Atherogenesis in Transgenic Mice with Human Apolipoprotein B and Lipoprotein (a)

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Abstract

The engineering of mice that express a human apoB transgene has resulted in animals with high levels of human-like LDL particles and through crosses with human apo(a) transgenics, high levels of human-like lipoprotein (a) (Lp[a]) particles. In this study, these animals have been used to compare the atherogenic properties of apo(a), LDL, and Lp(a). The presence of the high expressing apoB (apoBH) transgene was associated with a 2.5-fold increase in VLDL-LDL cholesterol (primarily in the LDL fraction) and a 15-fold increase in proximal lesions compared with non-transgenic mice ($P \leq 0.0001$), while the presence of the low expressing human apoB (apoBL) transgene was not associated with major changes in lipoprotein profiles or increases in aortic lesion size. Examination of aortas of apoBH mice demonstrated lesions along the entire length of the aorta and immunochemical analysis of the lesions revealed features characteristically seen in human lesions including the presence of oxidized lipoproteins, macrophages, and immunoglobulins. Unlike animals with the apoBL transgene, animals with the apo(a) transgene had significant increases in proximal aortic fatty streak lesions compared to non-transgenic control animals (threefold; $P < 0.02$), while animals with both transgenes, the apo(a)/apoBL double transgenics, had lesions 2.5 times greater than animals expressing the apo(a) transgene alone and eightfold ($P < 0.0006$) greater than nontransgenic animals. These murine studies demonstrate that marked increases in apoB and LDL resulted in atherosclerotic lesions extending down the aorta which resemble human lesions immunochemically and suggest that apo(a) associated with apoB and lipid may result in a more pro-atherogenic state than when apo(a) is free in plasma. (*J. Clin. Invest.* 1995. 96:1639–1646.) Key words: atherosclerosis • LDL • Lp(a) • mouse • transgene

Introduction

LDL has long been considered an important risk factor for cardiovascular disease. The atherogenic potential of elevated LDL is clearly indicated in epidemiological surveys and in individuals with defects of LDL removal from plasma such as familial hypercholesterolemia (1, 2). LDL is the major cholesterol-carrying lipoprotein in human plasma, while in mice, HDL is the major cholesterol-carrying lipoprotein. This difference in the species may contribute to the relative resistance of mice to atherosclerosis.

A further difference between humans and mice is the lack of apo(a) and Lp(a)[1] in the latter. Lp(a) consists of an LDL particle and an apo(a) molecule associated via a disulfide bond (3–5). Although first reported in 1963 (6) it was not until many years later that Lp(a) was also considered an independent risk factor for cardiovascular disease (see references 7 and 8 for reviews). The sequencing of apo(a) and an apo(a) cDNA demonstrated significant primary amino acid homology to plasminogen (9). This has lead investigators to suggest that the pro-atherogenic association of high Lp(a) concentrations may result from apo(a)'s sequence similarity to plasminogen leading to an inhibition of plasminogen's anti-thrombotic effects and anti-proliferative effects on smooth muscle cells (10, 11).

The absence of apo(a) in mice has in part been corrected through the creation of human apo(a) transgenic mice with high serum levels of apo(a) (12, 13). These animals have been shown in two studies to have higher diet induced atherogenic susceptibility compared with control animals despite little difference in lipids between the two groups (12, 14). These findings were somewhat surprising in light of the demonstration that human apo(a) does not tightly associate with mouse apoB in the plasma of these animals and unlike humans the apo(a) present is primarily in the lipid free fraction of the transgenics (13). Since little lipid free apo(a) is present in humans, the murine studies raise the possibility that the atherogenic properties of Lp(a) may be mediated largely through the apo(a) component of the Lp(a) particle.

The recent development of human apoB transgenic mice (15, 16) and subsequent breedings with apo(a) transgenic mice produced animals expressing both human transgenes. It has been shown in these mice that human apo(a) efficiently interacts with human apoB producing high plasma concentrations of hu-

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1. *Abbreviations used in this paper:* apoBH, high expressing human apolipoprotein B; apoBL, low expressing human apolipoprotein B; LC, lipoprotein containing; LF, lipoprotein free; Lp(a), lipoprotein(a).

man-like Lp(a) in the plasma of the doubly transgenic apo(a)/apoB animals. As a major component of LDL, apoB also presents itself as a potentially pro-atherogenic molecule. Elevated LDL in humans is believed to promote lesion formation through oxidative modification of LDL and uptake by scavenger receptors of macrophages in the vessel wall (17). The recent development of apoE and LDL receptor knockout mice has resulted in murine models for studying more severe forms of atherosclerosis than had been characterized previously in the naturally occurring atherosclerosis-susceptible C57BL/6 strain of mice. The apoE and LDL-receptor knockout mice, however, with plasma cholesterol levels over 1000 mg/dl have lipoprotein profiles significantly differing from the common profile present in humans with atherosclerotic heart disease. The apoB transgenic mouse differs from these two knockout models of human atherogenesis due to its "human-like" lipoprotein profile. In the present study we characterized mice with apo(a), low expression apoB and high expression apoB transgenes in various combinations. Apolipoproteins, lipoproteins and atherogenesis in mice containing these various human transgenes were examined.

