Tie1 attenuation reduces murine atherosclerosis in a dose-dependent and shear stress–specific manner

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Although the response of endothelial cells to the disturbed blood flow in the vicinity of atherosclerotic lesions is known to be distinct from that elicited by nonatherogenic laminar flow, the mechanisms involved are poorly understood. Our initial studies confirmed that expression of the endothelial receptor tyrosine kinase Tie1 was evident at regions of atherogenic flow in mature animals. We therefore hypothesized that Tie1 plays a role in the endothelial response to atherogenic shear stress. Consistent with this, we found that Tie1−/− mice bred to the apoE-deficient background displayed a 35% reduction in atherosclerosis relative to Tie1+/+“ApoE−/−” mice. Since deletion of Tie1 results in embryonic lethality secondary to vascular dysfunction, we used conditional and inducible mutagenesis to study the effect of endothelial-specific Tie1 attenuation on atherogenesis in ApoE−/− mice and found a dose-dependent decrease in atherosclerotic lesions. Analysis of primary aortic endothelial cells indicated that atheroprotective laminar flow decreased Tie1 expression in vitro. Attenuation of Tie1 was associated with an increase in eNOS expression and Tie2 phosphorylation. In addition, Tie1 attenuation increased IkBα expression while decreasing ICAM levels. In summary, we have found that shear stress conditions that modulate atherogenic events also regulate Tie1 expression. Therefore, Tie1 may play a novel proinflammatory role in atherosclerosis.

Introduction

Atherosclerotic lesions have a predilection for locales exposed to disturbed blood flow that can trigger inflammation and apoptosis (1–5). Disturbed flow is seen at the aortic sinus and at branch points such as bifurcations of the carotid, coronary, and renal arteries (2). In contrast, lesions rarely form along nonbranching segments of the descending aorta, where laminar flow is predominant and endothelial cells are protected against inflammatory activation and apoptosis (6, 7), and show increased expression of SOD and eNOS (8).

The force exerted by blood flow on the endothelium is termed shear stress. Mechanosensors of shear stress in endothelial cells include integrins, G protein–coupled receptors, PECAM-1, and the membrane lipid layer (5). The most recent additions to the list of shear stress mediators are the members of the Tie family of receptor tyrosine kinases (9, 10). A tyrosine kinase with immunoglobulin-like and EGF-like domains, Tie1 is expressed almost exclusively in endothelial cells (11). Ablation of Tie1 expression results in early embryonic lethality after day 13.5 as a result of severe edema, hemorrhage, and loss of microvessel integrity (12, 13). Puri et al. (12) and Sato et al. (13) have independently shown that Tie1 is not essential for vasculogenesis but is required during embryonic development for the integrity and survival of vascular endothelial cells. Although Tie1 is important for embryonic vessel development, its expression is also increased during pathological conditions in the adult (14–16). Recent studies have indicated expression of Tie1 in inflammatory tissue of rheumatoid arthritis and osteoarthritis patients (17, 18). Additionally, overexpression of Tie1 augmented the inflammatory markers VCAM-1, ICAM-1, and E-selectin (19). Conversely, analyses of Tie1 siRNA–transfected HUVECs showed a decrease in the levels of inflammatory markers, including TLR2 and IL-1β. These data suggest that Tie1 expression mediates proinflammatory responses in endothelial cells.

Tie1 expression in the adult correlates distinctively to vascular regions exposed to disturbed flow in both physiological and pathological conditions. In optic microvessels, Tie1 promoter activity is increased at bifurcations and downstream of branches (9). The effect of fluid shear on Tie1 has also been demonstrated in vitro, whereby expression is decreased with brief application of laminar flow and altered by acute changes in shear stress magnitude (10). Conversely, disturbed flow in vitro upregulates Tie1 promoter activity (9). Tie1 is commonly found associated with Tie2 (20–22). Overexpression of Tie1 with Tie2 results in a cooperative recruitment of both receptors to the cell border (23). Tie1 can be activated by an Ang1 chimeric protein (COMP-Ang1) as well as by native Ang1 and Ang4, but not by Ang2 or Ang3 (21). Notably, coexpression with Tie2 was required for Tie1 activation (21), and association of Tie1 with Tie2 has been reported to modulate Tie2 activation (22, 24, 25). In bovine aortic endothelial cells subjected to shear stress in vitro (10), Tie1 was cleaved, and the intracellular product was found to coimmunoprecipitate with the protein tyrosine phosphatase Shp2 (26), which indicates that Tie1 might transduce signals in the absence of ligand activation. To our knowledge, the role of Tie1 in shear stress–induced vascular diseases such as atherosclerosis has not previously been studied.

We hypothesized that Tie1 plays an important role in shear stress–induced atherosclerosis and that attenuation of Tie1 would ameliorate atherosclerotic burden. In the present study, we show that Tie1...
expression was induced in atherosclerosis-prone regions of the vasculature, characterized by disturbed flow. Moreover, we show that increasing shear stress under laminar flow conditions downregulated Tie1 promoter activity in vivo. We also report a dose-dependent reduction in atherosclerosis in Tie1-attenuated ApoE–/– mice. Furthermore, we devised what we believe to be a novel method of isolating murine aortic endothelial cells (MAECs), facilitating in vitro experiments in parallel with our in vivo model. In vitro laminar flow with high shear stress for 24 hours downregulated Tie1 levels. Additionally, these in vitro studies document that Tie1 deletion increased shear stress–mediated eNOS and Tie2 phosphorylation, while increasing IkBα and decreasing ICAM levels. Our findings suggest that Tie1 may be a key modulator of endothelial response to pathological shear stress and a novel target of therapy for atherosclerosis.

