Feedback Mechanism of Focal Vascular Lesion Formation in Transgenic Apolipoprotein(a) Mice*

Richard M. Lawn‡§, Andrew D. Pearle‡, Lawrence L. Kunz‡, Edward M. Rubin¶, Jill Reckless**, James C. Metcalfe**, and David J. Grainger**

From the ¥Falk Cardiovascular Research Center, Stanford University, Stanford, California 94305-5246, *NeoRx Corporation, Seattle, Washington 98119, the Lawrence Berkeley Laboratory, Berkeley, California 94720, and the **Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, United Kingdom

Apolipoprotein(a) (apo(a)), the distinguishing protein of atherogenic lipoprotein(a), directs accumulation of the lipoprotein(a) particle to sites in the arterial wall where atherosclerotic lipid lesions develop in man and in transgenic mice expressing human apo(a). It has been proposed that focal apo(a) accumulation in the transgenic mouse vessel wall causes the observed severe local inhibition of transforming growth factor-β (TGF-β) activity and the consequent activation of the smooth muscle cells, which subsequently accumulate lipid to form lesions if the mice are fed a high fat diet. We show that blocking formation of these vascular lesions by two independent mechanisms, tamoxifen treatment and increasing high density lipoprotein, also abolishes apo(a) accumulation, inhibition of TGF-β activity, and activation of smooth muscle cells. The data are consistent with a feedback mechanism in which an initial accumulation of apo(a) inhibits local TGF-β activity, leading to further accumulation of apo(a). Breaking the feedback loop prevents smooth muscle cell activation and therefore lipid lesion development.

An elevated plasma concentration of the lipoprotein Lp(a) causes focal deposition of Lp(a) in the vessel wall and represents a major independent risk factor for vascular diseases, including atherosclerosis, restenosis, and stroke (1–10). Lp(a) consists of a low density lipoprotein particle covalently linked to the additional protein, apolipoprotein(a) (apo(a)) (11). Plasma-derived Lp(a) accumulates in the vessel wall more readily than low density lipoprotein, reflecting the ability of its apo(a) component to bind to extracellular and cell surface targets (1, 2). The accumulation of Lp(a) occurs at focal sites in the vessel wall for reasons that are poorly understood, although several observations suggest that it occurs at sites of endothelial injury or dysfunction, where the “response to injury” hypothesis proposes that atherosclerotic lesions are initiated (12). For example, Lp(a) localizes in the vessel wall of rabbits where the endothelium has been physically injured by balloon catheter (13). In humans, Lp(a) is localized in atherosclerotic plaques and accumulates at sites of microvascular inflammation, where endothelial permeability is known to be increased (14). Once in the vessel wall, Lp(a) may further enhance initial endothelial dysfunction, as indicated by studies which show that high Lp(a) levels are associated with impaired endothelium-dependent vasodilation before atherosclerotic lesions are detectable by angiography (15, 16).

A potential mechanism by which apo(a) might promote endothelial dysfunction and atherogenesis is the inhibition of plasminogen activation, suggested by the sequence homology of apo(a) to plasminogen (17). Reduced plasmin activity would be expected to have two major atherogenic consequences, both of which have been confirmed in vivo. Reduced plasmin activity in the presence of apo(a) reduces clot lysis, which may stimulate lesion development by prolonging the lifetime of mural thrombi (18, 19). Reduced plasmin activity also results in reduced activation of transforming growth factor-β (TGF-β), a cytokine implicated in the maintenance of normal endothelial and smooth muscle cell phenotype and function (20–22). For example, the “protective cytokine” hypothesis postulates that TGF-β reduces lipoprotein permeability of the endothelium and expression of adhesion molecules for circulating leukocytes believed to initiate the earliest stage of plaque development (23). TGF-β also inhibits the processes of migration, de-differentiation, and proliferation of vascular smooth muscle cells, all associated with early lesion development (24–26). This may induce a positive feedback loop resulting in focal apo(a) accumulation (although data presented here and elsewhere suggest that active TGF-β inhibits the development of atherosclerosis, in the case where an artery has been mechanically injured, TGF-β may increase wall thickness by increasing extracellular matrix production, and overproduction of active TGF-β can be associated with tissue fibrosis (20, 27–31)).