Methods

Production of mice. Transgenic apo(a) mice (12, 13) and transgenic human-apoB mice (16) have previously been described. The apo(a) transgenic mice were originally prepared in the C57BL/6xSJL genetic background and maintained in the hemizygous state by continued crossing to F_1 C57BL/6xSJL mice for four generations. The apoB transgenic mice were prepared in the inbred FVB genetic background and hemizygous founder transgenic animals Tg 4 (low expressor, apoBL) and Tg 11 (high expressor, apoBH) (16) were bred with hemizygous apo(a) mice to produce mice that were nontransgenic, hemizygous for one transgene or doubly hemizygous with both transgenes. The mice from this breeding were then bred with litter mates to produce mice with neither, one, or both transgenes. The mice from this final breeding were used in this study and so were of a mixed genetic background of C57BL/6, SJL, and FVB. Mice were screened for the transgenes by immunodot blot using a biotinylated human apoB specific monoclonal antibody (provided by E. Krul, Washington University, St. Louis, MO) and a biotinylated apo(a) specific monoclonal antibody (mAb 4F3; Cappel, Durham, NC). The biotinylated antibodies were detected with an Extravidin-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO). All procedures were conducted on female mice, except where specifically stated.

Diets and lipid analysis. Mice were fed Purina mouse chow (No. 5001) until 10 wk of age when the animals were then fed an atherogenic diet consisting of 1.25% cholesterol, 0.5% cholic acid, and 15% fat for a further 18 wk (18). A blood sample was collected from the tail vein at 4 wk after initiation of the atherogenic diet. Total cholesterol and HDL cholesterol were determined using commercially available assay kits (Boehringer Mannheim) that were modified for use with a microtiter plate reader (19). HDL cholesterol was determined by selective precipitation of non-HDL lipoproteins by polyethylene glycol (20).

Lipoprotein and apolipoprotein analysis. Human-apoB plasma levels were determined by ELISA with a human apoB specific antibody (International Immunology, Murrieta, CA). Apo(a) levels were determined using a commercial ELISA test kit (Stategic Diagnostics, Newark, DE). Lipoproteins were separated from plasma by adjusting 25 μl of plasma to a density of 1.21 grams/ml with NaBr and to a volume of 230 μl. The sample was centrifuged in a 42.2 rotor (Beckman) at 40,000 rpm for 20 h and 25 μl of lipoprotein containing fraction was removed from the top of the tube and 25 μl of the lipoprotein-free fraction was collected from the bottom. The floating fraction was analyzed for LDL particle size on 2–16% acrylamide, non-denaturing gradient gels (21). Reduced samples were electrophoresed on pre-cast SDS/polyacrylamide (6% acrylamide; Schleicher & Schuell, Keene, NH) and transferred to nitrocellulose at 100 mA for 15 min using a semi-dry blotting apparatus (Millipore, Bedford, MA). Apo(a) was detected by blocking nonspecific binding with 3% gelatin in Tris buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) and then incubating the membrane with a goat polyclonal anti-human Lp(a) antibody (Biodesign, Kennebunkport, ME) in tris buffered saline with 1% gelatin and 0.05% tween 20. A rabbit anti-goat IgG secondary antibody conjugated to alkaline phosphatase (Sigma Chemical Co.) was then used to detect the primary antibody and color was developed using nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate reagents in 0.1 M Tris buffer (pH 9.5). Quantitation of Immunoblots was performed using an IS-1000 Digital Imaging System (Alpha Innotech Corporation).

Lesion development. After 18 wk of the atherogenic diet animals were sacrificed and their hearts and aortas were collected. Aortic sectioning, lipid staining, and lesion scoring were performed as previously described (22). Briefly, the heart and attached aorta were fixed in 10% phosphate buffered formalin and 10-μm thick sections were prepared, each separated by 10 μm. The first and most proximal section of the aorta was taken where the aorta becomes rounded and the aortic valves distinct. Sections were stained with oil-red O and haematoxylin and counterstained with Light Green. Lesion area as determined by oil red O staining was measured using a calibrated eyepiece at 100× magnification. The mean lesion area per section per animal was determined for each individual animal.

