4 male mice of each genotype, and the density < 1.215 g/liters fraction was subjected to FPLC; the triglycerides and cholesterol were measured in each fraction using the enzymatic assays described above. Plasma levels of human apoB were determined by a nephelometric method (BN II, Boehringer Mannheim) calibrated with World Health Organization–International Federation for Clincial Chemistry reference material (25), and plasma levels of apo(a) were measured using a double mAb-based enzyme-linked immunoassay (25). Analyses were performed on plasma samples stored for <2 mo at −80°C. The results are expressed as total Lp(a) mass. The plasma apo(a) level can be estimated by multiplying the Lp(a) level by 0.138.

Quantification of Aortic Fatty Lesion Area. Mice were anesthetized by using halothane followed by sodium pentobarbital and then were bled via retro-orbital sinus puncture. A thoracotomy and laparotomy were performed to expose the heart and aorta. A catheter was inserted into the left ventricle of the heart and the right atrium was punctured. Mice were perfused with 20 ml of Dulbecco's PBS until blood had been cleared from the circulation and then with 50 ml of fixative solution [4% (vol/vol) paraformaldehyde, 7.5% (wt/vol) sucrose, 430 mM Na_2EDTA, 10 mM butylated hydroxytoluene, and 1 mM sodium phosphate, pH 7.4]. The aorta was slit open *in situ* from the arch to the iliac bifurcation along the ventral midline, removed, and pinned out on a wax surface. The lipid-rich lesions were stained with Sudan IV (Fisher Scientific) destained using 80% ethanol, and then stored in fixative (8). The percentage of aortic surface area that stained with Sudan IV was determined as described (8).

Immunohistological Studies of Aorta. Aortic root segments were embedded in OCT compound (Sakura Finetek, Torrance, CA), frozen in liquid nitrogen, and sectioned with a cryostat to produce 10-μm sections for immunohistochemical evaluation. Cross-sections of the distal aortic sinus and proximal ascending aorta were stained using Oil Red O (26). Sections were immunostained for apo(a) and apoB as follows. Sections were incubated with 22 μg/ml rabbit polyclonal anti-human apo(a) antibody (Cortex, Irvine, CA) for 1 h or with a rabbit polyclonal anti-human LDL antibody (27) at a 1:250 dilution. This procedure was followed by a 1-h incubation with a secondary antibody, biotinylated goat anti-rabbit IgG (1.5 μg/ml). For apo(a) immunostaining, the tertiary reagent was a streptavidin-alkaline phosphatase conjugate (Histostain DS kit, Zymed), and the quaternary reagent was 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP-NBT). For apoB, the chromogenic steps included streptavidin-peroxidase and aminoethyl carbazole (Histostain DS kit, Zymed). Chromogen immunostains were photographed with a Nikon Optiphot 2 light microscope.

Statistical Analysis. The plasma levels of cholesterol, triglycerides, apoB and Lp(a), and the percent lesion areas were compared in the groups of mice by using the nonparametric Wilcoxon rank sum test (28). Correlations between the percent lesion areas and the plasma cholesterol levels were estimated by the Pearson product moment correlation. The statistical significance of the Pearson product moment correlation was assessed using a *t* test (29).

RESULTS

Ten male and 10 female mice of the following four genotypes were fed a chow diet from the time of weaning (≈21 days) until 6 mo of age: (*i*) *LDLR$^{-/-}$*, (*ii*) *LDLR$^{-/-}$;Tg(apoa$^{+/-}$)*, (*iii*) *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)*, and (*iv*) *LDLR$^{-/-}$;Tg(apoB$^{+/+}$); Tg(apoa$^{+/-}$)*. Ten *Tg(apoB$^{+/+}$)* mice (five males and five females) also were studied. The mean level of plasma cholesterol in the *LDLR$^{-/-}$* mice was ≈2.5-fold higher than in the *apoB$^{+/+}$* mice (310 ± 8 vs. 121 ± 8 mg/dl) (Table 1), and the triglyceride levels were lower in the *LDLR$^{-/-}$* mice (147 mg/dl vs. 119 mg/dl), although this difference was not statistically significant. The expression of apo(a) in the *LDLR$^{-/-}$* mice did not alter the level of plasma cholesterol (311 ± 9 vs. 310 ± 8 mg/dl) but produced a slight reduction in plasma triglycerides (94 ± 7 vs. 119 ± 8 mg/dl). The mean plasma level of Lp(a) was 27 mg/dl, which is almost twice as high as the plasma Lp(a) level in *LDLR$^{+/+}$* mice expressing the same apo(a) transgene (22). The mean plasma levels of Lp(a) were significantly higher in the male *LDLR$^{-/-}$; Tg(apoa$^{+/-}$)* and *LDLR$^{-/-}$;Tg(apoB$^{+/+}$);Tg(apoa$^{+/-}$)* mice than in their female counterparts ($P = 0.01$ and $P = 0.03$, respectively) (Table 1).

