TGF-β1 Limits Plaque Growth, Stabilizes Plaque Structure, and Prevents Aortic Dilation in Apolipoprotein E–Null Mice

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Objective—Impairment of transforming growth factor (TGF)-β1 signaling accelerates atherosclerosis in experimental mice. However, it is uncertain whether increased TGF-β1 expression would retard atherosclerosis. The role of TGF-β1 in aneurysm formation is also controversial. We tested whether overexpression of active TGF-β1 in hyperlipidemic mice affects atherogenesis and aortic dilation.

Methods and Results—We generated apolipoprotein E–null mice with transgenes that allow regulated overexpression of active TGF-β1 in their hearts. Compared to littermate controls, these mice had elevated cardiac and plasma TGF-β1, less aortic root atherosclerosis (P<0.002), fewer lesions in the thoracic and abdominal aortae (P<0.01), less aortic root dilation (P<0.001), and fewer pseudoaneurysms (P=0.02). Mechanistic studies revealed no effect of TGF-β1 overexpression on plasma lipids or cytokines, or on peripheral lymphoid organ cells. However, aortae of TGF-β1–overexpressing mice had fewer T-lymphocytes, more collagen, less lipid, lower expression of inflammatory cytokines and matrix metalloproteinase-13, and higher expression of tissue inhibitor of metalloproteinase-2.

Conclusions—When overexpressed in the heart and plasma, TGF-β1 is an antiatherogenic, vasculoprotective cytokine that limits atherosclerosis and prevents aortic dilation. These actions are associated with significant changes in cellularity, collagen and lipid accumulation, and gene expression in the artery wall. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: aneurysm • atherosclerosis • growth substances • inflammation • plaque

TGF-β1 is a pleiotropic cytokine that circulates in plasma and is produced by several cardiovascular cell types including smooth muscle and endothelial cells, monocytes, macrophages, and T cells.1,2 Several studies associate variations in TGF-β1 expression or signaling with atherosclerosis and aneurysm formation; however, the precise relationships between TGF-β1, atherosclerosis, and aneurysm formation are incompletely understood.

Human studies largely support an antiatherogenic role for TGF-β1, with most studies revealing a negative correlation between plasma TGF-β1 concentration and the presence or extent of atherosclerosis.3–6 Although some animal studies suggest that TGF-β1 could accelerate atherosclerosis by increasing vascular extracellular matrix accumulation and lipid retention,7–10 several studies in hyperlipidemic mice instead portray TGF-β1 as an antiatherogenic cytokine that limits atherosclerosis largely through immunosuppressive effects. Almost all of these murine studies involve systemic suppression of TGF-β activity, for example by injection of antibodies to TGF-β, infusion of a soluble TGF-β receptor, or transgene-mediated abrogation of TGF-β signaling in T cells.11–14 These studies show that less systemic TGF-β activity accelerates atherosclerosis, but they do not address whether enhancement of TGF-β1 signaling would be antiatherogenic. A recent gene-transfer study suggests that elevated systemic expression of TGF-β1 might suppress atherosclerosis15, however, because increased in vivo expression of TGF-β1 protein was not documented in this study, its interpretation is difficult.

The role of TGF-β signaling in regulating aortic lumen diameter and aneurysm formation is controversial. Evidence of increased TGF-β signaling was reported in aneurysmal aortae of humans with a familial aortic aneurysm syndrome and in a mouse model of Marfan syndrome.16,17 In the mice, aneurysm formation was prevented by injection of neutralizing antibodies to TGF-β.16 However, in a xenograft model of aneurysm formation, TGF-β1 facilitated aneurysm healing.18 Similarly, in mice deficient in the extracellular matrix glycoprotein Emilin1, increased aortic TGF-β signaling was associated with aortic constriction.19 Because these studies are...
either correlational or involve a severe inflammatory reaction, they do not allow confident prediction of the effect of chronic overexpression of TGF-β1 on native aortic diameter. Both aneurysm formation and aortic constriction seem plausible.

To determine the effect of enhanced TGF-β1 expression on atherosclerosis and aneurysm formation, we used tetracycline-suppressible transgenes (ie, the “tet-off” system) to achieve regulated systemic overexpression of a constitutively active form of TGF-β1 in ApoE−/− mice. We measured the effect of enhanced TGF-β1 expression on aortic lesion size and composition, aortic structure and diameter, T-cell accumulation, and expression of selected genes in the aortic wall.

Methods

Atherosclerosis Studies

Mice with tetracycline-regulated cardiac-specific expression of constitutively active TGF-β1 were bred into the C57BL/6 ApoE−/− background. Atherosclerosis studies were performed on 3 groups of female mice: doubly transgenic (αMHC-tTA/tetO-TGF-β1) mice on and off doxycycline (“DT-on” and “DT-off”) and singly transgenic αMHC-tTA mice off doxycycline (“ST-off”). Mice were killed at 18 weeks (after 12 weeks of Western diet).

TGF-β1 Protein, Plasma Lipids, and Peripheral Blood Cell Counts

TGF-β1 protein was measured by ELISA of mouse plasma or explant culture media. Plasma lipids were measured.

Tissue Processing and Histology

Atherosclerosis, aortic dilation, and pseudoaneurysm formation were evaluated using sections of aortic roots, computer-assisted planimetry, and both histochemical and immunohistochemical stains. Atherosclerosis was also measured on pinned aortas.

Immunoblot for Phospho-Smad2

Phosphorylation of Smad2 was assessed by Western analysis of extracts of hearts and aortas.