Results

Tie1 is expressed at regions of atherogenic disturbed flow. Previous studies have shown that Tie1 is ubiquitously expressed in various endothelia at birth, including those of heart, lungs, kidney, liver, and brain (27). To map the expression pattern of Tie1 in adult macrovasculature, we used a transgenic mouse with Tie1 promoter–driven LacZ expression (12). In this model, the LacZ gene is knocked into the Tie1 locus (Tie1lacZ) and placed under the control of endogenous Tie1 regulatory elements. As expected, Tie1-LacZ activity was ubiquitously expressed throughout the aorta and its branches in 4-week-old animals (Figure 1A), consistent with the role of an activated endothelium. Interestingly, in the 12-week-old adult mouse aorta, this expression was attenuated in the descending aorta (thoracic and abdominal segments). Whereas expression was induced in atherosclerosis-prone regions of the vasculature, characterized by disturbed flow. Moreover, we show that increasing shear stress under laminar flow conditions downregulated Tie1 promoter activity in vivo. We also report a dose-dependent reduction in atherosclerosis in Tie1-attenuated ApoE–/– mice. Furthermore, we devised what we believe to be a novel method of isolating murine aortic endothelial cells (MAECs), facilitating in vitro experiments in parallel with our in vivo model. In vitro laminar flow with high shear stress for 24 hours downregulated Tie1 levels. Additionally, these in vitro studies document that Tie1 deletion increased shear stress–mediated eNOS and Tie2 phosphorylation, while increasing IkBα and decreasing ICAM levels. Our findings suggest that Tie1 may be a key modulator of endothelial response to pathological shear stress and a novel target of therapy for atherosclerosis.

Results

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Tie1 suppression reduces distal aorta atherosclerosis progression in Apoe-null mice. To determine the role of Tie1 in atherosclerosis, Tie1+/−;Apoe−/− female mice (n = 49) fed a regular chow diet were evaluated for atherosclerotic plaque burden and compared with control Tie1+/--;Apoe−/− female littermates (n = 41). Female mice were chosen because of a clear gender-dependent difference in the progression of atherosclerosis (32). Mice were assessed at early (12 weeks old), intermediate (18 weeks old), and advanced (24 and 49 weeks old) stages of atherosclerosis. There were no statistically significant differences in serum cholesterol and triglyceride levels between experimental and control mice (Table 1). Semiquantitative RT-PCR analysis of descending aorta confirmed a 49% decrease in Tie1 mRNA expression of Tie1+/− mice (Figure 3A). Western blot assay of pulmonary endothelium showed a 40% reduction in Tie1 protein expression throughout the vascular endothelium (Figure 3B).

In the atherosclerosis-prone, disturbed-flow regions of the aorta, en face analysis showed a 40% reduction in Tie1 protein expression in serum cholesterol and triglyceride levels between experimental and control mice (Table 1). Semiquantitative RT-PCR analysis of descending aorta confirmed a 49% decrease in Tie1 mRNA expression of Tie1+/− mice (Figure 3A). Western blot assay of pulmonary endothelium showed a 40% reduction in Tie1 protein expression throughout the vascular endothelium (Figure 3B).

In the aortic sinus, we found no statistically significant differences in the extent of lesion area between Tie1+/−;Apoe−/− and Tie1+/−;Apoe−/− mice at any stage (12 weeks, 40,030 ± 15,311 vs. 41,179 ± 6,094 μm²; 18 weeks, 156,155 ± 40,275 vs. 152,210 ± 25,157 μm²; 24 weeks, 286,433 ± 16,160 vs. 334,760 ± 41,861 μm²; 49 weeks, 426,680 ± 40,877 vs. 529,025 ± 52,711 μm²; Supplemental Figure 3A). The trend toward increased lesions in Tie1+/−;Apoe−/− mice, the opposite of the effects seen in the distal aorta, is interesting but does not warrant further comment, given the lack of statistical significance.

Endothelial-specific Tie1 deficiency alleviates atherosclerosis progression in a dose-dependent manner. We next assessed the effect of endothelial-specific Tie1 deletion on development of atherosclerotic lesions in Apoe−/− mice. Since Tie1 deletion leads to early embryonic lethality (12, 13), we used Cre-Lox technology to delete Tie1 in a spatially and temporally defined manner. We used SCL-ER²-Cre mice to achieve endothelial-specific, tamoxifen-mediated induction of Cre expression (33). To determine an efficient tamoxifen induction dosage, SCL-ER²-Cre mice were bred to Rosa-LacZ reporter background. Using a protocol of 2 mg intraperitoneal tamoxifen injection every 48 hours, for 14 days, we were able to obtain robust, endothelial-specific, Cre-mediated deletion (evidenced by X-gal staining), as documented in the aortic valve endothelium and in the microvasculature of the endocardium (Figure 4A). Tie1−/− mice were then bred with SCL-ER²-Cre mice, and generalized endothelial-specific deletion was quantified by Western assay of pulmonary endothelium, which showed a 81% reduction in Tie1 protein levels (Supplemental Figure 4). In the resulting mice with a null Tie1 allele, a floxed Tie1 allele, and endothelial-specific Cre (Tie1−/−;SCL-ER²-Cre mice), tamoxifen treatment induced a 65% reduction in aortic endothelial Tie1 mRNA level (Figure 4B).
To assess the effect of Tie1 deletion on atherosclerosis progression, Tie1<sup>fl/fl</sup>/SCL-ER<sup>–Cre</sup> mice were bred onto the Apoe<sup>–/–</sup> background. Tie1<sup>fl/fl</sup>/SCL-ER<sup>–Cre</sup>;Apoe<sup>–/–</sup> female littermates were injected with tamoxifen as described above. Because tamoxifen administration has previously been associated with attenuation of atherosclerosis after chronic and unfettered oral intake (34), we first determined whether acute and limited exposure to tamoxifen had any effect in our experimental model. We noted a protective effect (46% decrease) 4 weeks after treatment (i.e., 12 weeks old; 1.41% ± 0.22% vs. 0.76% ± 0.11%, P < 0.01; Supplemental Figure 5), which was not maintained at the 16-week time point (i.e., 24 weeks old; 4.32% ± 0.54% vs. 3.64% ± 0.86%, P = NS). Tamoxifen treatment temporarily decreased serum cholesterol in Tie1<sup>fl/fl</sup>/Apoe<sup>–/–</sup> mice compared with untreated controls at 12 weeks of age (456.44 ± 40.58 vs. 291.43 ± 19.36 mg/dl, P < 0.001), but this effect was lost by 24 weeks (331.54 ± 28.54 vs. 345.20 ± 24.61 mg/dl, P = NS; Table 2). Tamoxifen treatment also reduced serum triglycerides of 12- and 24-week-old animals (12 weeks old, 185.8 ± 88.2 mg/dl, P < 0.05; 24 weeks old, 290.1 ± 115.7 mg/dl, P < 0.01; Table 2). However, the tamoxifen-induced reduction in serum triglycerides was not associated with an atheroprotective effect at 24 weeks.