Table 1. Characterization of plasma lipid and apolipoprotein levels (±SEM) in mice

Mice (n)	Plasma cholesterol, mg/dl	Plasma TG, mg/dl	Plasma human apoB, mg/dl	Plasma Lp(a), mg/dl	Lesion area, %
Tg(apoB$^{+/+}$) (10)	121 ± 8	147 ± 18	74 ± 13	—	0
Male (5)	120 ± 6	168 ± 33	54 ± 5	—	0
Female (5)	122 ± 12	135 ± 23	87 ± 21	—	0
LDLR$^{-/-}$ (20)	310 ± 8	119 ± 8	—	—	1.0 ± .2
Male (10)	323 ± 12	132 ± 13	—	—	0.6 ± .1
Female (10)	297 ± 11	106 ± 9	—	—	1.5 ± .4
LDLR$^{-/-}$;Tg(apoa$^{+/-}$) (20)	311 ± 9	94 ± 7	—	27 ± 2	1.4 ± .3
Male (10)	316 ± 16	109 ± 10	—	32 ± 3	1.4 ± .4
Female (10)	306 ± 9	80 ± 7	—	22 ± 2	1.3 ± .4
LDLR$^{-/-}$;Tg(apoB$^{+/+}$) (20)	799 ± 57	634 ± 45	260 ± 19	—	18.5 ± 2.5
Male (10)	745 ± 77	732 ± 70	281 ± 25	—	21.3 ± 4.6
Female (10)	853 ± 84	547 ± 46	239 ± 28	—	15.8 ± 1.9
LDLR$^{-/-}$;Tg(apoB$^{+/+}$);Tg(apoa$^{+/-}$) (20)	755 ± 61	642 ± 68	237 ± 31	25 ± 3	16.0 ± 1.7
Males (10)	738 ± 79	892 ± 86	257 ± 51	30 ± 4	16.3 ± 2.9
Female (10)	772 ± 75	414 ± 29	220 ± 38	19 ± 3	15.7 ± 2.1

Total plasma cholesterol, triglyceride, human apoB, and Lp(a) were measured from the indicated number (n) of male and female mice plasma obtained after a 4-h fast. The statistical analysis of comparisons between groups is provided in the text.

Expression of human apoB-100 in the *LDLR$^{-/-}$; Tg(apoa$^{+/-}$)* mice did not lead to additional increases in the mean level of plasma Lp(a) (25 ± 3 vs. 27 ± 2 mg/dl), even though the level of apoB rose markedly. This result suggests that the amount of apo(a) synthesized in these mice is rate-limiting for Lp(a) production. The *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* mice had a 2.6-fold increase in plasma cholesterol compared with *LDLR$^{-/-}$* mice (799 ± 57 vs. 310 ± 8 mg/dl). The most striking difference between the *LDLR$^{-/-}$; Tg(apoB$^{+/+}$)* mice and *LDLR$^{-/-}$* mice was in the level of plasma triglyceride, which was >5-fold higher in the *LDLR$^{-/-}$; Tg(apoB$^{+/+}$)* mice (634 ± 45 vs. 119 ± 8 mg/dl).

To determine the distribution of plasma cholesterol and triglycerides in the lipoproteins of these mice, FPLC analysis was performed on the density < 1.215 g/liter fraction of pooled plasma from 3–4 male mice of each genotype (Fig. 1). The results were compared with those obtained from analysis of age- and sex-matched wild-type (Fig. 1*A*) and *apoE$^{-/-}$* (Fig. 1*C*) mice. The lipoprotein profile of the *Tg(apoB$^{+/+}$)* mice (Fig. 1*B*) was similar to that reported (12, 13). As expected, the *LDLR$^{-/-}$* mice had an increase in the LDL-C peak and no change in the amount of HDL-C when compared with wild-type mice (Fig. 1 *D* vs. *A*). Expression of human apoB-100 in the *LDLR$^{-/-}$* mice resulted in a dramatic increase in the LDL cholesterol content and a fall in the cholesterol in the HDL fraction (Fig. 1*F*). The distribution of triglyceride tended to parallel that of the cholesterol in the *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* mice so that most of the triglyceride was in the LDL fraction, which is similar to chow-fed mice (12, 13) and rabbits (30) expressing a human apoB-100 transgene.