Sudan IV staining of the aortic tree. A detailed description of Sudan IV staining of the aortic tree has previously been reported (23). Briefly, a cannula was inserted into the left ventricle of the heart and the aorta was perfused with PBS containing 20 μmol/liter BHT and 2 mmol/liter EDTA, pH 7.4. The aorta was then fixed by perfusion with 4% paraformaldehyde, 5% sucrose, 20 μmol/liter BHT, and 2 mmol/liter EDTA, pH 7.4. After exposure of the aorta the minor branching arteries and adventitia were removed. The aorta was opened longitudinally, from the heart to the iliac arteries, while still attached to the heart and major branching arteries. The aorta was then removed and pinned out on a black wax surface. The aorta were fixed in formal sucrose overnight, stained with Sudan IV and the percentage of aorta staining for lesion was determined on 24-bit color images, using Optimas 4.0 image analysis software (Bioscan, Seattle, WA), and an Oculus TCX color frame grabber with 4 megabytes of frame buffer memory (Coreco, St-Laurent, Quebec) on an IBM-based computer.

Immunocytochemistry. Tissues that were fixed with formal sucrose as described above were also used for immunocytochemistry by embedding in paraffin and preparing 8-μm-thick sections. Tissue sections were immunostained for oxidized LDL using the guinea pig antiserum MAL-2 (1:250 dilution) (24) and for the presence of mouse macrophage scavenger receptors (1:250-1:500 dilution) (25). Immunoglobulins present within lesions were detected using biotinylated anti-mouse IgG and anti-mouse IgM antisera (Vector Laboratories, Inc. Burlingame, CA.) together, as previously described (23). Color was developed using an avidin-biotin-alkaline phosphatase system (Vector Laboratories). Controls included serial sections stained without primary antibody, or with nonspecific antibodies.

Statistical analysis. Significant differences between means were determined using the Mann-Whitney U test for nonparametric analysis.

Results

Plasma apo(a) and apoB. To determine if combined expression of the individual transgenes influenced the levels of apo(a) or apoB, plasma was assayed by ELISA for the concentrations of each protein. Our results indicate that the concentration of apo(a) in plasma was increased in the presence of the apoB

Table I. Plasma Concentrations of Human ApoB and Apo(a) in Transgenic Mice

Transgene(s)	*n*	Human apoB (mg/dl)	Apo(a) (mg/dl)
Nontransgenic	11	ND	ND
Apo(a)	12	ND	5.0 (0.91)
ApoBL	13	17.0 (1.1)	ND
ApoBL/apo(a)	10	14.3 (1.2)	8.7 (0.71)*
ApoBH	13	119.1 (10.9)	ND
ApoBH/apo(a)	12	109.7 (7.7)	14.3 (1.33)‡

Plasma was collected from mice, fasted overnight, after 18 wk of the atherogenic diet. Apo(a) and human apoB were determined by ELISA as described in Methods. Results are the means with standard error in parentheses. Apo(a) levels were determined relative to human Lp(a) reference standards. *ND,* Not Detectable. * $P = 0.005$ compared with apo(a) group; ‡ $P = 0.006$ compared with apoBL/apo(a) group.

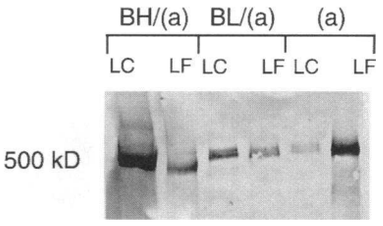

Figure 1. Apo(a) immunoblot of plasma fractions. Lipoprotein containing (LC, $d < 1.21$ gram/ml) or lipoprotein free (LF, $d > 1.21$ gram/ml) fractions of plasma from apo(a) transgenic mice with or without the apoB transgenes are presented. Plasma from mice fed the atherogenic diet for 18 wk was subfractionated and immunoblotted for apo(a) as described in Methods. *BH/(a)*, apoB high expressor with apo(a) transgene; *BL/(a)*, apoB low expressor with apo(a) transgene; *(a)*, apo(a) transgene only. Equal volumes of centrifuged plasma fractions were loaded into each lane.

transgene (Table I) and higher apoB levels were directly related to higher levels of apo(a). To ensure that immunoreactivity of apo(a) was not enhanced by the presence of apoB, plasma was electrophoresed under denaturing and reducing conditions and proteins were transferred to nitrocellulose membranes. Apo(a) was detected with a specific antibody and the intensity of the band was quantitated. The influence of apoB on apo(a) levels was consistent with the ELISA results (data not shown) demonstrating immunoreactivity was not influenced by apoB. Although apoB influenced the levels of apo(a) the reverse was not true. Plasma human apoB levels were unaffected by the presence of apo(a). The high expressor line had seven-fold higher apoB levels than the low expressor (Tg 4).