EXPERIMENTAL PROCEDURES

Lipid Measurements—Apo(a) transgenic mice contain human apo(a) cDNA controlled by the mouse transferrin promoter on a background of C57BL/6 × SJL strains (3, 32). They were fed a high fat diet (1.25% cholesterol, 7.5% saturated fat, 7.5% casein, 0.5% cholate; ICN Biomed., Aurora, OH) from 12 weeks of age with or without 15 μg of tamoxifen/g of food, equivalent to a dose of ~1 mg/kg/day, and sacrificed at 24 weeks of age. 10-μm frozen sections were prepared from the proximal aorta region following the sectioning strategy, and lipid lesion formation was measured following Oil Red O staining, as described previously (3). The mean lesion area per section was calculated for each individual and group of animals. Coded slides were examined blind. Total cholesterol and total triglycerides were measured in serum prepared from a terminal bleed from each mouse, and the amount of cholesterol in each lipoprotein subclass was measured following size fractionation of the lipoproteins in serum pooled from all mice in each group as described previously (20).

Quantitative Immunofluorescence—Frozen sections were prepared from the aortic sinus region of 24-week-old mice and fixed in ice-cold acetone for 90 s and analyzed in a blinded fashion. Relative amounts of...
apo(a), smooth muscle-α-actin, osteopontin, and active plus latent (α11) TGF-β were determined by quantitative immunofluorescence using specific antibodies as described previously (20, 33). Briefly, nonspecific protein binding to sections was blocked by incubation of the section with Tris-buffered saline containing 3% fatty acid-free bovine serum albumin (TBS + 3% bovine serum albumin) for 30 min at room temperature. Sections were then incubated with primary antibody (10 µg/ml in TBS + 3% bovine serum albumin) for 18 h at room temperature (4 °C for TGF-β), washed three times for 3 min each in TBS, then incubated with secondary antibodies conjugated to fluorescein or tri-methylrhodamine (10 µg/ml in TBS + 3% bovine serum albumin) for 4 h at room temperature. After three further washes in TBS and rinsing in MilliQ water, sections were allowed to air-dry before mounting using Citifluor AF-1. Fluorescence associated with the vessel wall was quantified using a Photonic Science extended linear range camera attached to a Nikon Diaphot inverted fluorescence microscope and Magiscan image analysis software as described (33). For each mouse, three sections were stained as above, and a further three adjacent sections were processed as above but with the primary antiserum omitted. For each section of the fluorescence, three randomly selected fields of view containing the aorta (assessed from a phase contrast image) were analyzed (a total of nine fields of view per mouse), and the mean fluorescence in the control sections (with primary antibody omitted) was subtracted from the mean fluorescence in the stained sections to obtain the mean specific fluorescence for each mouse. For each group of mice, the mean and standard error of the specific fluorescences (in arbitrary units) for each mouse within the group are presented. In every case, sections from all the mice analyzed were stained in a single batch. Under these conditions the mean specific fluorescence is linearly proportional to the amount of antigen present (33).

Where a mean area stained is also presented, the number of pixels staining more than 1 standard deviation above the mean fluorescence.
Lesion Formation in Apolipoprotein(a) Mice  

Effect of transgenic expression of apo(a) and apoAI on TGF-β, smooth muscle differentiation, and apo(a) localization

The relative quantity of various proteins in the aortic vessel wall of transgenic apo(a) mice, double transgenic mice expressing both apo(a) and apoAI, and wild type littermate controls is shown in arbitrary units (mean ± S.E.). For apo(a), the area of vessel wall (in mean pixels/section per animal) staining more than 1 standard deviation higher than the mean as a measure of focal apo(a) accumulation, is presented.

