Endothelial Kruppel-like factor 4 protects against atherothrombosis in mice

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The endothelium regulates vascular homeostasis, and endothelial dysfunction is a proximate event in the pathogenesis of atherothrombosis. Stimulation of the endothelium with proinflammatory cytokines or exposure to hemodynamic-induced disturbed flow leads to a proadhesive and prothrombotic phenotype that promotes atherothrombosis. In contrast, exposure to arterial laminar flow induces a gene program that confers a largely antiadhesive, antithrombotic effect. The molecular basis for this differential effect on endothelial function remains poorly understood. While recent insights implicate Kruppel-like factors (KLFs) as important regulators of vascular homeostasis, the in vivo role of these factors in endothelial biology remains unproven. Here, we show that endothelial KLF4 is an essential determinant of atherogenesis and thrombosis. Using in vivo EC-specific KLF4 overexpression and knockdown murine models, we found that KLF4 induced an antiadhesive, antithrombotic state. Mechanistically, we demonstrated that KLF4 differentially regulated pertinent endothelial targets via competition for the coactivator p300. These observations provide cogent evidence implicating endothelial KLFs as essential in vivo regulators of vascular function in the adult animal.

Introduction

Through the elaboration of numerous biological substances, ECs actively regulate fundamental physiological processes, such as regulation of blood coagulation, homing of immune cells, and barrier function. Studies over the past several decades have also identified key physiologic and pathologic phenotypic modulators of ECs. For example, stimulation of the endothelium with proinflammatory cytokines renders the endothelium dysfunctional, inducing a proadhesive and prothrombotic phenotype. In contrast, laminar flow induces critical genes that confer potent antithrombotic, antiadhesive, and antiinflammatory properties. The significance of fluid shear stress is evidenced by the observation that segments of the arterial tree exposed to laminar flow (e.g., straight regions of the vasculature) are less prone to the development of atherosclerotic lesions than are regions exposed to nonlaminar/disturbed flow (e.g., branch points). These observations have led to the current view that the balance of biochemical and biomechanical stimuli is the central determinant of vascular function under physiologic and pathologic conditions.

Given the importance of the endothelium in vessel homeostasis, there is great interest in identifying molecular pathways that mediate the effects of both biochemical and biomechanical stimuli. Prior studies from our group and others have identified 2 members of the Kruppel-like factor (KLF) family of transcription factors, KLF2 and KLF4, as being of particular interest. Both KLF2 and KLF4 are induced by laminar flow and in vitro studies regulate the expression of many flow-dependent endothelial gene products (e.g., eNOS or thrombomodulin [TM]). Furthermore, these studies also suggest that both KLF2 and KLF4 confer antiinflammatory effects by virtue of their ability to attenuate NF-κB activity and attendant expression of proadhesive (e.g., VCAM1) and prothrombotic (e.g., PAI1) targets (1, 2). These in vitro observations form the basis of the current view that KLF2 and KLF4 are atheroprotective factors. However, the in vivo role of either factor in endothelial biology and atherothrombosis has not been elucidated. In the present study we focused on KLF4 and, using in vivo EC-specific gain- and loss-of-function approaches, identified this factor as a key determinant of experimental vascular inflammation and thrombosis.

Results and Discussion

Endothelial KLF4 regulates experimental atherosclerosis. Floxed Klf4flxV E-cadherin–driven Cre mice (referred to herein as Klf4fl-Cre mice) on an Apoε−/− background showed significantly enhanced development of atherosclerosis after 20 weeks of high-fat diet (HFD) feeding (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI66056DS1). There was a profound deficiency of KLF4 in ECs isolated from these mice (Supplemental Figure 1, C and D). However, because VE-cadherin–driven Cre is known to leak into the hematopoietic compartment (Supplemental Figure 1C), BM transplant studies were performed. After irradiation, control Cre and Klf4fl-Cre mice (both on an Apoε−/− background) were reconstituted with Apoε−/− BM; the resulting EC-specific knockout mice are referred to herein as EC-Klf4−/− and EC-Klf4−/+ , respectively. These mice were assessed for atherogenesis following a 20-week HFD challenge. EC-Klf4−/− mice showed a modest increase in total cholesterol, driven by an increase in LDL (Supplemental Figure 1G).
Strikingly, EC-Klf4–/– mice demonstrated a higher atherosclerotic burden (Figure 1, A–C), as well as increased inflammatory cell infiltration (Figure 1, C and D, and Supplemental Figure 1E), compared with EC-Klf4+/+ controls.