To demonstrate the presence of apo(a) in the lipoprotein fraction of plasma when human apoB is also present, plasma from apoBL/apo(a) and apoBH/apo(a) mice was separated into the lipoprotein and non-lipoprotein containing fractions and analyzed for the presence of apo(a) by immunoblot. When immunoreactive material on the blot was quantitated 9% of total plasma apo(a) was found unbound to lipoproteins in the apoBH/apo(a) expressor line and 35% of total plasma apo(a) was found unbound to lipoproteins in the apoBL/apo(a) expressor line (Fig. 1). Virtually 100% of apo(a) in apo(a) transgenic mice was found unbound to lipoproteins. A separate analysis of unfractionated, nonreduced plasma by immunoblot analysis showed similar proportions of apo(a) present in the free form (data not shown) demonstrating that centrifugation artifacts were not responsible for the observed free component. Identification of free apo(a) present in the plasma of the apoB/apo(a) transgenic mice suggested that a certain fraction of apo(a) was unavailable for linkage in vivo or became dissociated after sample collection. Similar findings have been found in studies of apo(a) in human samples (26). However, it was clear from this analysis that most of the apo(a) present in the plasma of both the apoBH/apo(a) and apoBL/apo(a) mice was present bound to apoB, similar to that found in humans.

Plasma lipids. Plasma HDL cholesterol levels have previously been shown to be correlated with lesion development within inbred strains of mice (27), however the influence of LDL-cholesterol on lesion development has been difficult to study because of low levels of LDL found in mice. We have examined this relationship in the recent development of human apoB transgenic mice.

Non-HDL (VLDL-LDL) cholesterol levels were measured by subtraction of HDL cholesterol from total plasma cholesterol. When fed the atherogenic diet, the apoBL, apo(a) and apoBL/apo(a) mice had similar VLDL-LDL cholesterol levels that were increased ~ 50% compared with nontransgenic control mice (Fig. 2). The increase of VLDL-LDL cholesterol observed in the apo(a) mice fed the atherogenic diet is in contrast to that previously reported by us for animals with this transgene and may suggest effects of differing mouse genetic backgrounds between the separate studies. Compared with control mice the apoBH and apoBH/apo(a) mice had an ~ 150% increase in VLDL-LDL cholesterol when fed the atherogenic diet.

Plasma lipoproteins, from animals fed the atherogenic diet, examined by gradient gel electrophoresis showed LDL as the major lipoprotein species in apoBH transgenic mice, but VLDL predominated in mice without the apoBH transgene (Fig. 3), including the apoBL mice. It was also determined by analysis of ultracentrifugal fractions of plasma that > 70% of VLDL-LDL cholesterol was found in the LDL region from apoBH mice compared with 20% in nontransgenic mice when both groups were fed the atherogenic diet (data not shown). This data support previous studies of mice fed a chow diet showing LDL levels were increased due to the high expressing apoB transgene (15, 16) and shows that LDL is the major cholesterol-carrier in these mice. Both the apoBH and apoBH/apo(a) transgenic mice had significantly lower HDL cholesterol levels than control mice but these changes were not observed in the presence of the apoBL or apo(a) transgenes.

Lesion development. Significant differences in fatty streak lesion area were noted in the different groups of transgenic mice after 18 wk of the high fat diet. In agreement with previous studies, the apo(a) mice developed three-fold more lesions than the nontransgenic mice ($P < 0.02$) (Table II). The apoBL transgene in combination with the apo(a) transgene was used to produce mice with significant plasma levels of Lp(a) in an environment where the contribution of the apoB component to overall changes in the lipoprotein profile and atherogenesis of the transgenic animal would be minimal. There was a small increase in lesions observed in the apoBL mice compared with controls but this failed to reach significance. However, when the apoB transgene was present with apo(a) in apoBL/apo(a) mice the number of lesions increased considerably (2.5-fold, $P = 0.035$; and 8-fold, $P = 0.0006$) compared with the apo(a)