Results and Discussion

Previous cell culture studies showed that apo(a) inhibited the plasmin-dependent activation of TGF-β and consequently promoted the migration and proliferation of smooth muscle cells (26, 35). Studies of transgenic mice expressing the human apo(a) gene identified sites of very high focal accumulation of apo(a), mainly near the luminal surface of the vessel. At these sites, activation of TGF-β was severely inhibited, and there was greatly enhanced expression of osteopontin, an in vitro protein marker of smooth muscle cell activation (20, 36, 37). Correlated increases in apo(a) accumulation and osteopontin expression and a decrease in active TGF-β occurred progressively with age but without formation of lipid lesion when the mice are fed a chow diet (Fig. 1). However, if the apo(a) mice are subsequently fed a high fat diet, then lipid lesions are formed at the focal sites of apo(a) accumulation. This pathway is shown in red in Fig. 1. This lipid accumulation may be due to the increased propensity of activated smooth muscle cells to accumulate lipoproteins compared with contractile cells, resulting from up-regulation of oxidized low density lipoprotein (“scavenger”) receptors (38–40). In this particular mouse model, analysis of the cellular response to apo(a) is simplified by the observation that most of the lipid-filled “foam cells” are of smooth muscle origin.

There are few macrophages as detected by electron microscopy (Fig. 2) or by immunostaining with Mac 1 and a panel of other monocyte/macrophage antibodies. However, since it has been reported that TGF-β inhibits scavenger receptor activity in macrophages (41), lipid accumulation in this cell type might also be enhanced by the pathway of apo(a) deposition and TGF-β suppression, as proposed here.

To test whether lesions develop according to the proposed pathway (Fig. 1c in red), we inhibited the pathway at two points. Mice overexpressing the high density lipoprotein (HDL) component, apolipoprotein A-I, have elevated levels of HDL, which is thought to reduce lipid accumulation by reverse cholesterol transport and anti-oxidant activity. Thus, double transgenic mice expressing apo(a) plus apolipoprotein A-I should have lipid accumulation directly blocked. Treatment with tamoxifen is a second method of preventing vascular lesions recently described for C57BL/6 mice (42). Tamoxifen is a mixed antagonist/agonist of estrogen that is widely used in breast cancer therapy. It is known to induce the synthesis of TGF-β by a number of cell types, including smooth muscle cells.
Lesion Formation in Apolipoprotein(a) Mice

The development of lipid-filled vascular lesions, as well as the plasma lipoprotein profile and expression of various proteins, was measured in 24-week-old apo(a) transgenic mice on a high fat diet from 12 weeks of age with or without tamoxifen (TMX). Apo(a) plasma concentration in transgenic mice was 9.3 ± 0.9 mg/dl and remained unchanged by tamoxifen or diet treatments; n = 8 for each group. All values are reported as mean ± S.E. except for the cholesterol in lipoprotein fractions, where a single determination on pooled serum was made for each group.

| High fat diet | High fat diet + TMX |
|--------------|---------------------|
| **TMX (mg/g food)** |          |
| Weight after experiment (g) | 28 ± 2 | 22 ± 1a |
| Lesions per mouse | 6.8 ± 2.3 | 1.4 ± 0.9a |
| Lesions area (μm²/mouse) | 11,644 ± 4238 | 992 ± 375a |
| (a + 1) TGF-β (arbitrary units) | 47 ± 7 | 60 ± 7b |
| Active TGF-β (arbitrary units) | 23 ± 2 | 51 ± 6b |
| Osteopontin (arbitrary units) | 66 ± 4 | 14 ± 2b |
| SM-α-actin (arbitrary units) | 114 ± 7 | 182 ± 13b |
| Apo(a) (arbitrary units) | 26 ± 2 | 8 ± 1b |
| Apo(a) area (pixels/section) | 2170 ± 380 | 70 ± 110b |
| Total cholesterol (mg/dl) | 71 ± 5 | 65 ± 4b |
| VLDL-cholesterol (mg/dl) | 17 | 16 |
| LDL-cholesterol (mg/dl) | 17 | 19 |
| HDL-cholesterol (mg/dl) | 37 | 30 |
| Total triglycerides (mg/dl) | 91 ± 15 | 115 ± 11b |

*p < 0.05 Mann-Whitney U-test.

Tamoxifen thus elevates TGF-β activity and blocks smooth muscle cell activation. If the proposed pathway shown in Fig. 1c is correct, blockade at various points should abolish effects occurring later, but not earlier, in the pathway.