Peripheral Blood Cell Counts, Lymphocyte Markers, and Plasma Cytokines

Blood cells were counted by an outside laboratory. Flow cytometry was performed on splenocytes and lymph node cells. Plasma cytokines were measured by cytofluorometric bead assay.

Aortic Gene Expression

Aortic RNA was extracted, reverse transcribed, and amplified.

Statistical Analysis

Normally distributed data with equal group variances are presented as mean±SEM and analyzed using ANOVA and unpaired Student t test. Other data are presented as median (25% to 75% range) and analyzed with Kruskal–Wallis ANOVA and Mann–Whitney rank-sum test.

Results

Increased TGF-β1 Expression and Signaling in DT-Off Mice

DT-off mice had doxycycline-regulated, cardiac overexpression of active TGF-β1, and elevated total cardiac and plasma TGF-β1 (Figure 1A and 1B). As with the DT-off ApoE+/− mice reported previously, no active TGF-β1 was detected in any plasma sample. Elevated plasma total TGF-β1 was easily detected 4 weeks after doxycycline withdrawal and continued for at least 12 weeks. The 10-fold increase in plasma TGF-β1 in DT-off mice is within the range associated with a relative absence of severe multivessel coronary artery disease in humans.

DT-off hearts had evidence of increased TGF-β1 activity and signaling, shown by increased cardiac phosphorylated (phospho)-Smad2 in the absence of any change in total Smad2 protein (Figure 1C). Immunohistochemical staining of aortic root sections revealed surprisingly high baseline levels of both phospho-Smad2 and nucleus-localized Smad3 in
control aortae, with no further increase in aortae of DT-off mice (supplemental Figure I and data not shown). Similarly, Western blotting of extracts of DT-off aortae did not show increased phospho-Smad2 compared to controls (supplemental Figure II).

TGF-β1 Overexpression Limits Atherosclerosis, Aortic Root Dilation, and Pseudoaneurysm Formation

DT-off mice had significantly smaller aortic root intimal plaques (20% to 25% reduction; \( P \leq 0.002 \) versus each of the control groups; Figure 2A). DT-off mice also had less total aortic surface atherosclerosis than either of the 2 control groups. This finding was of borderline significance versus the ST-off mice (\( P = 0.05 \)); but not versus the DT-on group (\( P = 0.3 \); Figure 2B). The DT-off mice had less atherosclerosis in all segments of the aorta (Figure 2 and data not shown), but this difference reached statistical significance only in the abdominal aorta versus the ST-off mice (50% reduction; \( P = 0.02 \); Figure 2B). To measure lesion initiation, we counted the number of individual lesions in the abdominal and thoracic aortae. This analysis revealed a large and statistically significant antiatherogenic effect of TGF-β1 overexpression: TGF-β1–overexpressing mice had fewer lesions in both the thoracic and abdominal aorta (approximately 30% decrease in thoracic aorta; \( P \leq 0.01 \) versus both control groups; approximately 50% decrease in abdominal aorta; \( P = 0.005 \) versus both control groups; Figure 2E and 2F).

Overexpression of TGF-β1 in DT-off mice also decreased aortic root circumference and cross-sectional area. Aortic roots of DT-off mice had a 10% decrease in circumference and a 20% decrease in calculated area within the internal elastic lamina (\( P < 0.001 \) versus both control groups; Figure 3A, Table). Aortic roots of DT-off mice also had significantly fewer pseudoaneurysms (75% reduction; \( P = 0.02 \) versus both control groups; Table, Figure 3B and 3C). Total aortic luminal surface area was not different between DT-off mice and controls. However, mice on doxycycline had slightly less total aortic surface area (6% decrease; \( P = 0.05 \) versus all mice off doxycycline; Table).

Figure 2. Aortic atherosclerosis. TGF-β1-overexpressing (DT-off) mice and littermate controls (DT-on and ST-off) were fed a high-fat diet for 12 weeks. Atherosclerosis was quantified by measuring intimal area on sections of aortic roots (A), percent Sudan IV-stained lesion area on pinned aortae (B through D), and counting individual lesions on pinned aortae (E and F). Data points represent individual aortae; bars are group medians.

Figure 3. Aortic root circumference and pseudoaneurysm formation (A). Aortic root circumference was measured at the level of the internal elastic lamina in TGF-β1-overexpressing mice (DT-off) and littermate controls (DT-on and ST-off). Data points represent individual aortae; bars are group medians. Aortic root pseudoaneurysm (B and C). Section is from a control (ST-off) mouse, after 12 weeks on high-fat diet. The pseudoaneurysm (box in B, enlarged in C) is defined as disruption of all medial elastic laminae with protrusion of intimal plaque contents abluminal to the external elastic lamina. Movat stain; Bars: 500 μm (B) and 100 μm (C).
**Effects of TGF-β1 Overexpression on Cardiac and Systemic Parameters**

Heart weight, body weight, heart/body weight ratios, and cardiomyocyte diameter were not affected by TGF-β1 overexpression (Table). DT-off hearts had mild perivascular and epicardial fibrosis and occasional patches of interstitial fibrosis (Figs. III and IV). There were no significant differences in plasma triglycerides and FPLC profiles also did not differ among the groups (Table). Blood pressure of DT-off mice was not significantly different from controls (Supplemental Figure V).

Total peripheral leukocyte and lymphocyte counts were not altered in DT-off mice versus either control group (Table). Three independent FACS analyses of splenocytes and lymph node cells revealed no consistent alterations in the number or proportion of CD4+ T cells, CD69+ cells, or CD11b+ macrophages relative to controls (Supplemental Figure V).