Next, to determine the effect of endothelial KLF4 overexpression on atherogenesis, we generated transgenic mice expressing human KLF4 (referred to herein as EC-KLF4 Tg mice) and nontransgenic WT controls and confirmed KLF4 expression (Supplemental Figure 1D). Following crossing onto an Apoe–/– background, EC-KLF4 Tg and WT mice were fed HFD for 16 weeks, then assessed for lesion burden by Sudan IV staining. No significant difference in lipids was observed between WT and EC-KLF4 Tg lines (Supplemental Figure 1G). However, sustained expression of KLF4 significantly reduced atherosclerotic lesion area (Figure 1, E–G) as well as inflammatory cell infiltration (Figure 1, G and H, and Supplemental Figure 1F).

Endothelial KLF4 is an essential regulator of vascular inflammation and thrombosis in vivo.

Endothelial KLF4 is regulated by biomechanical and biochemical stimuli. As shear stress, cytokines, and oxidized lipids are important in atherothrombosis, we first assessed the effect of these stimuli on KLF4. KLF4 mRNA expression was significantly higher in HUVECs exposed to atheroprotective versus atheroprone flow (Figure 3A), consistent with in vivo porcine data showing enhanced KLF4 expression in atheroprotected (i.e., descending thoracic aorta) versus atheroprone (i.e., aortic arch) sites (3). Next, we subjected HUVECs to the oxidative stress often seen in organisms at risk for atherogenesis — oxidized LDL in the presence of atheroprone flow. As expected, VCAM1 expression was increased under these conditions; interestingly, so was that of KLF4 (Supplemental Figure 3A), consistent with previous reports from our laboratory showing that

To extend these observations in vivo, we performed thrombosis studies using the Rose Bengal laser injury method. No significant difference in tail bleeding time (Supplemental Figure 2, A and B) or complete blood counts (Supplemental Table 1) was observed between Klf4fl-Cre or EC-KLF4 Tg mice and their respective controls. However, EC-Klf4–/– mice exhibited a strongly prothrombotic response to injury (Figure 2B). Conversely, EC-KLF4 Tg mice exhibited a prolonged time to thrombosis (Figure 2D). Collectively, the observations in Figures 1 and 2 identified endothelial KLF4 as an essential regulator of vascular inflammation and thrombosis in vivo.
endothelial KLF4 expression is enhanced by proinflammatory stimuli (2). These results were strikingly similar to the gene expression profile in aortas of mice fed HFD for 16 weeks (Supplemental Figure 3B). In contrast, and consistent with our prior reports, KLF2 levels were reduced in this inflammatory milieu.

KLF4 differentially regulates key endothelial targets. Next, we sought to determine how altering KLF4 levels affects the expression of key endothelial targets in the setting of biomechanical and biochemical stimuli. Intriguingly, overexpression of KLF4 induced atheroprotective genes and reduced proadhesive/prothrombotic targets, even in the presence of atheroprotective shear patterns or cytokines (Figure 3, B and C). In contrast, depletion or deficiency of KLF4 reduced expression of eNOS and TM under basal conditions (Supplemental Figure 3C) and in the setting of atheroprotective flow (Figure 3D). Furthermore, KLF4 deficiency resulted in heightened expression of VCAM1 and PAI1 under basal conditions and after cytokine stimulation (Supplemental Figure 3, D and E). These changes in the setting of KLF4 deficiency were observed despite a nearly 2-fold induction of the related transcription factor KLF2 (Supplemental Figure 3F).