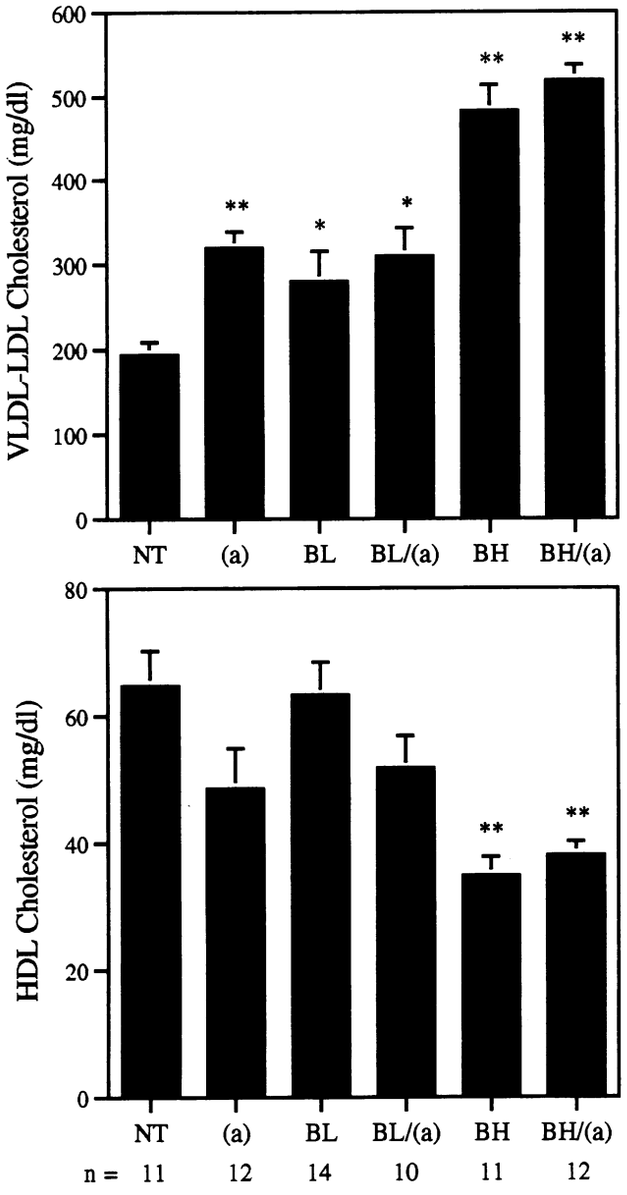

Figure 2. Plasma lipoprotein cholesterol concentration. Plasma VLDL-LDL and HDL cholesterol levels were determined for mice fed the atherogenic diet for 4 wk. Mice were fasted overnight before blood collection. Results are the means with standard error in parentheses. * $P < 0.05$ or ‡ $P < 0.001$ compared with nontransgenic group. *NT,* non-transgenic; *(a),* apo(a) transgenic; *BL,* apoB low expressor transgenic; *BH,* apoB high expressor transgenic.

group and the non-transgenic groups. Thus the addition of the apoB transgene to the apo(a) background was associated with a 70% increase in apo(a) levels, no change in VLDL-LDL cholesterol, but a 160% increase in lesion development.

Male mice have previously been shown not to be as responsive to diet induced atherogenesis as female mice (27), so in the interests of conciseness we have chosen to present data just for females. However, we have also examined male mice and found the same trends in lesion development across the different transgenic groups compared with female mice. Of particular

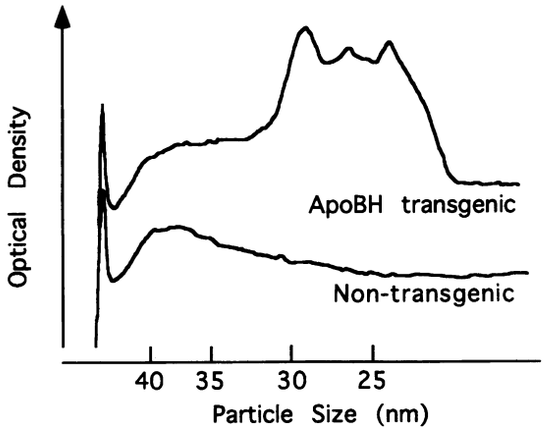

Figure 3. Gradient gel electrophoresis of plasma lipoproteins. Plasma was collected from control and apoBH transgenic mice that had been fed the atherogenic diet for 20 wk, The $d < 1.21$ g/ml lipoprotein fraction was separated from plasma as described in the Methods section. Equal volumes (10 μl) of the lipoprotein fraction were loaded onto 2–16% acrylamide gradient gels. Lipoproteins were detected by Coomassie staining of gels and scanned by densitometry. Representative scans of non-transgenic and apoBH transgenic mice are presented.

note was that male apoBL/apo(a) mice showed an 86% increase in mean lesion area of 3,652±614 μm^2 (n = 12) compared with 1,960±737 μm^2 (n = 10) for apo(a) mice ($P < 0.05$). Although the lesion areas were always less pronounced in male mice, all of the same statistical comparisons remained true as for female mice.

It was expected that the apoBH transgenic mice would show increased diet induced atherogenesis as a result of high LDL-cholesterol levels. The apoBH mice did develop significantly more lesions than the non-transgenic (15-fold, $P < 0.0001$) or apoBL mice (12-fold). The introduction of the apo(a) transgene into these mice increased the number of lesions by 40% compared with the apoBH mice but this did not achieve statistical significance. In this case, the excessively high background of atherogenic apoB lipoproteins and atherogenesis in

Table II. Lesion Areas of Mouse Aortas

Transgene(s)	n	Mean lesion area (μm^2) (± SEM)
Nontransgenic	11	891 (356)
Apo(a)	12	2932 (753)
ApoBL	14	1106 (324)
ApoBL/apo(a)	10	7781 (2646)
ApoBH	11	13462 (1877)
ApoBH/apo(a)	12	19203 (3500)

Areas of Oil-red O lipid staining material from the aortic origin of mice fed the atherogenic diet for 18 wk were quantitated as described in Methods. Results are the means with standard error. Significance levels by the Mann-Whitney U test were: nontransgenic compared with apo(a), $P < 0.02$; nontransgenic compared with apoBL, not significant; non-transgenic compared with apoBL/apo(a), $P < 0.0006$; nontransgenic compared with apoBH, $P < 0.0001$; apo(a) compared with apoBL/(a), $P = 0.035$.

the apoBH mice (fourfold greater lesion area compared with the apo(a) transgenic mice) may be obscuring the influence of an Lp(a) particle in the apoBH/apo(a) mice.