Consistent with our previous studies, en face analysis of the aorta from Tie1-deleted animals showed a statistically significant reduction in atherosclerotic lesions. In 12-week-old mice with early disease progression, we found a 68% decrease in lesions of Tie1-deleted mice compared with controls (0.76% ± 0.114% vs. 2.44% ± 0.46%, P = 0.0005; Figure 4D). The decrease in atherosclerosis persisted in mice up to 24 weeks of age, when Tie1 deletion resulted in a 70% decrease in plaque progression compared with controls (3.644% ± 0.865% vs. 1.108% ± 0.207%, P < 0.006; Figure 4, C and E). However, similar to the atherosclerosis data in Tie1-heterozygous mice, we observed no significant difference in the degree of atherosclerosis at the aortic sinus between control Tie1<sup>fl/fl</sup>/Apoe<sup>–/–</sup> and Tie1<sup>fl/fl</sup>/SCL-ER<sup>–Cre</sup>;Apoe<sup>–/–</sup> mice (12 weeks old, 16,607 ± 5,004 vs. 21,126 ± 4,274 μm<sup>2</sup>; 24 weeks old, 22,816 ± 3,019 vs. 18,587 ± 33,856 μm<sup>2</sup>; Supplemental Figure 3B). Tie1 deletion had no effect on serum cholesterol levels compared with controls when both groups were treated with tamoxifen (Table 2). Serum triglycerides in Tie1-deleted mice treated with tamoxifen were slightly increased compared with tamoxifen-treated controls, but similar to those of untreated controls (172.9, 147.9, and 186.8 mg/dl, respectively; Table 2). These results suggest that deletion of endothelial Tie1 has a protective effect attenuating the rate of atherosclerosis progression independent of effects on serum cholesterol or triglyceride levels.

### Table 1

<table>
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<td>300.1</td>
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Tie1 reduction caused no discernible effect; no significant differences were found between groups.
shear stress after 24 hours (Supplemental Figure 7F). These results demonstrated that MAECs isolated from immorto mice display the same characteristics of many primary endothelial cell lines.

Next, we isolated endothelial cells from homozygous Tie1 heterozygosity reduces advanced-stage atherosclerosis burden. (A and B) Quantitation of Tie1 levels in Tie1+/− mice of (A) aortic endothelia by RT-PCR (49% reduction, \( P < 0.01, n = 4 \)) and (B) pulmonary tissue by Western blot (40% suppression, \( P < 0.05 \)). (C and D) Effect of reduced Tie1 expression on atherosclerosis. (C) Representative images of Sudan IV–stained aorta (aortic arch and thoracic and abdominal aorta) from 49-week-old Tie1+/+;Apoe−/− and Tie1+/−;Apoe−/− mice. (D) Atherosclerotic lesion area in Tie1+/+;Apoe−/− versus Tie1+/−;Apoe−/− mice at 12, 18, 24, and 49 weeks of age. Data points denote individual animals; horizontal bars indicate group average. *\( P < 0.05; **P < 0.01.\)

Discussion

Our present findings provide major insights into the role of Tie1 in atherosclerosis. First, Tie1 expression was accentuated in regions of atherogenic shear stress, such as those with disturbed flow in the adult aorta. Second, there was dynamic downregulation of Tie1 expression in vitro and in vivo under conditions of laminar flow with high shear stress. Third, in conjunction with the expression of Tie1 at atherosclerosis-prone areas, we found that Tie1 reduction alleviated atherosclerosis progression in a dose-dependent manner. Finally, our in vitro and in vivo data support a proinflammatory role for Tie1.

Previous in vivo studies have focused on the role of Tie1 in early stages of embryonic development. Here, we showed that the expression pattern of Tie1 in the adult macrovasculature was different from that of the immature mouse. In the aorta of a young

Figure 3

Tie1 heterozygosity reduces advanced-stage atherosclerosis burden. (A and B) Quantitation of Tie1 levels in Tie1+/− mice of (A) aortic endothelia by RT-PCR (49% reduction, \( P < 0.01, n = 4 \)) and (B) pulmonary tissue by Western blot (40% suppression, \( P < 0.05 \)). (C and D) Effect of reduced Tie1 expression on atherosclerosis. (C) Representative images of Sudan IV–stained aorta (aortic arch and thoracic and abdominal aorta) from 49-week-old Tie1+/+;Apoe−/− and Tie1+/−;Apoe−/− mice. (D) Atherosclerotic lesion area in Tie1+/+;Apoe−/− versus Tie1+/−;Apoe−/− mice at 12, 18, 24, and 49 weeks of age. Data points denote individual animals; horizontal bars indicate group average. *\( P < 0.05; **P < 0.01.\)
4-week-old mouse, we observed pervasive endothelial Tie1 expression consistent with aortic immaturity. However, in the 12-week-old adult mouse, Tie1 expression decreased specifically in the descending aorta. We noted strong X-gal staining at locales subjected to atherogenic disturbed flow (39), such as the aortic arch and its branch vessels, and bifurcations of the vertebral and renal arteries. LacZ expression was also observed at the aortic sinus and the aortic valves, areas characterized by disturbed flow.