Expression of apo(a) in the *LDLR$^{-/-}$* (Fig. 1*E*) and *LDLR$^{-/-}$; Tg(apoB$^{+/+}$)* (Fig. 1*G*) mice did not significantly affect the FPLC lipid profiles, although both groups of *Tg(apoa$^{+/-}$)* mice had proportionally more triglyceride in the VLDL fraction, suggesting that apo(a) expression was associated with higher plasma levels of triglycerides. However, the mean plasma level of triglycerides in the entire sample of male *LDLR$^{-/-}$;Tg(apoa$^{+/-}$)* mice actually was lower than in the *LDLR$^{-/-}$* mice (109 ± 10 vs. 132 ± 13 mg/dl) (Table 1). To determine the distribution of apo(a) among the lipoproteins, immunoblot analysis of the FPLC fractions was performed with an anti-apo(a) antibody (data not shown). In the *LDLR$^{-/-}$; Tg(apoB$^{+/+}$);Tg(apoa$^{+/-}$)* mice, almost all of the immunodetectable apo(a) was present in fractions 8–17, which corresponded to LDL-sized particles (Fig. 1); only a trace amount of apo(a) was detected in the VLDL fraction, and no apo(a) was found in the density > 1.215 g/liter fraction. Finding apo(a) in the LDL fraction of *LDLR$^{-/-}$;Tg(apoB$^{+/+}$);Tg(apoa$^{+/-}$)* mice was not surprising because apo(a) is disufide-linked with human apoB in mice expressing both transgenes (12, 13). Similar immunoblot analysis from plasma of the *LDLR$^{-/-}$;Tg(apoa$^{+/-}$)* mice demonstrated that almost all the apo(a) was in the LDL fractions, with trace amounts in the VLDL fractions (data not shown). Previously, we demonstrated that human apo(a) does not form a covalent attachment to mouse apoB (18) but that mouse apoB immunoprecipitates with apo(a) in the *Tg(apoa$^{+/-}$)* mice (31). Taken together, these findings are consistent with apo(a) circulating noncovalently associated with mouse apoB particles in the

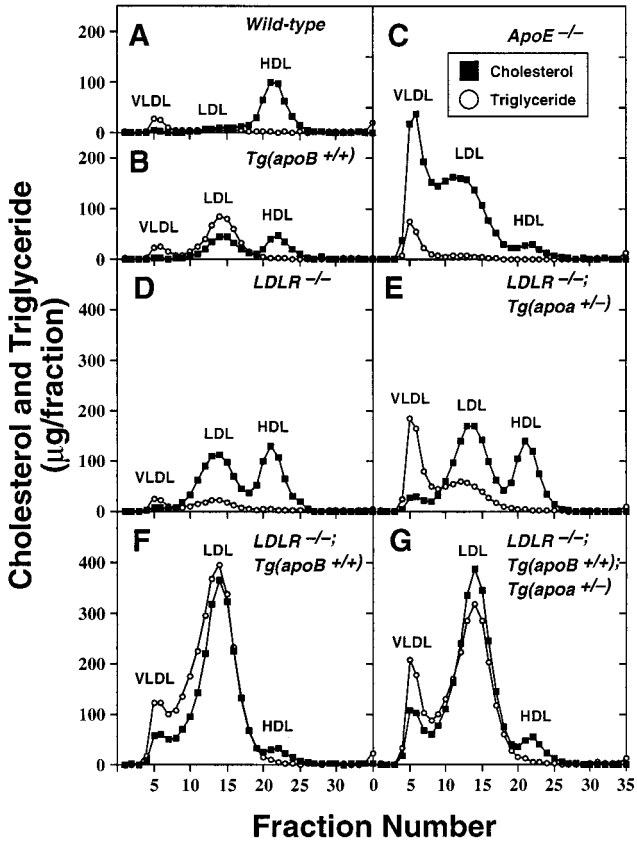

FIG. 1. Venous plasma was pooled from 3–4 male mice of the indicated genotype, and lipoproteins were isolated by ultracentrifugation. The wild-type mice were age- and sex-matched C57BL/6 mice. The top fraction (density < 1.215 g/liter) was subjected to FPLC using a Superose 6 column (Sigma). Thirty-five fractions were collected, and plasma cholesterol and triglycerides were measured in each fraction as described in *Materials and Methods*.