**TGF-β Overexpression Alters Aortic Lipid and Collagen Accumulation, T-Cell Abundance, and Gene Expression**

Because we found no systemic effects of TGF-β1 overexpression that could account for the highly significant reductions in atherosclerosis, aortic root dilation, and pseudoaneurysm formation in DT-off mice versus both control groups (Figures 2 and 3, Table), we looked for effects of increased plasma TGF-β1 on cellularity, matrix accumulation, lipid content, and gene expression in the aortic wall. Of the 2 control groups available for these studies, DT-on mice—but not ST-off mice—were exposed to several weeks of doxycycline, a compound that can affect vascular collagen deposition and cellularity, lipid accumulation, gene transcription, and cytokine expression.25–30 As noted above, there was a small borderline-significant effect of doxycycline on total aortic surface area. Therefore, for these experiments we used ST-off mice as littermate controls that differed from DT-off mice only in exposure to elevated TGF-β1.

After 12 weeks on high-fat diet, plaques in DT-off mice had a significantly different composition than plaques in ST-off mice. DT-off plaques had less lipid (52 versus 61% of plaque volume) and more collagen (4.7 versus 1.5% of plaque volume; \( P < 0.002 \) for both; Figure 4A and 4B). These findings are of particular interest because they contrast with in vitro and correlational studies that associate TGF-β1 effects on vascular matrix with retention of lipid and worse atherosclerosis.31,32 The groups did not differ in percentage of intimal plaque volume occupied by macrophages or smooth muscle cells (Table). DT-off intimal plaques also had less evidence of immune system activation. After 6 weeks on diet, intimal T-cell accumulation was decreased in DT-off versus ST-off plaques (2.2 [1.1 to 2.6] versus 5.2 [2.7 to 6.6] intimal

**Figure 4. Aortic root intimal plaque lipid and collagen content.** Aortic root sections from TGF-β1-overexpressing mice (DT-off) and littermate controls (ST-off), all fed a high-fat diet for 12 weeks, were analyzed for: plaque lipid content, determined by oil red O stain (A) and plaque collagen content, measured by picrosirius red birefringence (B). Data points represent individual aortae; bars are group medians.
T cells per section; \( P=0.03 \); Figure 5A), and I-A expression was reduced by 80% (2.8 [1.8 to 8.5] versus 12 [6.2 to 16] intimal I-A–expressing cells per section; \( P=0.04 \); Figure 5B). I-A is a mouse MHC-II gene product expressed primarily by antigen-presenting cells such as macrophages, and most of the I-A–positive cells appeared to be macrophages.

We also measured aortic expression of genes encoding selected cytokines, matrix metalloproteinases (MMPs), and MMP inhibitors (supplemental Table I). These analyses revealed 40% to 50% less mRNA for TNF-α, MIP-1α, and MIP-1β in DT-off versus ST-off aortae (\( P=0.015 \) for all). IFN-γ mRNA was reduced by 40%; however, this was of only borderline significance (\( P=0.09 \)). mRNA for IL-10, MIP-3α, and MIP-3β were not significantly altered (\( P=0.4 \) for all). In DT-off mice, aortic MMP-13 expression was reduced by 70% (\( P=0.01 \)), and TIMP-2 expression was increased by 230% (\( P=0.03 \)). Expression of MMP-9 was reduced by 70% and MMP-14×25%; these changes were of borderline significance (\( P=0.1 \)). Expression of MMP-2, -3, and -12, TIMP-1, and TIMP-3 were not significantly altered (\( P=0.2 \) for all).

**Discussion**

We generated Apoe\(^{-/-}\) mice with regulated cardiac-specific overexpression of active TGF-β1. These mice had: (1) Smaller aortic root intimal lesions, with less lipid and more collagen; (2) Fewer atherosclerotic lesions in their thoracic and abdominal aorta; (3) Unaltered peripheral blood leukocyte levels, peripheral lymphoid organ population, and plasma cytokine levels; (4) Less evidence of immune activation within intimal plaques including fewer T cells and lower I-A expression; (5) Decreased aortic expression of TNF-α and the T-cell chemoattractant cytokines MIP-1α and MIP-1β; (6) Less aortic root dilatation and pseudoaneurysm formation; and (7) Lower aortic expression of MMP-13 and higher expression of TIMP-2. These data show that elevated plasma TGF-β1 limits atherosclerosis and prevents aneurysm formation. They also suggest that elevated plasma TGF-β1 acts directly on cells in the aortic wall.

Before initiating this study, we attempted both constitutive and conditional overexpression of TGF-β1 in the mouse vasculature. Constitutive overexpression of TGF-β1 was embryonically lethal,\(^{31}\) and conditional postnatal vascular overexpression of TGF-β1 was unsuccessful.\(^{20}\) Therefore, we developed a conditional cardiac overexpression system that produces high plasma levels of TGF-β1.\(^{20}\) We reasoned that this system, applied in Apoe\(^{-/-}\) mice, would deliver cardiac-derived active TGF-β1 locally to the adjacent aortic root lesions and potentially deliver blood-born TGF-β1 to the entire arterial tree. We anticipated that this model would test the hypothesis, derived from correlational human studies,\(^{3-6}\) that elevated plasma TGF-β1 suppresses atherogenesis.