The lesions that developed in the apoBL/apo(a), apoBH and apoBH/apo(a) mice were by far the most severe of the groups analyzed in this study and were both qualitatively more severe as well as several fold larger, based upon proximal aortic fatty streak formation, than those seen in C57BL/6 mice fed the same diet for a similar time period (E. Rubin and J. Verstuyft, unpublished observation). We have examined Sudan IV stained flat preparations of aortas from the heart to the iliac bifurcation in several apoBH mice to evaluate the nature of lesion development in these mice. The extent of lesion development showed variability from animal to animal of the same genotype. However, in apoBH transgenic mice, lesions were typically observed in the aortic arch, at branch points of the intercostal arteries, and throughout the abdominal aorta (Fig. 4 *B*). This is in sharp contrast to the non-transgenic control mice from this study (Fig. 4 *A*) and C57BL/6 mice (Tangirala, R., E. M. Rubin, and W. Palinski, manuscript submitted for publication) which either did not develop lesions or developed small lesions limited to the proximal aorta, in the latter case. To evaluate for the presence of oxidized lipoproteins within the atherosclerotic lesions, the MAL-2 antibody, which is directed against an epitope of oxidatively modified lipoproteins, was used to immunostain lesions from apoBH mice. Evidence of malondialdehyde-lysine adducts were observed in lesions from the apoBH transgenic mice (Fig. 4 *C*) and macrophage infiltration was also a feature of these lesions as evidenced by the presence of macrophage scavenger receptors (Fig. 4 *D*). In addition, IgG and IgM, which are commonly seen as well known components of atherosclerotic lesions were also found in lesions of apoBH mice (Fig. 4 *E*). Some of the lesions analyzed in the apoBH transgenic mice had fibrous caps, necrotic cores, cholesterol crystals, and medial involvement. Similar observations of lesion development along the length of the aorta and oxidized lipoprotein and macrophage involvement in lesions were seen in apoBH/apo(a) transgenic mice but not in the control mice (data not shown).

Discussion

The human apoB transgene in the mouse was extremely atherogenic when expressed at high levels. This was perhaps not surprising given the increase in LDL cholesterol observed in these mice and the well reported effects of elevated LDL cholesterol in humans and experimental animals (1, 2, 28). Naturally occurring murine models of atherogenesis such as the C57BL/6 strain develop lesions primarily at the aortic root, under typical experimental conditions. However, LDL-receptor deficient and apoE knockout mice develop large lesions at the aortic root and throughout the aorta, in the setting of plasma cholesterol levels exceeding 1000 mg/dl (23, 29–31). The development of lesions at branch points of arteries along the aorta and not just at the root of the aorta in high expressing apoB transgenic mice is consistent with the distribution pattern of lesions observed in apoE and LDL-receptor deficient mouse models, yet in the context of a lipoprotein profile similar to that seen in the vast majority of patients with atherosclerosis.

The most commonly attributed mechanism through which LDL is hypothesized to result in lesion formation is via modification and uptake by scavenger receptors of macrophages in the vessel wall; receptors that are not regulated by intracellular cholesterol levels (32). Malondialdehyde (MDA)-lysine is an oxidation specific epitope that develops as a result of lipid peroxidation and is associated with oxidized but not native LDL (24, 33). The demonstration of these epitopes within lesions of apoBH mice is similar to that reported for apoE deficient mice, WHHL rabbits and humans (23, 24). Furthermore, lesions of humans and rabbits, as well as apoE deficient mice, contain immunoglobulins and immuno-competent cells such as macrophages (34). In rabbits and humans some immunoglobulins present in lesions are directed against oxidized LDL (35), possibly leading to the attraction of macrophages, so the observations of immunoglobulins and macrophages in the lesions of apoBH mice is supportive of similar mechanisms of lesion development in these transgenic mice. These results support the usefulness of the apoBH transgenic mouse as an atherosclerosis model with a "human-like" lipoprotein profile.

The development of lesions in apo(a) transgenic mice in this study confirmed previous findings (12, 14) of the atherogenic properties of this molecule, and have extended these studies to include an analysis of both free apo(a) and the Lp(a) particle. A question arising from the prior studies is; to what degree do the atherogenic properties of apo(a) in isolation contribute to the lesion development associated with high Lp(a) levels in humans? To address this question we bred human apoB transgenic mice with apo(a) transgenic mice to produce mice capable of forming the Lp(a) particle. In addition to a high expressing apoB transgenic line we chose a low expressing line to breed with the apo(a) mice to minimize the effect of excess apoB contributing to non-Lp(a) atherogenic lipoproteins.