LDLR$^{-/-}$; *Tg(apoa*$^{+/-}$*)* mice. The almost 2-fold higher plasma level of Lp(a) in the *LDLR*$^{-/-}$;*Tg(apoa*$^{+/-}$*)* mice compared with previously reported *LDLR*$^{+/+}$;*Tg(apoa*$^{+/-}$*)* mice (18–22) suggests that apo(a) is cleared together with LDL by the LDLR in these mice.

Mice were sacrificed at 6 mo of age, and the entire aortas were pinned out flat and stained with Sudan IV to reveal neutral (sudanophilic) lipids. The distribution of sudanophilia in the aortas from representative male mice of each genotype is shown in Fig. 2. The *Tg(apoB*$^{+/+}$*)* mice had no detectable sudanophilia (data not shown). Only very small focal regions of sudanophilia were present in the aortas of the *LDLR*$^{-/-}$ mice (Fig. 2*A*). Expression of apo(a) in *LDLR*$^{-/-}$ mice was not associated with a significant change in the amount or distribution of sudanophilia (Fig. 2*B*). In contrast, expression of human apoB-100 in an *LDLR*$^{-/-}$ background was associated with a dramatic increase in sudanophilic area throughout the aorta and extending into the external iliac arteries (Fig. 2*C*). Of particular interest is the presence of extensive and large lesions in the posterior segments of the aortas of *LDLR*$^{-/-}$ mice expressing human apoB-100 (Fig. 2 *C* and *D*). No increase in sudanophilia was seen with coexpression of apo(a) (Fig. 2*D*). In these analyses, the intensity of sudanophilic staining varies and is not a reliable indicator of the thickness of the lesions.

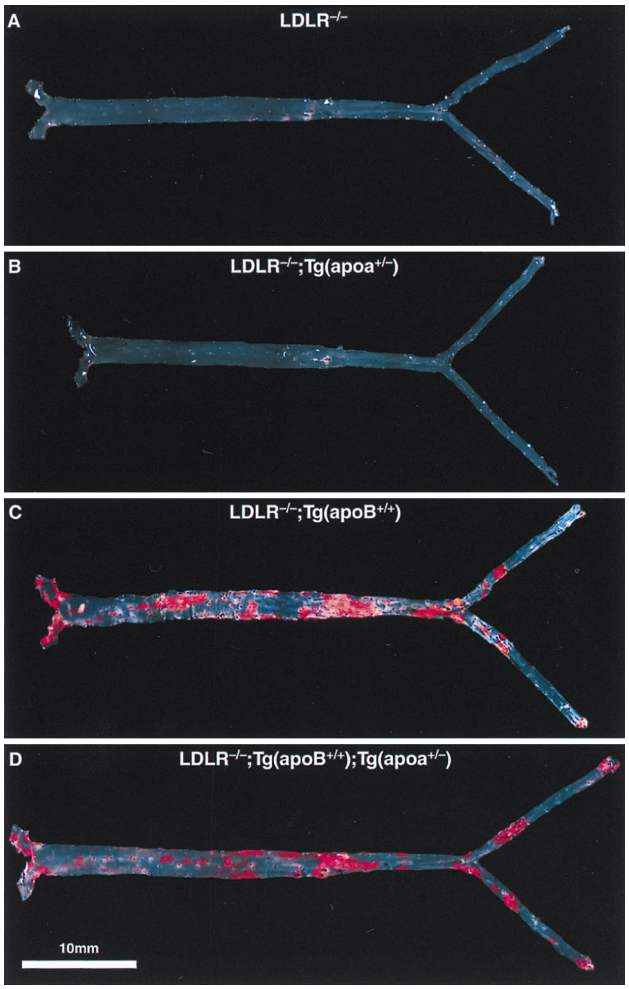

FIG. 2. Sudan IV-stained aortas from genetically modified mice that were fed a mouse chow diet for 6 mo. Representative aortas from *LDLR*$^{-/-}$ (*A*), *LDLR*$^{-/-}$;*Tg(apoa*$^{+/-}$*)* (*B*), LDLR$^{-/-}$;*Tg(apoB*$^{+/+}$*)* (*C*), and LDLR$^{-/-}$;*Tg(apoB*$^{+/+}$*)*;*Tg(apoa*$^{+/-}$*)* (*D*) groups of mice were pinned out and stained with Sudan IV, which stains neutral lipids red. The lesion area in each aorta shown was as follows: 1.03% (*A*), 2.63% (*B*), 21.76% (*C*), and 16.86% (*D*). The scale bar represents 10 mm.