**Figure 4**

Endothelial-specific Tie1 deletion reduces atherosclerosis burden. (A) Representative H&E-stained aortic valve showing endothelial-specific LacZ expression (arrowheads) from tamoxifen-treated SCL-ER<sup>T</sup>-Cre;Rosa26R-LacZ mouse. Original magnification, ×100. (B) RT-PCR analysis of aortic Tie1 levels from tamoxifen-treated Tie1<sup>−/−</sup>;SCL-ER<sup>T</sup>-Cre mice (65% reduction, P < 0.05). (C) Representative Sudan IV–stained distal aorta from Tie1<sup>−/−</sup>;SCL-ER<sup>T</sup>-Cre;ApoE<sup>−/−</sup> and Tie1<sup>fl/fl</sup>;ApoE<sup>−/−</sup> mice demonstrated lesion reductions in Tie1-deleted mice. (D and E) Mean aortic lesion area in tamoxifen-treated Tie1<sup>−/−</sup>;SCL-ER<sup>T</sup>-Cre;ApoE<sup>−/−</sup> and Tie1<sup>fl/fl</sup>;ApoE<sup>−/−</sup> mice analyzed at (D) 12 weeks (68% reduction, P < 0.0005) and (E) 24 weeks (70% reduction, P < 0.006). (F–H) Atherosclerotic lesion areas in Tie1-deleted mice, as assessed in 3 regions of the aorta, showed reduction of atherosclerosis in all regions of disturbed flow. Insets show respective regions of lesion analysis. Shown are mean atherosclerotic lesion areas at (F) lesser curvature of the aortic arch (12 weeks, 0.066% ± 0.023% vs. 0.038% ± 0.014%, P = NS; 24 weeks, 1.20% ± 0.379% vs. 0.206% ± 0.048%, 82% reduction, P < 0.01); (G) aortic arch branch arteries, including brachiocephalic, left common carotid, and left subclavian arteries (12 weeks, 0.211% ± 0.055% vs. 0.086% ± 0.026%, 56% reduction, P < 0.05; 24 weeks, 1.29% ± 0.283% vs. 0.568% ± 0.119%, 59% reduction, P < 0.05); and (H) descending aorta (12 weeks, 0.487% ± 0.074% vs. 0.118% ± 0.032%, 70% reduction, P < 0.01; 24 weeks, 1.13% ± 0.291% vs. 0.333% ± 0.097%, 75% reduction, P < 0.01). Data points denote individual animals, and horizontal bars indicate group average. *P < 0.05; **P < 0.01; ***P < 0.001.
The effect of altered shear stress on Tie1 expression was demonstrated in vivo by the use of vascular casts. Cheng et al. previously demonstrated that these in vivo shear stress–modifying casts altered the shear forces in the carotid arteries of Apoe-null mice (31). Lowered shear stress upstream of the cast was found to induce formation of unstable plaques, and the immediate downstream region of the cast formed stable plaques in the presence of oscillatory flow. In our Tie1-LacZ reporter mice, we found persistence of Tie1 promoter–driven LacZ expression also in these immediate vicinities around the cast. Furthermore, Cheng and colleagues reported increased eNOS expression and detected no atherosclerotic lesions within the boundaries of the tapered cast (30). Interestingly, we found a dramatic reduction in Tie1 promoter activity after only 7 days of implanting tapered casts in Tie1-LacZ mice. Attenuation of LacZ expression was distinctly confined to the boundaries of the tapered cast, where shear stress increases from 10 to 25 N/m². Hence, the attenuation of Tie1 expression may be caused, at least in part, by both the higher shear stress magnitude and laminar flow conditions.

Interestingly, the in vivo response of endothelial cells to the same magnitude of shear stress may be context dependent, with endothelial cells from different vascular beds having different set points for response to shear stress. There is significant variability in the absolute wall shear stress of vascular regions within a given species, as well as between similar regions in the same species (reviewed in ref. 40). The shear stress of the mouse abdominal aorta is approximately 9 N/m² (41), and in our studies, little Tie1 expression was detected in the regions of laminar shear, but Tie1 expression persisted in regions of nonlaminar flow. By comparison, the average shear stress of the carotid arteries in mice has been calculated to be 15 N/m² (31), and Tie1 was expressed in carotid endothelial cells under normal conditions. However, when shear stress in the carotid arteries was increased by placement of vascular casts to 25 N/m², a magnitude comparable to the level of shear stress produced in most in vitro studies, there was a marked attenuation in Tie1 expression. Clearly, shear stress is not the only factor regulating Tie1 expression, but our data suggest that expression of Tie1 is downregulated in vitro and in vivo by shear stress profiles that are thought to be atheroprotective.

Table 2
Plasma cholesterol and triglycerides for tamoxifen-treated Apoe−/− mice

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<th>Genotype</th>
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<th>n</th>
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<td>Tie1fl/fl;SCL-ERt−/−Cre;Apoe−/−</td>
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<td>293.9</td>
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<td>24 weeks old</td>
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<td>345.2</td>
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<td>Yes</td>
<td>331.5</td>
<td>188.6†</td>
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Tamoxifen temporarily decreased cholesterol levels at 12 weeks (i.e., 4 weeks after treatment), but the effect was lost by 24 weeks. Tamoxifen treatment decreased triglycerides at 12 and 24 weeks. Tie1 deletion decreased triglycerides. *P < 0.05, †P < 0.01 vs. untreated Tie1fl/fl;SCL-ERt−/−Cre;Apoe−/−. *P < 0.05 versus tamoxifen-treated Tie1fl/fl;Apoe−/−.

Since we found expression of Tie1 at regions of atherogenic shear stress, and a previous study reported Tie1 expression on both the surface and the subendothelium of atherosclerotic lesions (9), we postulated that attenuation of Tie1 may modulate atherosclerosis progression. We found that moderate reductions in Tie1 (Tie1−/−;Apoe−/− mice) elicited 35% and 38% reductions in atherosclerotic lesions of 24- and 49-week-old mice, respectively. Remarkably, further reductions of Tie1 by up to 80% in Tie1−/−;SCL-ERt−/−Cre;Apoe−/− mice resulted in 68% and 70% reduction in lesions of 12- and 24-week-old mice, respectively. Hence, using 2 different mouse models, our results demonstrated that Tie1 attenuation conferred amelioration of atherosclerosis progression.

To our knowledge, this is the first report of an in vivo biological function for Tie1 in the adult mouse. Whereas Tie1 plays a requisite role in prenatal vascular integrity (12, 42), our results suggest that loss of Tie1 in late adult stages confers an atheroprotective effect. This divergence in function may be due in part to the complex shear stress magnitudes and flow profiles experienced pre- and postnatally. Kontos et al. previously showed that Tie1 is capable of activating Akt to promote cell survival (43); Tie1 is also required for the maintenance of various adult vascular endothelial cell populations (12). Hence, Tie receptors may possess context-dependent roles (44) that are not only defined by endothelial heterogeneity, but also influenced by the physiologic environment that changes during vascular maturity.