Elevation of plasma TGF-β1 in Apoe\(^{-/-}\) mice decreased the size and number of aortic atherosclerotic lesions and altered lesion composition toward a less inflammatory and more stable plaque phenotype. These results were not predictable from previous studies that almost exclusively used loss-of-function approaches to investigate the role of TGF-β signaling in murine atherosclerosis.\(^{11-14,32}\) There are 3 major reasons for this. First, in general, it is not possible to predict the effect of an excess of a plasma factor solely from the effect of its deficiency. Second, in at least 2 of the TGF-β loss-of-function studies, deficient TGF-β signaling caused systemic immune stimulation including splenocyte activation and elevated plasma cytokine levels.\(^{12,13}\) These systemic effects rather than loss of a specific downstream effect of TGF-β signaling might be responsible for accelerated atherosclerosis. Third, 3 of the loss-of-function studies used germ-line manipulations that would alter TGF-β signaling during development, potentially yielding abnormal immune systems in the adult mice.\(^{12,13,32}\) Atherosclerosis studies performed in mice with abnormal immune systems might yield results that are not generally applicable. To avoid developmental perturbations, we used a conditional TGF-β overexpression approach that cannot affect immune system development because it is “turned on” only in adults. The lack, in the present study, of measurable effects of TGF-β1 overexpression on plasma cytokines and peripheral lymphoid organ cells suggests that we also avoided potentially confounding effects on systemic immunity. Nevertheless, we acknowledge the possibility that TGF-β1 overexpression in the DT-off mice could have caused immune system perturbations or other systemic effects that we failed to detect.

We investigated several potential mechanisms through which elevated plasma TGF-β1 could suppress atherosclerosis. After noting the absence of detectable effects of TGF-β1 overexpression on plasma lipids, cytokines, leukocytes, and peripheral lymphoid organ cells, we looked for evidence of increased TGF-β signaling in the aorta. Although we did not find increased aortic phospho-Smad2 (discussed below), we did find that TGF-β overexpression significantly altered aortic T-cell abundance, lipid and collagen content, and aortic wall expression of I-A, TNF-α, MIP-1α, MIP-1β, TIMP-2, and MMP-13. These specific effects on T cells, collagen, I-A, cytokines, TIMP-2, and MMP-13 are all plausibly related to TGF-β overexpression because each of them is a well-described consequence of increased TGF-β activity.\(^{13,35-37}\) Moreover, suppression of T cells, I-A and TNF-α expression, and upregulation of TIMP-2 are all associated experimentally with decreased atherosclerosis.\(^{13,38,39}\) In addition, elevated aortic expression of MIP-1α and MIP-1β is correlated with plaque growth in Apoe\(^{-/-}\) mice.\(^{40}\) Taken together, our data identify several plausible molecular and cellular mechanisms.

**Figure 5.** Cellular markers of inflammation in aortic root intima. Sections of aortic roots of TGF-β1–overexpressing mice (DT-off) and littermate controls (ST-off), all fed a high-fat diet for 6 weeks, were stained for expression of CD3 (a T-cell marker; A) and I-A expression (B). Data points represent mean values for aortae of individual mice; bars are group medians.
through which elevated TGF-β1 could suppress atherosclerosis at the level of the artery wall. Although we cannot exclude that plasma TGF-β1 acts at an extravascular site with indirect effects on atherosclerotic lesion growth, the simplest explanation of our results is that high levels of plasma TGF-β1 suppress atherosclerosis via direct actions on gene expression and consequently on cellularity and lipid accumulation in the artery wall.

We were surprised to find increased phospho-Smad2/3 in hearts but not in aortae of DT-off mice. Three potential explanations for this are: (1) insensitivity of Western analysis and immunostaining in a setting of high baseline phospho-Smad2/3; (2) compensatory downregulation of phospho-Smad2/3 via a negative feedback loop in response to TGF-β1–mediated phenotypic changes; and (3) Smad2/3-independent TGF-β1 signaling. Further studies are required to determine whether the DT-off mice have altered vascular cell signaling and, if so, which pathways are activated and when. These studies would also be aimed at identifying specific vascular cell types that respond to elevated plasma TGF-β1 and would likely include confocal microscopy to colocalize cell-type–specific antigens and markers of activated TGF-β signaling. Unfortunately, cell-type-specific deletion of TGF-β signaling cannot easily be used to investigate which vascular cells are responsive to TGF-β1, because deletion of TGF-β signaling in immune cells causes a lethal systemic inflammatory disease and loss of TGF-β signaling in SMC or EC is embryonically lethal. Overexpression of TGF-β1 also prevented aortic root dilation and pseudoaneurysm formation. These vasculoprotective effects are all logical consequences of TGF-β1 stimulation of aortic TIMP-2 expression and suppression of MMP-13 expression. This TGF-β1–mediated protection against aortic root dilation contrasts with a study in fibrillin-1–null mice in which aortic root dilation was associated with increased aortic wall TGF-β1 signaling and a report that familial aortic aneurysm syndromes are associated with type I and type II TGF-β receptor mutations that cause a paradoxical increase in TGF-β1 signaling. However, other studies are congruent with our finding that TGF-β1 can prevent aortic medial destruction and aneurysm formation. For example, overexpression of TGF-β1 in aortic xenografts stabilized already-formed aneurysms, downregulated metalloproteinase expression, decreased T-cell infiltration, and promoted vascular healing. Moreover, mice lacking Emilin1 had increased vascular TGF-β1 signaling that led to generalized blood vessel narrowing. It is difficult to reconcile all of these studies, which appear to show opposite effects of increased TGF-β signaling on aortic diameter. Further mechanistic studies are needed to explain these apparently context-specific effects of TGF-β1 on aortic diameter.