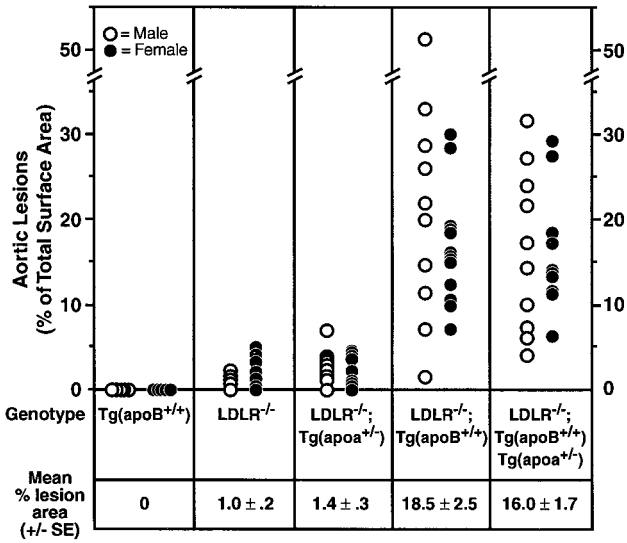

FIG. 3. Extent of sudanophilia in the aortas of Tg(apoB$^{+/+}$), *LDLR*$^{-/-}$, *LDLR;Tg(apoa*$^{+/-}$*)*, *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)*, and *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)*;*Tg (apoa*$^{+/-}$*)* mice. Mice were weaned at ≈21 days and then fed a low fat chow diet until 6 mo of age. The mice were sacrificed and the percent Sudan IV staining of the total aortic surface area was determined as described in *Materials and Methods*.

To compensate for size differences among murine aortas, the sudanophilia was expressed as a percentage of total aortic surface area (Fig. 3). Less than 1% of the surface area was sudanophilic in the *Tg(apoB*$^{+/+}$*)* mice. Very low levels of neutral lipid staining were present in the *LDLR*$^{-/-}$ mice, and the percent mean lesion areas were not significantly different in the *LDLR*$^{-/-}$ and *LDLR*$^{-/-}$;*Tg(apo(a))* mice (1.0 ± 0.2 vs. 1.4 ± 0.3%). Female *LDLR*$^{-/-}$ mice had a higher mean lesion area than their male counterparts (1.5±.4 vs. 0.6 ± 0.1; $P = 0.08$), but this was not true when the two sexes of the *LDLR*$^{-/-}$;*Tg(apoa*$^{+/-}$*)* mice were compared (1.3 ± 0.4 vs. 1.4 ± 0.4%; $P = 1$). The mean percent lesion area was higher in male *LDLR*$^{-/-}$;*Tg(apoa*$^{+/-}$*)* than in *LDLR*$^{-/-}$ mice (1.4% ± 0.4 vs. 0.6 ± 0.1%), although the difference was not statistically significant ($P = 0.07$).

The percent lesion areas varied over a wide range in the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice, probably in part because of the mixed genetic background of these animals. No significant difference was found in either the mean lesion areas (or the distribution of lesions) in the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice compared with *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)*;*Tg(apoa*$^{+/-}$*)* mice (18.5 ± 2.5% vs. 16.0 ± 1.7; $P = 0.47$). The lesion area was related directly to the plasma cholesterol level in both groups of *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice ($r = 0.5$; $P = 0.01$). The correlation between plasma cholesterol level and percent lesion area was 0.8 ($P < 0.001$) when all the mice of different genotypes were analyzed together.

Histochemical and immunocytochemical studies were performed in a subset of animals from each genotypic group. Tiny, superficial lesions associated with the valve roots were present in the aortas of the *LDLR*$^{-/-}$;*Tg(apoa*$^{+/-}$*)* mice, and only trace amounts of apo(a) immunoreactivity were seen associated with these lipid accumulations as well as on the endothelial surface of the aorta. In contrast, multiple lipid-laden lesions with fibrous caps (that stained positive with an α-actin antibody) were present in the aortic roots of the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice, whether or not they also expressed apo(a) (data not shown).