We also noted that attenuation of Tie1 did not significantly alter the extent of atherosclerotic lesions, as assessed by quantification of lipid accumulation in the aortic valve region. However, there was a clear decrease in macrophage infiltration in this region. Interestingly, Tie1 attenuation elicited a trend of increased lesions at the aortic sinus, an effect opposite to that observed in the distal aorta. Previous studies have demonstrated a predilection of lesions for specific locales of the distal aorta in PECAM-knockout mice on either the Apoe-deficient or the LDL receptor–deficient background (45, 46). The aortic valve also experiences a unique and complex shear stress profile not evidenced elsewhere in the vasculature. Peacock previously described the flow profile in the aortic sinus as turbulent, composed of spinning vortices superimposed with random motion of fluid particles (28). In contrast, the flow profile at aortic bifurcations distal to the aortic sinus has been described as recirculatory (4), characterized by bidirectional blood flow with the resultant effect of a low time-averaged shear stress (2). Additionally, Butcher et al. demonstrated that the shear stress–induced transcriptional response of valvular endothelial cells is vastly different from aortic endothelial cells. More than 400 genes were differentially expressed, and overall, the inflammatory response of valvular endothelial cells were found to be less pronounced than that of aortic endothelial cells (47). Hence, Tie1 may regulate atherosclerosis progression in a shear stress–specific manner, discriminating between different flow profiles characteristic of unique anatomical and physiological locations within the vascular system and further highlighting the context-dependent response of heterogeneous endothelial populations.

To further investigate the effect of shear stress on Tie1 expression in vitro, we devised a method of isolating genetically modified MAECs that maintain a high percentage of cells displaying unique set points for response to shear stress. There is significant variability in the absolute wall shear stress of vascular regions within a given species, as well as between similar regions in the same species (reviewed in ref. 40). The shear stress of the mouse abdominal aorta is approximately 9 N/m² (41), and in our studies, little Tie1 expression was detected in the regions of laminar shear, but Tie1 expression persisted in regions of nonlaminar flow. By comparison, the average shear stress of the carotid arteries in mice has been calculated to be 15 N/m² (31), and Tie1 was expressed in carotid endothelial cells under normal conditions. However, when shear stress in the carotid arteries was increased by placement of vascular casts to 25 N/m², a magnitude comparable to the level of shear stress produced in most in vitro studies, there was a marked attenuation in Tie1 expression. Clearly, shear stress is not the only factor regulating Tie1 expression, but our data suggest that expression of Tie1 is downregulated in vitro and in vivo by shear stress profiles that are thought to...
endothelial characteristics. The culture of MAECs is a technically
challenging process, often resulting in low yields with poor propagation
properties. We circumnavigated these problems through
the generation of conditionally immortalized aortic endothelial
cells from Tie1fl/fl:SCL-ERt2-Cre immortal mice and selecting with
PECAM by flow-assisted cell sorting. Jat et al. first reported the
derivation of conditionally immortalized cell lines from the H-2Kβ-
tsA58 transgenic mouse (48); subsequently, several groups have
successfully isolated a variety of cell lines from this mouse (49–51).
In this model, the large Tag of a temperature-sensitive strain
tsA58) of the simian virus 40 (SV40) is fused with the major
histocompatibility complex promoter H-2Kβ, which is active in
a wide range of tissues and is induced by IFNs. The presence of the
inducible tsA58 Tag allows for the extended propagation of
MAECs under permissive conditions (33°C, IFN-γ). MAECs
transferred to normal culture conditions (37°C) were confirmed to be
endothelial by their characteristic cobblestone morphology, for-
mation of microtubules on Matrigel, expression of CD31, and
uptake of DiI-AcLDL. Additionally, MAECs aligned to the direc-
tion of laminar flow after only 24 hours (52). Our results indicate
that immortalized MAECs display the characteristics germane to
many primary endothelial cell lines.

We achieved 50% deletion of Tie1 in vitro, concordant with in
vivo tamoxifen-induced deletion in Tie1fl/fl:SCL-ERt2-Cre mice. We
surmise that Cre expression under the SCL promoter may not be
sufficient for extensive deletion of biallelic floxed Tie1 and antici-
pate that enhanced deletion could be achieved by development of
Tie1−/SCL-ERt2-Cre cells. Marron et al. previously reported cleavage
of Tie1 with phorbol ester or VEGF (35), while Chen-Konak and
colleagues reported changes in levels of Tie1 intracellular fragment
after brief alterations in shear stress (10). Upon administration of
4OHT, we also noted a concomitant decrease in levels of cleaved
Tie1 intracellular fragment. Whereas deletion of Tie1 resulted in
a 50% reduction in full-length Tie1 receptor, the cleaved Tie1 frag-
ment was decreased by 74%. The significance of this observation is
currently being explored.

Our in vitro experiments in MAECs showed that 24 hours of
laminar flow at 20 dynes/cm² suppressed Tie1 expression and
increased eNOS activity, while oscillatory flow at 5 dynes/cm² for
a similar duration augmented expression of Tie1. Using bovine
aortic endothelial cells, Chen-Konak et al. found that brief in vitro
application of shear stress at 10 dynes/cm² temporarily decreased
Tie1, but expression levels returned to baseline after 2 hours (10).
Therefore, our in vitro model recapitulated our in vivo data show-
ing increased Tie1 expression at regions of disturbed flow. Stabili-
ity of eNOS may be regulated by ROCK1 and ROCK2 (53, 54).
We showed that genetic deletion of Tie1 augmented eNOS activ-
ity in vivo and in vitro while downregulating the transcription of
ROCK1 and ROCK2 in vivo. Hence, our data suggest that Tie1
may signal upstream of the ROCK-eNOS signaling pathway.

An octameric negative shear stress response element that down-
regulates Tie1 expression has also been found (10). Shear stress
response elements are targets of the NF-κB transcription factor,
which in turn can also be regulated by shear stress (55). Recent
studies have shown that siRNA silencing of Tie1 in vitro reduced
expression of inflammatory markers (56), and overexpression stud-
ies have shown that siRNA silencing of Tie1 in vitro reduced
expression of inflammatory markers both in vivo and in vitro.