Without the apo(a) transgene the low expressing apoB transgenic background was ineffective at initiating the development of lesions. This was true even though plasma VLDL-LDL cholesterol increased by ~ 50% in apoBL mice compared with non-transgenic mice suggesting that a threshold level of apoB may be required before a significant increase in lesions is observed. Although mouse apoB plasma levels were not measured in this study, levels were previously shown not to be influenced by the presence of the human apoB transgene (15, 16). The increase in VLDL-LDL cholesterol therefore represents a mix of human apoB and mouse apoB particles. Lesions in the combined apoBL/apo(a) transgenic mice were increased over 8-fold compared with the nontransgenic controls and 2.5-fold compared with apo(a) mice, suggesting that linkage of apo(a) with apoB may enhance the atherogenic potential of the apo(a) transgene. The parallel findings observed in male mice further reinforces one of the major findings of this study, namely, that the transgenic combination of apoBL and apo(a) promotes lesion development beyond the effect of the apo(a) transgene alone. Several possibilities exist as to how the linkage of apo(a) and apoB leads to increased lesion development.

One possibility is that strong association of apo(a) with lipoproteins, when human apoB is present, may result in a particularly atherogenic lipoprotein particle as indicated by the fact that apoBH/apo(a) mice developed the most extensive lesions (Table II), even though the particles that formed as a result of the apoB transgene alone are atherogenic at high levels as demonstrated in the high expressing apoB transgenic mice of this study. When cholesterol-rich particles are associated with apo(a), cholesterol may be targeted directly to sites where the