Another limitation of our study is that we could not—using only this line of tetO-TGF-β1 mice—determine the minimum level of TGF-β1 overexpression necessary to retard atherosclerosis and aneurysm formation. Identification of this minimum level will require generation of new lines of tetO-TGF-β1 mice that express TGF-β1 at lower levels and would include efforts to quantify TGF-β1 activity and signaling in target tissues such as the aorta.

In summary, elevated plasma TGF-β1 in Apoe−/− mice retards atherosclerosis, stabilizes plaque structure, prevents aortic dilation, decreases vascular inflammation, and limits pseudoaneurysm formation. In humans, the inverse correlation of plasma TGF-β1 and atherosclerosis could represent a cause-and-effect relationship. Enhancement of TGF-β1 signaling in the artery wall may be a promising approach to prevention and treatment of atherosclerosis and aneurysm formation in humans.

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Disclosures

None.

References


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Methods

Transgenic Mice
We obtained mice expressing a cardiac alpha-myosin heavy chain promoter-driven tetracycline transactivator (αMHC-tTA) from Dr. Glenn Fishman (New York University).\(^1\) We generated mice transgenic for a tetracycline-regulated TGF-β1 allele (tetO–TGF-β1) that expresses a constitutively active mutant of rat TGF-β1.\(^2\) Mature rat TGF-β1 is 100% identical to mouse TGF-β1 at the amino acid level.\(^3\) Mice were housed in specific-pathogen-free facilities and all animal protocols were approved by the Institutional Animal Care and Use Committee.

Atherosclerosis Studies
The αMHC-tTA and tetO-TGF-β1 alleles were each bred for more than 9 generations with C57BL/6 Apoe\(^{-/-}\) mice (Jackson Laboratory). Singly transgenic (αMHC-tTA or tetO–TGF-β1) Apoe\(^{-/-}\) mice were crossed to obtain doubly transgenic (αMHC-tTA/tetO–TGF-β1) Apoe\(^{-/-}\) mice and singly transgenic littermate controls. Atherosclerosis studies were performed on 3 groups: doubly transgenic mice on and off doxycycline (“DT-on” and “DT-off”) and singly transgenic αMHC-tTA mice off doxycycline (“ST-off”). This experimental design included controls for overexpression of TGF-β1, presence of doxycycline, as well as any nonspecific effects of the αMHC-tTA. All mice were genotyped by Southern analysis\(^2\) or PCR of tail-tip DNA. PCR primers were: αMHC–tTA forward, 5’-GAATTCAGGCTCGCCTGCAG-3’; reverse, 5’-TCAGACCAGAGTTTCTCCATCCC-3’; tetO-TGF-β1 forward, 5’-TAGTGAACCCGTCAGATCG-3’; reverse, 5’-TTGGGACTGATCCCATTG-3’.

To prevent expression of the conditional TGF-β1 allele, pregnant females and their pups were fed doxycycline-containing chow (Bioserve) until initiation of the high-fat
diet. At 6 wks of age, all mice were begun on a high-fat “Western” diet containing 21% fat and 0.15% cholesterol by weight (TD88137; Harlan-Teklad). DT-on mice continued to receive doxycycline in drinking water (1 mg/ml, Sigma) until study termination. Mice were killed at 18 wks of age (after 12 wks of Western diet).

**Measurement of TGF-β1 Protein**

To confirm doxycycline-regulated expression of TGF-β1 protein in fat-fed, Apoe<sup>−/−</sup> mice, TGF-β1 protein was measured by ELISA (Promega) of mouse plasma (4 and 12 wks after doxycycline withdrawal) and media conditioned by hearts explanted 6 wks after doxycycline withdrawal.<sup>2</sup> The ELISA detects either total or active TGF-β1 depending on whether samples are treated with acid. Blood was drawn from the retro-orbital sinus via heparinized capillary tubes into centrifuge tubes containing 0.5 M EDTA and plasma was separated by centrifugation at 14,000 rpm for 5 min. Heart explant cultures were performed by placing minced sections of hearts into DMEM without phenol red for 18 hrs. The medium was frozen at -80 °C, then assayed.

**Plasma Lipids**

After a 4-hr fast, plasma was obtained for individual measurements of total cholesterol and triglycerides. Fast protein liquid chromatography (FPLC) was performed on fasting plasma samples that were pooled from 2 – 7 mice of the same genotype.<sup>4</sup> Cholesterol values are from mice in the atherosclerosis study. Triglycerides and FPLC profiles are from other mice.

**Tissue Processing and Histology**

Mice aged 18 wks (12 wks of Western diet) were perfused with saline for 2 – 3 min via cardiac puncture followed by perfusion for 3 – 5 min with 10% buffered formalin. Aortae were excised, pinned, and stained with Sudan IV.<sup>5</sup> Aortic roots were placed into
OCT compound. Sixty 8-μm-thick serial cryosections were cut from each aortic root, beginning at the level of attachment of the aortic valve cusps.\textsuperscript{5} Serial sections at 4 – 5 levels, each 80 μm apart, were stained with hematoxylin and eosin (H & E), oil red O, Movat pentachrome, and picrosirius red. Birefringence of picrosirius red-stained collagen fibrils stained was visualized with polarized light. Aortic roots were also harvested from a separate cohort of 12-wk-old mice (6 wks on Western diet), embedded in paraffin, and cut in 8-μm-thick sections.