Finally, the effect of Tie1 deletion on the progression of athero-
sclerosis is unlikely to be exclusively related to decreases in Tie1-
mediated signaling. As mentioned above, Tie1 and Tie2 have been
shown to interact physically (21, 23, 24), but the in vivo biologi-

Figure 5
In vivo deletion of Tie1 promotes antiinflammatory effect. Analyses of descending aorta mRNA from tamoxifen-treated Tie1fl/fl and Tie1−/−;SCL-
ERt2-Cre mice. Semiquantitative RT-PCR assay of (A) Tie1 (P < 0.001), (B) eNOS (P < 0.01), (C) ROCK1 (P < 0.001), (D) ROCK2 (P < 0.002),
(E) ICAM (P < 0.05), and (F) VCAM (P < 0.001). n = 3 per group. *P < 0.05.
The relevance of these interactions has not been demonstrated. Attenuation of Tie1 has been shown to upregulate Tie2 phosphorylation (22), as has laminar flow (38). Our results showed that the combination of shear stress and Tie1 deletion augmented the increased phosphorylation of Tie2. Interestingly, increased Tie2 signaling has recently been associated with a decrease in atherosclerosis (57). A recent fluorescent resonance energy transfer–based proximity assay has documented Tie1-Tie2 complex formation at the cell surface and demonstrated that these interactions are inhibitory for Tie2 signaling (25). We did not observe a difference in the expression of either Ang1 or Ang2 in response to Tie1 attenuation following 24 hours of in vitro laminar flow (data not shown). Therefore, it is likely that the increased phosphorylation of Tie2 under these conditions (Figure 6K) may be explained, at least in part, by the relief of inhibitory interactions normally imposed by association with Tie1.

Figure 6
In vitro deletion of Tie1 and endothelial cell response to shear stress. (A–E) Treatment of Tie1fl/fl-SCL-ER-T-Cre MAECs with 4OHT activated Cre and reduced Tie1 expression. Representative immunofluorescent images showing Cre nuclear localization following 4OHT treatment (B) compared with untreated controls (A). Scale bars: 100 μm. (C) RT-PCR analysis of Tie1 mRNA levels. Western blot analyses of full-length protein levels (D) and cleaved Tie1 (Cl Tie1) 45-kDa endodomain levels (E). (F) Western blot analysis of full-length Tie1 receptor after 24 hours of shear stress showed that laminar flow at 20 dynes/cm² (Lss20) decreased Tie1 expression. (G–K) Western blot analyses of Tie1-deleted (i.e., 4OHT-treated) versus untreated control cells, comparing laminar shear–induced eNOS expression (G), eNOS phosphorylation (H), ICAM expression (I), IκBα expression (J), and Tie2 phosphorylation (normalized to Tie2) (K). *P < 0.05.
In summary, we found that Tie1 was expressed in specific regions experiencing atherogenic shear stress, and reduction of Tie1 expression attenuated atherosclerosis progression in a shear stress–specific and dose-dependent manner. Our in vitro shear stress studies further suggest a proinflammatory role for Tie1, which we believe to be novel. This prophylactic alleviation of atherosclerosis burden by the reduction of Tie1 expression and signaling points to Tie1 as an attractive candidate for targeted therapeutic approaches.

**Methods**

**Animal breeding and tamoxifen injections.** Tie1<sup>fl/fl</sup> mice were a gift from M.C. Puri (University of Toronto, Toronto, Ontario, Canada; ref. 12), and SCL-ER<sup>Cre</sup>-Cre mice were a gift from J.R. Gothert (University of Duisburg-Essen, Essen, Germany; ref. 33). Tie1<sup>Δ/Δ</sup>;Apoe<sup>−/−</sup>, Tie1<sup>−/−</sup>;Apoe<sup>−/−</sup>, Tie1<sup>Δ/Δ</sup>;SCL-ER<sup>Cre</sup>-Cre, and Tie1<sup>−/−</sup>; SCL-ER<sup>Cre</sup>;Apoe<sup>−/−</sup> mice were bred in our laboratory. All mice used in this study were bred onto a pure C57BL/6 background and were maintained in microisolator cages on a rodent chow diet (Purina Mills Inc.) and autoclaved water ad libitum. Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Tamoxifen injections were performed on 6-week-old mice. Intraportal injections of 2 mg tamoxifen (catalog no. TS648; Sigma-Aldrich) dissolved in 10% ethanol and 0.1 ml sunflower oil (catalog no. S0007; Sigma-Aldrich) were administered once every 2 days (7 injections total).

**Generation of Tie1 mutant alleles.** To generate Tie1<sup>Δ/Δ</sup> mice, a loxP site and a neomycin resistance cassette were introduced within the first intron of Tie1, and another loxP site was introduced just upstream of the minimal promoter region. This strategic placement of loxP sites would allow for excision of the Tie1 minimal promoter and exon 1 upon Cre activation (58).

**Detection of β-galactosidase.** Whole tissue was collected in HBSS; fixed in 0.2% glutaraldehyde, 2 mM MgCl<sub>2</sub> and 5 mM EGTA solution; and stained in 1 mg/ml X-gal, 2 mM MgCl<sub>2</sub>, 5 mM EGTA, 0.01% deoxycholate, 0.02% NP-40, and 0.1 M phosphate buffer (pH 7.3) solution overnight at 30°C. The stained tissues were postfixed in 4% paraformaldehyde, cleared in a glycerol gradient, and photographed in 100% glycerol with a dissecting photomicroscope. Serial cryosections of aortic sinus adjacent to oil red O–stained sections were stained as described above, postfixed with 4% paraformaldehyde, dehydrated in ethanol, cleared in xylene, counterstained with eosin, and mounted in permount.