When transgenic mice overexpressing apolipoprotein A-I are crossed with the apo(a) mice, the plasma HDL concentrations are increased 2-fold compared with apo(a) only mice, and there is a 95% reduction in lipid vascular lesion formation as expected (45). However, the double transgenic mice also showed reduced vessel wall staining of apo(a), with a reduction in focal apo(a) accumulation of about 95% compared with the apo(a) only mice. Also abolished was smooth muscle cell activation that is measured by osteopontin increase and α-actin decrease (Table I).

In the second manipulation, apo(a) mice were fed a high cholesterol diet with or without 15 μg of tamoxifen per gram, equivalent to a tamoxifen dose of 1 mg/kg body weight per day (see legend to Table II). This had the anticipated result of increasing TGF-β concentration in serum and in the vessel wall. After 12 weeks, aortic sections were assayed for lipid lesion development, apo(a), and TGF-β activity. Tamoxifen reduced the number of lipid lesions and lesion area by 80 and 92%, respectively (Table II). Tamoxifen had no effect on the circulating levels of apo(a) in the transgenic mice (where apo(a) cDNA is controlled by the transferrin promoter) but surprisingly reduced the average concentrations of apo(a) in the vessel wall by 69% and area of focal apo(a) accumulation by 97% (Table II). There was 2-fold increase in the mean active TGF-β levels in the aortic wall, and there were no focal sites of very low TGF-β activity. Tamoxifen treatment also reduced osteopontin and increased smooth muscle α-actin, consistent with the increased local concentration of active TGF-β (Table II).

As in the previous study of C57BL/6 mice (42), tamoxifen treatment caused a reduction in weight gain of apo(a) mice, and there was a small but significant increase in triglycerides and decrease in total cholesterol (Table II). However, most of the decrease in cholesterol occurred in the cardioprotective HDL fraction, and the ratio of HDL to total cholesterol was virtually identical for both groups. The effects of the drug on the lipoprotein profile would not, therefore, be expected to reduce lesion formation. The elevation of TGF-β activity is a plausible mechanism by which the cardioprotective effect of tamoxifen is exerted. But irrespective of the mechanism of action of tamoxifen, we did not expect it to inhibit apo(a) accumulation as well as vascular lesions. Yet, tamoxifen prevents apo(a) accumulation, the inhibition of TGF-β activity, smooth muscle cell activation, and lipid lesion formation. These data support a causal linkage between these effects.

We have shown that when we inhibited two steps in the lesion development pathway, all steps were inhibited. The data are consistent with a mechanism for atherogenesis by apo(a), which is driven by a positive feedback loop between focal apo(a) accumulation and TGF-β inhibition at specific sites in the vessel wall, as indicated by the circulatory pathway in Fig. 1c. Apo(a) probably begins to accumulate at sites where the integrity or function of the endothelium is impaired. As a result of the apo(a) accumulation, TGF-β activity is locally depressed within the vessel wall, and the protective effect of TGF-β is diminished, resulting in further accumulation of apo(a). In support of this mechanism, TGF-β has been shown to reduce adhesion of neutrophils to endothelial cells and protect the endothelium in models of ischemic reperfusion, while its absence in knockout mice results in rampant inflammation (48–51). Hence, reduced levels of active TGF-β not only lead to smooth muscle cell activation but may promote inflammatory damage to the endothelium and increased permeability of the endothelial barrier to circulating lipoproteins such as Lp(a). In the presence of excess lipid in the circulation of the apo(a) transgenic mice fed a high fat diet, lipid accumulates in activated smooth muscle cells at the sites of very low TGF-β activity. We have therefore modified our original linear pathway by providing evidence for a positive feedback mechanism driving vascular lesion development.

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3 It should be noted that in this model, tamoxifen does not alter the circulating levels of apo(a), since this derives from transgenic expression of apo(a) cDNA driven by the transferrin promoter. However, tamoxifen has recently been shown to lower the concentration of circulating Lp(a) in humans (46) by inhibiting transcription of the apo(a) gene (47). Hence, tamoxifen and its analogs may exert a doubly protective effect against the cardiovascular risk of elevated Lp(a).