Immunohistochemical staining was performed on frozen sections, using a rat antibody to mouse MOMA-2 (Serotec), a biotinylated mouse anti-mouse I-A\textsuperscript{b} antibody (BD Pharmingen) or a mouse anti-human alpha smooth muscle actin [(R & D Systems), conjugated to horseradish peroxidase (HRP) using the HRP Conjugation Kit (Serotec)]. Control antibodies were rat IgG2b (Serotec), mouse IgG\textsubscript{2a} [BD Pharmingen; biotinylated using the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce)], or mouse IgG (Vector Laboratories; conjugated using the HRP conjugation kit), respectively. Bound MOMA-2 was detected with goat anti-rat IgG\textsubscript{2} conjugated to HRP (Kierkegaard & Perry) and diaminobenzidine. Bound anti-I-A\textsuperscript{b} and anti-smooth muscle actin were detected with streptavidin-horseradish peroxidase (BD Pharmingen or Zymed) and diaminobenzidine (BD Pharmingen) or AEC (Zymed). Sections were counterstained with hematoxylin. Immunohistochemical staining was also performed on sections of paraffin-embedded aortic roots, using rabbit antibodies to CD3\textsubscript{ε} (Dako), phospho-Smad2 (Cell Signaling #3101 and Chemicon #AB3849) and Smad3 (Zymed #51-1500). Rabbit IgG (Dako) was used as a control. Bound primary antibodies were detected either with the goat anti-rabbit IgG VectaStain ABC kit (Vector Laboratories) and diaminobenzidine or as described.\textsuperscript{6} The Chemicon anti-phospho-Smad2 and the anti-Smad3 antibody were both tested previously for specificity using western analysis.\textsuperscript{6} Sections stained for CD3\textsubscript{ε} were counterstained with hematoxylin; phospho-Smad2 and Smad3-stained sections were not counterstained.
Morphometry

Total luminal surface area and Sudan IV-stained area of pinned aortae were quantified using computer-assisted color thresholding and planimetry (ImagePro). Areas were measured separately for the aortic arch, thoracic aorta, and abdominal aorta. In addition, as an independent measure of lesion initiation, we counted the number of individual lesions in the thoracic and abdominal aortae. We could not acquire reproducible (i.e., from observer to observer) data for lesion initiation in the aortic arch, most likely because lesions there are largely confluent, requiring subjective judgments about whether adjacent lesions arose separately or not. The analysis of pinned aortae was done by two observers blinded to genotype. The observers’ measurements were highly correlated (Spearman rank-order correlation > 0.85 for lesion area; > 0.77 for lesion number; \( P < 0.0001 \) for all). The means of the two observers’ measurements were used for final data analysis.

Aortic root atherosclerosis and pseudoaneurysm formation were measured on 4 – 5 evenly spaced step sections per aortic root. We used computer-assisted thresholding and planimetry to measure: aortic root circumference at the level of the internal elastic lamina; aortic root area bound by the internal elastic lamina; aortic root intimal plaque area; and the area of each intimal plaque that stained with oil red O, picrosirius red, or MOMA-2. The area within the IEL of the aortic root was calculated using the measured IEL circumference and the formula: area = circumference\(^2\) ÷ 4\(\pi\). We also recorded the presence of aortic root pseudoaneurysms, defined as disruption of all of the medial elastic laminae by an intimal plaque that extended abluminally beyond a line drawn between the adjacent, disrupted ends of the external elastic lamina. For each data set, measurement values correlated highly between two independent observers.

To determine whether TGF-β1 overexpression caused cardiomyocyte hypertrophy, we measured cardiomyocyte diameter using hematoxylin & eosin-stained sections of hearts of DT-off and ST-off mice. Photographs were taken of 5-10 fields per
mouse at 400x. Photographs were downloaded into Image Pro, calibrated with a micrometer. Cardiomyocyte thickness was measured at midnuclear level on 50 cells per mouse and mean thickness was calculated for each mouse.

**Immunoblot for Phospho-Smad2**

Hearts and aortae of 12-wk-old mice were excised, snap-frozen in liquid nitrogen, pulverized, and homogenized in lysis buffer including a mixture of protease inhibitors [25 mM Hepes, 150 mM NaCl, 10% Glycerol, 5 mM EDTA, 1% Triton X-100, 1 mM Na Orthovanadate, 50 mM NaF, 1 ng/μl Pepstatin A, and 2 mM 4-(2-aminoethyl)-bezenesulfonylfluoride]. We used protein extracts from aML cells (a hepatic cell line from TGF-α transgenic mice) treated with 5 ng/ml of TGF-β1 (R&D Systems) or vehicle as positive and negative controls, respectively. \(^7\) Protein concentration of lysates was measured by BCA assay (Pierce). Equal amounts of protein (100 μg for hearts, 60 μg for aortae) were separated by 10% SDS-PAGE, and transferred to nitrocellulose membranes (Hybond-ECL, Amersham). Equal protein loading and transfer was confirmed by Ponceau Red stain of membranes. Membranes were blocked for 1 hr with TBS-T (0.05% Tween 20) containing 5% skim milk, and probed with rabbit anti-human phospho-Smad2 (1:1000 dilution, Cell Signaling #3101). Bound antibody was detected with horseradish peroxidase-linked anti-rabbit secondary antiserum (1:1000 dilution, Abcam) and enhanced chemiluminescence (ECL kit, Amersham). The membranes were stripped (Restore Western Blot Stripping Buffer, Pierce) and reprobed with mouse anti-human Smad2 (detects total Smad2; 1:1000 dilution; Cell Signaling #3103). Bound antibody was detected with horseradish peroxidase-linked donkey anti-mouse secondary antibody (1:10,000 dilution, Jackson Immunoresearch).

**Peripheral Blood Cell Counts**
Blood was drawn from 12- and 18-wk-old mice. Cell counts were performed by an outside laboratory (Phoenix Central Laboratory).