**Analysis of aortic lesions.** The extent of atherosclerotic lesions was evaluated by 2 alternate methods: cross-sections of the aortic sinus and the whole aorta pinned out en face (59). Aortas were flushed through the left ventricle, and the entire aorta was dissected and pinned out for en face analysis as described previously (59). In addition, to quantify atherosclerotic lesions in the aortic sinus, the heart was embedded in OCT medium and frozen. 6-μm cryosections of the aortic valve region was collected and processed for oil red O staining. Lesions in the aorta were analyzed using Imaging System KS 300 (Kontron Elektronik GmbH). The mean atherosclerotic lesion area for each animal was determined by taking the average of 15 alternate sections for each mouse. To quantify atherosclerotic plaques in the aorta en face, the aorta was fixed in 4% paraformaldehyde and dissected free of fat. The aorta was stained in Sudan IV and analyzed using the Imaging System KS 300. Lesions in the distal aorta were evaluated in 2 ways. First, the percent lesion area for the whole aorta was calculated as total lesion area relative to total surface area. Second, lesions were assessed according to 3 regions: (a) lesser curvature of the aortic arch; (b) greater curvature of the aortic arch, including the brachiocephalic trunk, left common carotid artery, and left subclavian artery; and (c) descending aorta, including the thoracic, abdominal, and lumbar regions. For each region, the lesion area was calculated as a percentage of total aorta surface area.

**Immunocytochemistry.** The localization of mouse macrophages in the arterial lesions was examined by immunocytochemistry using 5-μm cryosections of the aortic sinus fixed in acetone at 4°C. The sections were immersed in PBS (pH 7.2), blocked in goat serum dilution (containing 4% BSA and 2% goat serum), and incubated overnight at 4°C with monoclonal rat antibody MOMA-2 (Accurate Chemical & Scientific Corp.). The sections were treated with goat biotinylated antibody rat IgG (BD Biosciences—Pharmingen) for 45 minutes at 37°C, then incubated with avidin-biotin complex labeled with alkaline phosphatase (Vector Laboratories). Enzyme was viewed with Fast Red TR/Naphthol AS-NX substrate (Sigma-Aldrich) and counterstained with hematoxylin. Nonimmune rabbit or rat serum was used in the place of primary antibody as a negative control. Photomicroscopy was performed on a Zeiss Axioskop microscope with Plan-FLUAR objectives. Area of staining was normalized to total plaque area previously calculated for each mouse.

**Shear stress–modifying cast.** Casts were manufactured at the Vanderbilt Physics Machine Shop with medical grade polyetherketone (Invibio Inc.) using single lip micro cutters (provided by Alignment Tools Pte Ltd.). Casts with nonconstricting bores were used as controls. As described by Cheng et al., the tapered casts induce lowered shear stress (10 N/m²) immediately upstream, increased shear stress inside the cast and oscillatory shear stress with vortex flow immediately downstream (30, 31). The tapered region increases shear stress from 10 to 25 N/m².

8 mice 12 weeks of age were assigned randomly to 2 groups. Shear stress in the right common carotid artery was altered by cast placement. Each animal was anesthetized with isoflurane, and the anterior cervical triangle was accessed by a sagittal anterior neck incision. Both halves of the cast were placed around the right common carotid artery and fixed with a suture. Wounds were closed, and the animals were allowed to recover. Animals with control and tapered casts were sacrificed 7 days after surgery.

**Immunoblotting.** Mouse carotid arteries were processed for whole-mount immunostaining with VE-cadherin after β-gal staining. Tissue samples were blocked in 10% goat serum at room temperature for 30 minutes and incubated with rabbit antibody to VE-cadherin (catalog no. 160840; Cayman Chemical) diluted in blocking solution at 4°C overnight.

Sections were treated with goat biotinylated antibody to rabbit IgG (BD Biosciences—Pharmingen) for 45 minutes at 37°C. Then, sections were incubated with avidin-biotin complex labeled with peroxidase (Vector Laboratories). Enzyme was viewed with 3,3′-diaminobenzidine (DAB) substrate (Sigma-Aldrich).

**Protein isolation and Western blotting.** Tissue samples or cells were lysed on ice using a lysis buffer (50 mM Tris, 50 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 5 mM NaF, and 1X β-glycerophosphate) supplemented with complete protease inhibitor cocktail (Roche). After centrifugation, the supernatant was collected. Protein concentrations were determined with Protein Assay Reagent (BioRad Laboratories). The nuclear pellet was rinsed 3 times in lysis buffer and incubated at 100°C with 2X sample buffer to extract nuclear proteins. Lysates were resolved by SDS-PAGE electrophoresis and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare). Blots were blocked with 5% nonfat milk and probed with rabbit antibodies to Tie1 (catalog no. sc342; Santa Cruz), NOS3 (catalog no. sc654; Santa Cruz), phospho-NOS3 (catalog no. sc12972; Santa Cruz), Tie2 (catalog no. sc342; Santa Cruz), ICAM (catalog no. sc1511; Santa Cruz), IkBβ (catalog no. sc1643; Santa Cruz), phospho-Tie2 (catalog no. AF2720; R&D Systems), and mouse antibody to...
β-actin (catalog no. A5316; Sigma-Aldrich) overnight at 4°C. After washing with 0.1% Tween-20–supplemented PBS (PBST), blots were incubated with secondary antibodies IRDye 800CW goat anti-rabbit IgG and IRDye 680CW goat anti-mouse IgG for 1 hour at room temperature with gentle agitation. Blots were washed and scanned on the Odyssey Infrared Imaging System (LI-COR Biosciences) and densitometry analyses were performed using Odyssey Software.