The distributions of apo(a) and human apoB immunoreactivity were compared in adjacent sections from the proximal aorta of an *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)*;*Tg(apoa*$^{+/-}$*)* mouse (Fig. 4). The luminal aspect of the lesion, as well as the core, stained intensely with anti-human apoB-100 antibody (red, Fig. 4 *A* and *C*). The apo(a) immunoreactivity (dark blue, Fig. 4 *B* and *D*) colocalized with

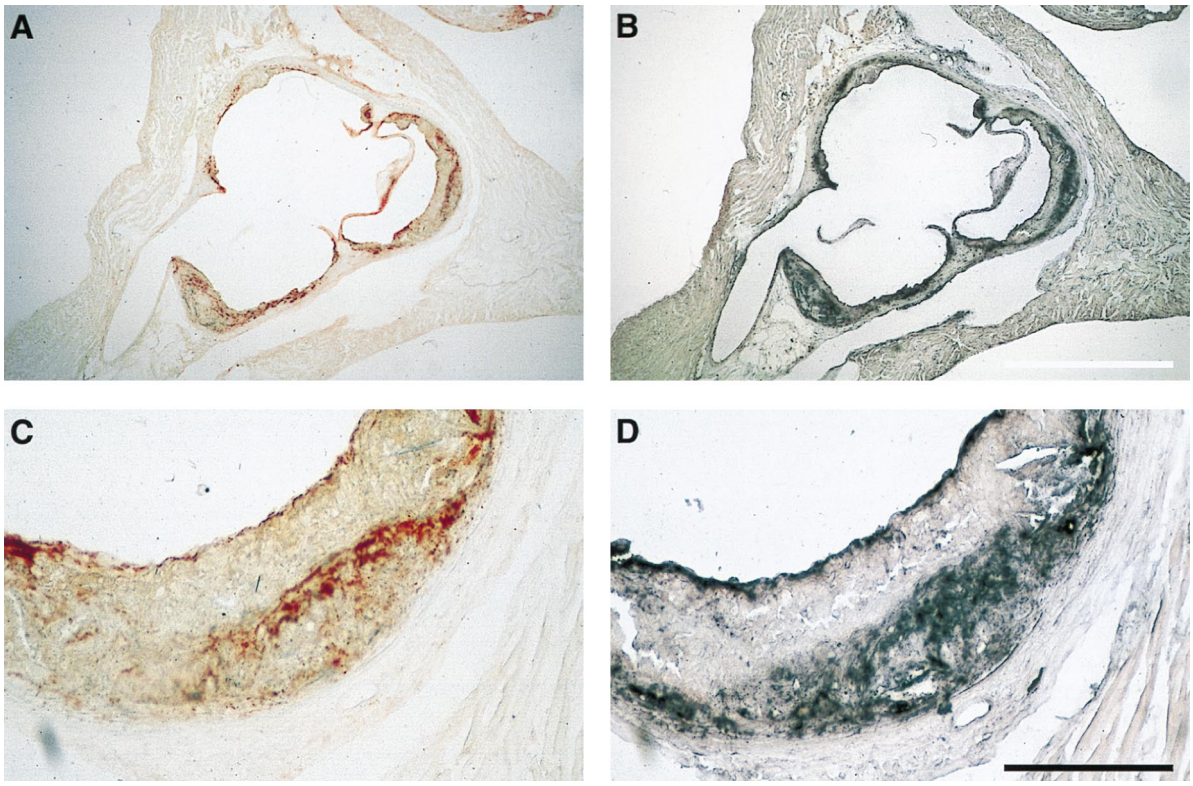

FIG. 4. Immunostained lesions from serial sections of the aortic root of a LDLR$^{-/-}$;*Tg(apoB$^{+/+}$)*;*Tg(apoa$^{+/-}$)* male mouse. (*A* and *B*) Low power images showing the distribution of apoB (*A*) and apo(a) (*B*) in adjacent sections. (C and *D*) High power images detailing the distribution of apoB (*C*) and apo(a) (*D*) in the same lesion. Scale bars represent 1 mm (*A* and *B*) and 200 μm (*C* and *D*).

apoB-100 staining but also extended into the tunica media. Whether apo(a) staining in the media is caused by local production of apo(a) or plasma apo(a) infiltrating into this region will require further study. No immunoreactive apo(a) was detected in the tunica media in the absence of an overlying atherosclerotic plaque in either the *LDLR$^{-/-}$;Tg(apoB$^{+/+}$);Tg(apoa$^{+/-}$)* or the *LDLR$^{-/-}$;Tg(apoa$^{+/-}$)* mice (data not shown). Thus, whether any apo(a) is produced in the arterial wall, it occurs only in the atherosclerotic plaques.