**FACS Analysis**

Only ST-off mice were used as controls for the FACS analysis, plasma cytokine, and aortic gene expression experiments. ST-off mice were used as controls instead of DT-on mice to avoid having doxycycline ingestion as an additional, uncontrolled variable between the (TGF-β1-overexpressing) DT-off mice and the control mice.8-10

Spleens and lymph nodes (inguinal and axillary) from 18-wk-old mice were excised and disrupted between glass slides. Cells were washed in PBS, centrifuged at ~3000g x 5 min, resuspended in RPMI 1640 on ice, and counted. Biotinylated (bio), FITC-, PE-, peridinin chlorophyl protein (PerCP)-, and allophycocyanin-conjugated (ACP) monoclonal antibodies to CD4 (L3T4), CD8α (53–6.7), CD25 (7D4), CD25 (PC-61), CD62L (MEL-14), CD69 (H1.2F3), CD44 (Ly-24), CD-19 (MB19-1), and I-A^b (AF6-120.1) were purchased (BD Biosciences and eBioscience). To detect intracellular cytokines, cells were first incubated with anti-CD3 (2C11, 15µg/ml) and monensin (2 µM GolgiStop) at 37 °C in 7% CO2 for 4 hr. Intracellular staining was performed using the Cytofix/Cytoperm procedure (BD Biosciences) and the following antibodies: anti-mouse IL-2 (JES6-5H4), IL-4 (11B11), IL-10 (JES5-16E3), IFN-γ (XMG1.2), TNF-α (MP6-XT22) (eBioscience). Flow cytometry was performed on a FACScan (Becton Dickinson).11,12

**Plasma Cytokines**

Blood (~ 200 µl) was obtained from the retro-orbital sinus of 18-wk-old mice as described above, and treated with 5 µl 0.5 mM EDTA. Plasma was separated by centrifugation at ~5000 g for 5 min and stored at -80 °C. After thawing, plasma was separated from the lipid layer and concentrations of IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-
5, IL-6, IL-10, IL-12p40/p70, and GM-CSF were determined by cytofluorometric bead assay (Biosource) using a LiquiChip plate reader (Qiagen). All samples were assayed in duplicate for both DT-off (n = 10) and ST-off (n = 9) mice. After subtraction of background fluorescence in samples and standards, cytokine plasma concentrations were calculated with reference to standard curves constructed for each cytokine from standards provided by the manufacturer. Plasma levels of IL-4, IL-6, IL-10, and GM-CSF were below the limit of detection in almost all samples and were therefore not compared.

RT-PCR Analysis of Aortic Gene Expression
Aortae excised from 18-wk-old mice were pulverized with mortar and pestle on dry ice, resuspended in Qiagen lysis buffer, homogenized with a Polytron, and treated with Proteinase K (Invitrogen) for 20 min at 55 °C. RNA was extracted using RNeasy kit (Qiagen), treated with DNAse (Qiagen), and resuspended in RNAse-free water. RNA quantity and quality were evaluated with a ND1000 spectrophotometer (NanoDrop Technologies) and a Bioanalyzer 2100 electrophoresis system (Agilent Technologies), respectively. RNA samples with evidence of degradation were excluded from RT-PCR. One μg of total RNA was reverse transcribed using 2 μg of random hexamers (GE Healthcare) and Superscript 11 (Invitrogen) according to the manufacturer’s instructions. For PCR reactions, specific primers and fluorogenic probes for TIMPs 1, 2, 3, MMPs -2, -3, -9, -12, -13, -14, TNF-α, MIP-1α, MIP-1β, MIP-3α, and MIP-3β were designed as described. Expression of IFN-γ was analysed using the Gene Expression Assay (Applied Biosystems; assay ID Mm01168133_g1). RNA from concanavalin A-treated mouse splenocytes was a positive control for IFN-γ expression. PCR reactions were performed using the 7500 Fast Real Time PCR system (Applied Biosystems).13

Blood Pressure Measurement
Blood pressure transmitters (Data Science International) were surgically implanted into
11–13-wk-old mice via the left common carotid artery, with the catheter tip terminating in the aortic arch. Mice were placed in individual cages, fed a high-fat diet and water (with or without doxycycline) *ad libitum*, and allowed to recover for 3 d after surgery. Systolic, diastolic, and mean arterial pressure (MAP) were then measured for 2 min periods—at 15 min intervals—over 5 d. Measurements made from 6 a.m. to 6 p.m. were used to compare blood pressure among groups.

**Statistical Analysis**

Normally distributed data from groups with equal variances were analyzed by unpaired Student’s t test and are presented as mean ± SEM. Otherwise, data were analyzed with the Mann-Whitney rank-sum test and are presented as median (25–75% range). Not all possible pairwise comparisons were made.
References


**Supplemental Figure Legends**

**Supplemental Figure I.** Immunohistochemistry for phospho-Smad2. Aortic root sections were stained with an antibody to the phosphorylated form of Smad2 (Chemicon). Sections are from mice overexpressing TGF-β1 (DT-off) and from control ST-off mice. Sections were from 12-wk-old mice, 6 wks after withdrawal of doxycycline and initiation of a Western diet. Panels illustrate staining of intimal macrophages (A, B), smooth muscle cells (C, D) and endothelial cells (E, F). Arrows indicate some of the stained nuclei in each panel. Because there was no counterstain, all stained nuclei are positive for phospho-Smad2. Similar results were obtained with a different antibody to phospho-Smad2 (Cell Signaling) and with an antibody to Smad3, except that the Smad3 antibody stained cytoplasm as well as nuclei. For macrophages and smooth muscle cells, the frequency of stained nuclei was highly consistent among all of the sections, whereas the frequency of positive endothelial cells was more variable. For this reason, we counted phospho-Smad2 positive endothelial cells (5 mice per group, 3 sections per mouse). There was no difference between the groups (DT-off: 32 ± 5; ST-off: 37 ± 10 phospho-Smad2 positive endothelial cells/section; P = 0.3). M = vascular media; IEL = internal elastic lamina. Size bars = 20 μm.