**MAEC isolation.** The immorto mouse is a transgenic mouse generated by the introduction of thermolabile SV40 TAg, tsAS8 (48). In this model, the SV40 large TAg of temperature-sensitive strain tsAS8 is fused with the major histocompatibility complex promoter H-2Kβ. Expression of the promoter and the large TAg protein is only evident when immorto mouse-derived cells are cultured at a permissive temperature (33°C). The addition of IFN-γ was also used to further enhance promoter activity (48). A 4- to 8-week-old immorto mouse was anesthetized with isoflurane and sacrificed by cervical dislocation. The lumbar aorta was punctured, and 5 ml of HBSS was perfused through the left ventricle to flush out blood. The heart, lung, and aorta block was excised and transferred to a dish containing HBSS and antibiotics. The heart and lungs were removed. The rest of the aorta was incubated at 37°C for 15 minutes with filtered 10 mg/ml collagenase type II (Worthington Biochemicals) and 1x antibiotics dissolved in dispase (Roche). The adventitia was gently removed, and the remaining intima layer comprising the endothelium and internal elastic lamina was cut open longitudinally and incubated at 37°C for 30 minutes in filtered 20 mg/ml collagenase type II solution and 1x antibiotics dissolved in dispase. The aorta was dissociated by pipetting several times in the dissolving solution and filtered through a 100 μm sterile cell strainer (Fisher Scientific). Culture media (MCDB 131 [ Gibco, Invitrogen], 10% FBS [HyClone], 10 U/ml heparin [Sigma-Aldrich], 2.75 nM hydrocortisone [Sigma-Aldrich], 0.2% bovine brain extract [Hammond Celltech], 10 U/ml recombinant murine IFN-γ [Peprotech], and 1x antibiotics [Gibco, Invitrogen]) was added to the cell suspension and centrifuged at 375 g for 10 minutes. The cell mixture was then plated onto a fibronectin coated culture dish and propagated at 33°C in a mixture of 5% carbon dioxide and 95% oxygen. MAECs were isolated from wild-type and Tie1Δfl/fl-Cre immorto mice.

**Flow-assisted cell sorting.** MAECs were released from the plate by incubating with Accutase (Invitrogen) for 10 minutes at 37°C. Cells were collected by centrifugation at 375 g for 10 minutes at 4°C and resuspended in HBSS (supplemented with 1% FBS and 1x antibiotics). The cells were then incubated with APC-conjugated CD31 (catalog no. 551262; BD Biosciences) at 1 mg/1 × 10⁶ cells for 30 minutes on ice with occasional agitation. MAECs were washed 3 times in HBSS and filtered through a 40-μm cell strainer (Fisher Scientific). Propidium iodide (Invitrogen) was added at 1:1000 dilution prior to sorting. CD31⁺ cells were collected in culture medium, centrifuged, and plated onto collagen-coated (Rat tail collagen type I; BD Biosciences) dishes.

**MAEC characterization.** MAECs were rinsed with PBS, briefly fixed with methanol at −20°C, and blocked with 10% normal goat serum (Jackson ImmunoResearch) for 1 hour at room temperature. Immunostaining was performed at 4°C overnight with either CD31 (catalog no. 553370; BD Biosciences) or VE-cadherin (catalog no. 160840; Cayman Chemical) and counterstaining with goat anti-rat Alexa Fluor 488 (BD Biosciences) or goat anti-rabbit Alexa Fluor 488. MAECs were also cultured on Matrigel-coated (BD Biosciences) dishes overnight and observed for network formation. Dil-AcLDL (Biomedical Technologies) was added to the media, centrifuged, and plated onto collagen-coated (Rat tail collagen type I; BD Biosciences) dishes.

**Induction of Cre activation by 4OHT and immunocytochemistry.** MAECs from Tie1Δfl/fl-SCL-ERα-Cre mice were cultured at 37°C in complete media (as described above) without IFN-γ and treated with 5 μM 4OHT (Sigma-Aldrich) every 2 days for 6 days. To ascertain ERα-Cre nuclear localization, 4OHT-treated cells were rinsed once with HBSS, fixed with cold 100% methanol for 3 minutes, and rinsed twice with HBSS. Cells were blocked with 5% goat serum, incubated overnight at 4°C with Cre antibody (Abcam, AB24608), rinsed, and immunostained with Alexa Fluor 488–conjugated goat anti-rabbit secondary antibody (BD Biosciences).

**Shear stress experiments.** Shear stress experiments were performed using a custom cone-and-plate shear stress viscometer design. An inverted servo motor (ElectroCraft) was attached to a plexiglass cone with 0.5° angle. A Motomatic II motor controller (Reliance Electric, ElectroCraft) regulated velocity in laminar flow, and a digital function generator (Instek) was used to produce a sinusoidal waveform modulating oscillatory flow. A digital oscilloscope (Tektronix) was used in parallel to monitor the output magnitude and waveform. The servomotor and cone were lowered onto the culture dish by a step-controlled base stand. Laminar shear was attained with unidirectional motion of the cone, while oscillatory shear was achieved by bidirectional motion at 5 dynes/cm². Shear stress experiments were performed for 24 hours in complete media in a sterile, 5% CO₂ incubator at 37°C.

**RNA extraction and real-time RT-PCR.** RNA from tissue or cells was isolated using TRIReagent ( Molecular Research Center), and SuperScript III First-Strand System (Invitrogen) was used to synthesize first-strand cDNA. Quantitative real-time RT-PCR was performed using iT SYBR Green Supermix (BioRad) on the iQ5 Real Time PCR Detection System (BioRad). Experiments were performed in triplicate using glyceraldehyde-3-phosphate dehydrogenase as the housekeeping gene to normalize the data. The relative change in gene expression was determined using the critical threshold (Ct) and the calculation 2^-ΔΔCt. Primers used were as follows: Tie1, 5′-GTGCCACACATTGGAGACTG-3′ and 5′-CAGGACACAGCGTT- GTGA-3′; glyceraldehyde-3-phosphate dehydrogenase, 5′-CAGTGCTATGGAAG-3′; IFN-γ, 5′-AAGAGAACCCACGTTGAGGT-3′ and 5′-GGGGCAACGTTGACATA- AAC-3′; ICAM, 5′-GAAGGTGGTTCTTCTGAGGC-3′; VCAM, 5′-AAGAGAACCCACGTTGAGGT-3′; glyceraldehyde-3-phosphate dehydrogenase, 5′-CAGTGCTATGGAAG-3′; IFN-γ, 5′-AAGAGAACCCACGTTGAGGT-3′; ICAM, 5′-GAAGGTGGTTCTTCTGAGGC-3′; VCAM, 5′-AAGAGAACCCACGTTGAGGT-3′.; Nos3, 5′-TGAAGATCTCTGCTCCTACTCATG-3′; and 5′-AGCTCTCAAGGCGATACCAAGAATTGTT-3′.

**Statistics.** All statistical differences in this study were determined by a 2-tailed, unpaired Student’s t test with 95% confidence intervals, using Prism 4.0 software (Graphpad Inc.). Statistical significance was accepted for P values less than 0.05. Graphical data representations are presented as mean ± SEM.

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