DISCUSSION

In this paper, we show that high level expression of human apoB-100 in *LDLR$^{-/-}$* mice is associated with the development of complex and extensive atherosclerotic lesions involving ≈15–20% of the aortic intimal surface. Lesion development does not require consumption of a high fat, high cholesterol diet, as is necessary for atherosclerotic lesion formation in *LDLR$^{-/-}$* (11) and *Tg(apoB$^{+/+}$)* (14, 15) mice. The *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* mice have dramatically elevated plasma levels of cholesterol and triglyceride, which are contained predominantly in the IDL/LDL fraction, and markedly reduced plasma levels of HDL-C. Coexpression of apo(a) in the *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* mice is not associated with a significant increase in the amount of lipid staining of the intimal aortic surface.

The "en-face" method used to quantify lesions shows the surface distribution of the lesions as well as their total surface areas. Unlike earlier mouse atherosclerosis models in which the lesions predominate in the aortic root (3, 8), the *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* mice have smaller lesions in the aortic root than in the posterior half of the aorta. The extensive lesions in the abdominal and terminal segments of the aortas of the *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* and *LDLR$^{-/-}$;Tg(apoB$^{+/+}$);Tg(apoa$^{+/-}$)* mice (Fig. 2) would have been missed completely if only the aortic root had been analyzed. A disadvantage of the en-face method is that it provides little information regarding the thickness of the lesions. However, there appears to be a good correlation between the relative lesion areas determined using the en-face method and the so-called "Paigen" method in which multiple sections from the proximal aorta are analyzed (8, 32).

Overall, there was a significant correlation between percent lesion area and the plasma levels of cholesterol, as was reported previously for other genetically modified mice (8). The surface area of lipid staining varied over a wide range, especially in the two groups of *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)*. It is likely that the mixed genetic backgrounds of these animals contribute to the impressive variation in lesion amount in these mice. Efforts are now being directed to develop *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* mice in a genetically homogeneous background, which should reduce the amount of variation within each mouse strain.

Chow-fed, *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* mice provide an alternative animal model to the *apoE$^{-/-}$* mouse for the study of atherosclerosis. The vascular lesions in both the *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* and *apoE$^{-/-}$* mice share many pathological hallmarks of human atherosclerotic plaques, including extensive lipid deposition with associated fibrous cap formation. Both strains develop complex atherosclerotic lesions distributed throughout the aorta and extending into large peripheral arteries. Although no direct comparison between chow-fed *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* mice and *apoE$^{-/-}$* mice has been performed, aortas of chow-fed, 6-mo-old, *apoE$^{-/-}$* mice were analyzed previously in the same laboratory by using an identical methodology (8); the mean lesion area in the *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* mice was 4- to 5-fold greater than that found previously in *apoE$^{-/-}$* mice (8). The distribution of lesions in the aortas of the *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* and *apoE$^{-/-}$* mice appear to differ (3, 8). The *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* mice had proportionally more lesion in the distal abdominal aorta and iliac vessels than the *apoE$^{-/-}$* mice. Additional experiments will be required to compare systematically the distribution of lesions in *apoE$^{-/-}$* and *LDLR$^{-/-}$;Tg(apoB$^{+/+}$)* mice of the same genetic background and in the same experiment. If, however, our impressions are confirmed, different classes of

atherogenic lipoprotein (i.e., LDL vs. β-VLDL) may have predilections for different regions of the vasculature in mice.