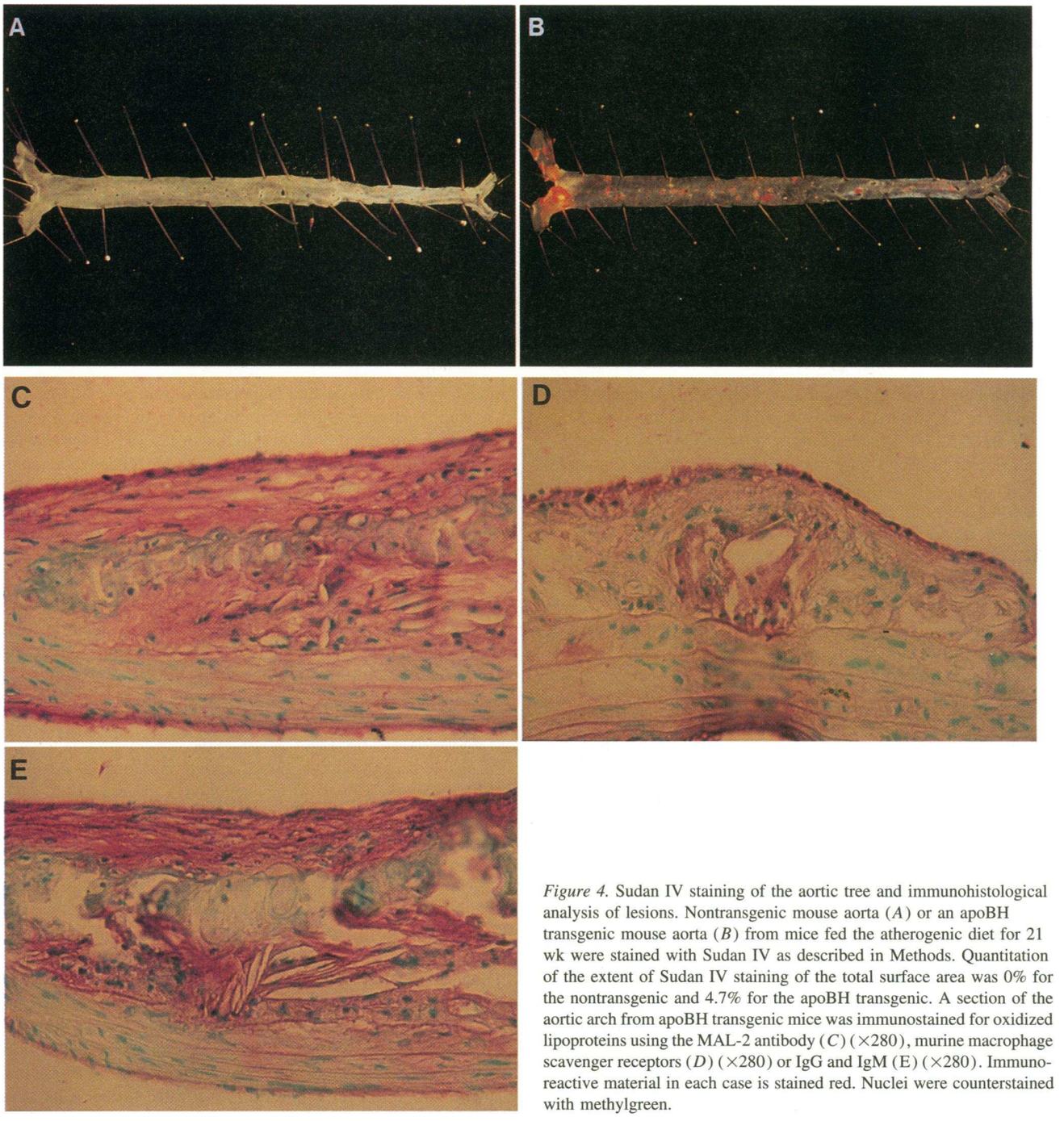

Figure 4. Sudan IV staining of the aortic tree and immunohistological analysis of lesions. Nontransgenic mouse aorta (*A*) or an apoBH transgenic mouse aorta (*B*) from mice fed the atherogenic diet for 21 wk were stained with Sudan IV as described in Methods. Quantitation of the extent of Sudan IV staining of the total surface area was 0% for the nontransgenic and 4.7% for the apoBH transgenic. A section of the aortic arch from apoBH transgenic mice was immunostained for oxidized lipoproteins using the MAL-2 antibody (*C*) (×280), murine macrophage scavenger receptors (*D*) (×280) or IgG and IgM (E) (×280). Immunoreactive material in each case is stained red. Nuclei were counterstained with methylgreen.

atherogenic properties of apo(a) are also active, thereby combining two atherogenic mechanisms at a single site in the vessel wall. Free or associated with lipoproteins, apo(a) may impact on the vasculature through the inhibition of the formation of plasmin from plasminogen by a variety of mechanisms. These include competition with plasminogen for binding to cell surface plasminogen receptors or inhibiting tissue-type plasminogen activator thus decreasing thrombolytic and anti-smooth muscle proliferative properties of plasminogen (10, 11). Therefore, damage to the vessel wall due to apo(a)'s effect on plasminogen coupled with the targeted delivery of lipid to the same sites via the lipid rich Lp(a) particle may contribute to the increased atherogenicity of Lp(a) versus apo(a).

A second factor that may lead to increased atherogenesis when human apoB and apo(a) are expressed together is the increased plasma levels of apo(a) when human apoB is also present in the plasma. The increased atherogenesis may be independent of an association with apoB and just reflect the atherogenic properties of increased apo(a) levels. Although studies have suggested that production rather than clearance rates are the most important determinants of apo(a) concentration in humans (36, 37), variability in the levels of apo(a) (Lp[a])

in the transgenic mice are not likely to be determined at the level of production because of the lack of regulatory elements and the identical cDNA transgene in all mice. Human apoB has been shown to have a reduced interaction with the mouse LDL receptor (38) so it is conceivable that in our apoB/apo(a) transgenic model, apo(a) was increased in proportion to increased apoB because of inefficient clearance by the mouse LDL receptor of human apoB (and Lp[a]) particles and possibly competition of Lp(a) with apoB containing particles for clearance. The influence of apoB plasma levels on Lp(a) levels in humans is suggested by evidence that abetalipoproteinemic patients who lack plasma apoB tend to have lower apo(a) concentrations (39). In contrast to these studies is the demonstration that individuals with defective apoB and diminished LDL-receptor binding capacity do not have elevated Lp(a) levels (8) suggesting that Lp(a) removal from plasma may be independent of the LDL receptor. Regardless of the mechanism by which apo(a) and Lp(a) are cleared from the plasma, the association of apo(a) with apoB clearly results in increased plasma concentrations of apo(a) both in humans and transgenic mice.

The mice used in this study were littermates and so, although genetically mixed, were similar but not identical. The highly significant differences in atherogenesis susceptibility ($P < 0.0001$) and VLDL-LDL cholesterol levels ($P < 0.0001$) between the non-transgenic group and apoBH transgenic group makes it extremely unlikely that these observed differences are simply due to segregating alleles of atherosclerosis susceptibility. In addition, the five-fold greater level of lesions seen in the apoBH transgenic mice compared with C57BL/6 mice (the inbred strain most susceptible to atherosclerosis) examined under identical experimental regimes (Verstuyft and Rubin, unpublished results) indicates that the genetic susceptibility to atherogenesis of the C57BL/6 background is insufficient to produce the values found in the apoBH mice of this study. The FVB inbred mice have been shown to be resistant to diet-induced atherogenesis (Verstuyft and Rubin, unpublished results).

In conclusion, marked elevations of human apoB and LDL in the mouse, as in humans, are associated with increased susceptibility to atherosclerosis. Human apoB in transgenic mice also has the capacity to increase the plasma concentration of apo(a) and consequently Lp(a). Through its effect on apo(a) levels alone, or by associating apo(a) with cholesterol-rich lipoproteins thus making a more atherogenic particle, human apoB can promote the atherogenicity of the apo(a) transgene. The severity of lesions which developed in mice with high levels of apoB, LDL and Lp(a), coupled with a lipoprotein profile not unlike that of humans with heightened atherosclerosis susceptibility, suggest the usefulness of these strains of transgenic mice for addressing issues relevant to human atherosclerosis.

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