**Supplemental Figure II.** Western blot of aortic extracts to detect total and phosphorylated Smad2. Aortae from TGF-β1-overexpressing mice (DT-off) and littermate controls (DT-on and ST-off) were studied after 6 wks of high-fat diet (all mice) and (for DT-off and ST-off mice) after 6 wks off doxycycline. Western blot of protein extracts was probed sequentially with antibodies against phospho-Smad2 and total Smad2. Control lanes contain extracts of cells treated with TGF-β1 (+) or vehicle (−). This experiment was repeated once with similar results.
**Supplemental Figure III.** Mild cardiac fibrosis in TGF-β1-overexpressing mice. Sections of hearts of a control ST-off mouse (A, C) and a DT-off (TGF-β1-overexpressing) mouse (B, D). Mice were 18 wks old and had been on high-fat diet for 12 wks. Mild perivascular (arrows) and epicardial (arrowheads) fibrosis is present in the DT-off heart. Masson trichrome stain, size bar = 100 μm.

**Supplemental Figure IV.** Plasma FPLC profiles. Plasma was obtained from TGF-β1-overexpressing mice (DT-off) and littermate controls (DT-on and ST-off) after 12 wks of high-fat diet. Lipoprotein distributions for cholesterol (A) and triglycerides (B) are indicated with horizontal bars. Data points are the mean ± SD of samples from 9 – 13 mice (n) per group, analyzed in 2 – 4 pools (p) per group.

**Supplemental Figure V.** Mean aortic blood pressure. Blood pressure was measured with indwelling catheters placed in the proximal aortae of TGF-β1-overexpressing mice (DT-off, n = 3) and littermate controls (DT-on, n = 2; ST-off, n = 3). All mice were fed a high-fat diet for 12 wks. Tracings represent the mean of three days of mean arterial pressure (MAP) readings for each mouse, obtained at 15-min intervals for 12 hrs per day. Group mean arterial blood pressures did not differ significantly: 99 ± 8 (DT-off) versus 107 ± 7 mmHg (controls); P = 0.2.
**Supplemental Table I.** mRNA Expression in Aortae of DT-off and ST-off Mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>DT-off (High TGF-β1)</th>
<th>ST-off (Low TGF-β1)</th>
<th>P value (DT-off vs. ST-off)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>3.3 ± 2.4</td>
<td>7.0 ± 3.7</td>
<td>0.01</td>
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<tr>
<td>IFN-γ</td>
<td>2.8 (1.1 – 3.8)</td>
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<tr>
<td>IL-10</td>
<td>0.27 (0.19 – 0.83)</td>
<td>0.97 (0.21 – 1.7)</td>
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</tr>
<tr>
<td>MMP-2</td>
<td>0.91 ± 0.65</td>
<td>0.84 ± 0.79</td>
<td>0.8</td>
</tr>
<tr>
<td>MMP-3</td>
<td>0.87 (0.5 – 1.2)</td>
<td>1.2 (0.44 – 3.3)</td>
<td>0.4</td>
</tr>
<tr>
<td>MMP-9</td>
<td>1.2 (0.74 – 2.0)</td>
<td>3.8 (1.1 – 5.7)</td>
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</tr>
<tr>
<td>MMP-12</td>
<td>4.6 ± 4.0</td>
<td>8.0 ± 6.7</td>
<td>0.2</td>
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<tr>
<td>MMP-13</td>
<td>0.93 (0.74 – 1.8)</td>
<td>3.0 (2.2 – 4.6)</td>
<td>0.01</td>
</tr>
<tr>
<td>MMP-14</td>
<td>1.8 (1.3 – 1.9)</td>
<td>2.4 (1.2 – 4.6)</td>
<td>0.1</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>1.4 ± 0.72</td>
<td>1.6 ± 1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>6.1 ± 4.2</td>
<td>2.6 ± 2.9</td>
<td>0.03</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>5.3 (4.1 – 7.4)</td>
<td>6.7 (4.4 – 10)</td>
<td>0.5</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>2.2 (1.4 – 2.7)</td>
<td>3.6 (2.4 – 5.7)</td>
<td>0.015</td>
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<tr>
<td>MIP-1β</td>
<td>4.7 (3.3 – 5.2)</td>
<td>8.4 (7.2 – 10)</td>
<td>0.005</td>
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<tr>
<td>MIP-3α</td>
<td>2.7 (2.0 – 4.2)</td>
<td>3.8 (2.0 – 7.5)</td>
<td>0.5</td>
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<tr>
<td>MIP-3β</td>
<td>1.9 (1.6 – 2.8)</td>
<td>2.6 (1.9 – 2.8)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Data are Ct values normalized to 18S and are mean ± S.D. or median (25 – 75% range) if data were not normally distributed with equal variances between groups.

*n* = 10 – 11 aortae per group.
Supplementary Figure I

DT-off

ST-off

A

B

C

D

E

F
Supplementary Figure V

MAP (mm Hg)

Hour

6am 9 12 3 6pm

High TGF-B1
- DT-off

Low TGF-B1
- DT-on
- ST-off