Both *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* and *apoE*$^{-/-}$ mice have dramatically elevated plasma cholesterol levels and very low concentrations of plasma HDL-C. However, the distribution of cholesterol among the other lipoprotein classes differs significantly between the two genotypes. Cholesterol in the *apoE*$^{-/-}$ mouse is predominantly in the VLDL and IDL size fractions (1, 2), whereas, in the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice, the cholesterol peak is shifted to the IDL and LDL size range, which is a pattern more closely mimicking the atherogenic lipid profile in humans. A major difference between *apoE*$^{-/-}$ *and LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice is the plasma level of triglyceride. Plasma triglyceride levels are normal in *apoE*$^{-/-}$ mice (1, 2) but are significantly increased in *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice. Triglycerides in the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice are predominantly in particles within the IDL/LDL size range on FPLC analysis (Fig. 1). The large amount of triglyceride in the IDL/LDL fraction of these mice cannot be ascribed solely to the absence of either the LDLR or cholesterol ester transfer protein. Similar elevations in plasma LDL-triglyceride are present in mice and rabbits (8, 12, 13, 30) expressing a human apoB-100 transgene, both of which have normal LDLR function. Coexpression of cholesterol ester transfer protein in mice expressing a human apoB-100 transgene mice does not alter significantly the lipid composition of LDL (33). The high levels of triglyceride-rich LDL in the plasma of these mice is most likely due to increased hepatic synthesis of apoB-containing lipoproteins stimulated by the overexpression of apoB. The absence of the LDLR may result in a larger proportion of newly synthesized lipoprotein particles being released into the plasma, and these particles may not be as accessible to lipolysis because of yet-to-be defined differences in their lipid or protein composition.

The *LDLR*$^{-/-}$;*Tg(apoa*$^{+/-}$*)* mice have ≈2-fold higher plasma levels of apo(a) and cholesterol than some *Tg(apoa*$^{+/-}$*)* mice shown previously to have a significant increase in aortic lesion area (19–21). Despite these higher levels of apo(a), we found no association between the expression of apo(a) in the *LDLR*$^{-/-}$ mice and the extent of sudanophilic lesions in the aorta. A possible explanation for the discrepancy between our results and those of some prior studies (19–21) is that different methodologies were used to quantify lesion burden. In the earlier studies, the mean lesion area was determined in a statistically relevant sample of sections from the aortic root. Because the *Tg(apoa*$^{+/-}$*)* mice have very small accumulations of lipids in their aortas (19–21), it is possible that the en face method used in this study does not detect the small lesions associated with apo(a) expression.

The comparison between the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* and *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)*;*Tg(apoa*$^{+/-}$*)* mice is more clinically relevant than the comparision between the *LDLR*$^{-/-}$ and *LDLR*$^{-/-}$;*Tg(apoa*$^{+/-}$*)* mice because the apo(a) in the "triple threat" mice circulates just as it does in humans, i.e., covalently attached to LDL. Apo(a) expression had no effect on percent lesion area in the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice (Fig. 3). It is possible that the very high plasma levels of LDL in these mice overwhelm any atherogenic effect of Lp(a), although in humans the combination of high plasma levels of LDL and Lp(a) appears to be particularly atherogenic (23). It also is possible that the plasma levels of apo(a) in these mice are not high enough to see any independent effect of Lp(a). It is noteworthy, however that despite the presence of large amounts of immunodetectable apo(a) within the lesions of the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)*;*Tg(apoa*$^{+/-}$*)* mice, no obvious differences were found in the morphology of their vascular lesions when compared with the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice. It has been proposed that apo(a) is atherogenic in the mouse because it interferes with plasminogen and TGFβ-1 activation (34, 35). No thrombotic lesions were identified in the apo(a) and Lp(a) transgenic mice. Nor was any qualitative increase in smooth muscle proliferation appreciated in the subset of mice expressing apo(a) or Lp(a), although this needs to be analyzed in a more systematic quantitative fashion.

The most important observation in this study is that *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice, which have dramatically elevated plasma levels of IDL/LDL and reduced HDL, develop large, complex atherosclerotic lesions on a chow diet within a useful experimental time frame. This mouse model provides an important alternative to the *apoE*$^{-/-}$ mice for the study of atherosclerosis. The *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* mice develop much more extensive aortic lesions (≈4 fold greater) and have an atherogenic lipoprotein profile that is more typical of that seen in humans than do the *apoE*$^{-/-}$ mice, although the triglyceride content of the LDL is much higher than that seen in man. A disadvantage of using the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$) mice is the difficulty of introducing other gene modifications into the *LDLR*$^{-/-}$;*Tg(apoB*$^{+/+}$*)* background; additional crosses are required to achieve homozygosity at three loci. A possible strategy to obviate this problem would be to insert the human apoB-100 transgene into the mouse LDLR locus. Such a mouse would provide an even more powerful tool for the study of atherosclerosis.

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