Next, we sought to determine the molecular basis for KLF4’s ability to simultaneously induce some target genes and inhibit others. For example, induction of eNOS and TM was sustained even in the setting of cytokine stimulation, when targets such as VCAM1 were reduced (Figure 3C). KLF4 is known to interact with p300, a key coactivator required for optimal transcriptional activity of many factors, including KLF4 and NF-κB. Furthermore, a recent study showed that KLF4 can alter p300 recruitment to NF-κB targets in myeloid cells (4), possibly via interaction of KLF4 with p300 — to induce the VCAM1 promoter, an effect that was largely dependent on the presence of the P3ID (Figure 3F). Conversely, KLF4 inhibited the ability of p65 — both alone and in combination with p300 — to induce the VCAM1 promoter, an effect that was largely dependent on the presence of the P3ID (Figure 3G). Collectively, these studies support the idea that KLF4 can differentially regulate EC gene expression through coactivator competition.

In the early 1970s, the pioneering efforts of Jaffe and colleagues describing EC isolation ushered in the modern era of vascular biology (6). Studies over the ensuing 3 decades by numerous groups led to the appreciation that ECs sense, integrate, and transduce a variety of mechanical and biochemical signals to orchestrate vessel function. These insights fueled the view that the endothelium orchestrates vascular function in health and disease. Consequently, much effort has been invested in the identification of key regulators of EC gene expression and function. An important advance to this end was the identification of KLFs as flow- and cytokine-regulated factors that control endothelial gene expression and function in vitro. In particular, 2 members of this gene family — KLF2 and KLF4 — emerged as potentially important. Compelling insights gleaned largely from in vitro studies led to rapid acceptance that KLFs were master regulators of EC biology. Recently, Atkins et al. showed that systemic hemizygous deficiency of KLF2 augmented experimental atherogenesis (7). However, the in vivo role of these factors, specifically in endothelial biology in the adult organism, have been lacking. The central finding of the present study was that endothelial-specific deficiency of KLF4 rendered mice susceptible to atherothrombosis, whereas KLF4 sufficiency had a protective effect. These observations provide the strongest evidence to date implicating endothelial KLFs in general, and KLF4 in particular, as essential regulators of endothelial and vascular function in vivo in the adult animal. While our study provides compelling evidence for the importance of endothelial KLF4, the relative contribution of KLF4 and KLF2 in EC biology remains an open question. In vitro studies to date suggest that there is significant overlap between the endothelial targets and functional effects of KLF4 and KLF2.
For example, both could induce eNOS/TM and inhibit cytokine-mediated endothelial proinflammatory activation. Hemizygous loss of KLF2 leads to enhanced KLF4 expression in lung tissue (7), which raises the possibility that these factors may compensate for one another. In this regard, our findings were particularly noteworthy. Depletion of KLF4 led to an increase in KLF2 levels (Supplemental Figure 3F). However, as evidenced by the cellular studies and the atherothrombotic phenotype, this increase was insufficient to compensate for loss of endothelial KLF4. Thus, although KLF2 and KLF4 may have partially overlapping functions, they are clearly nonredundant. It may be that differential effects of KLF2 and KLF4 on downstream gene targets, particularly under inflammatory conditions, are related to the differential regulation of the KLFs themselves. Whereas KLF2 was repressed by proinflammatory stimuli, KLF4 expression was enhanced, perhaps as an adaptive response. Future efforts comparing endothelial-specific deficiency of KLF2 with those observed here for KLF4 are clearly required. Such efforts, along with compound deletion of both factors, may provide important insights into the full spectrum of KLF function in endothelial biology and vascular homeostasis.

**Methods**

**Mice.** EC-Klf4<sup>−/−</sup> mice were generated by mating floxed Klf4 mice (gift from K. Kaestner, University of Pennsylvania, Philadelphia, Pennsylvania, USA; ref. 8) with VE-cadherin–driven Cre mice backcrossed into the C57BL/6 background (B6.Cg-Tg [Cdh5-cre] 7Mlia/J; The Jackson Laboratory). EC-KLF4 Tg mice were generated in our laboratory using the human KLF4 coding sequence under control of the VE-cadherin promoter (gift from K. Walsh, Boston University, Boston, Massachusetts, USA). All mice used in the in vivo atherogenesis studies were on an ApoE<sup>−/−</sup> background.

**BM transplantation.** Recipient mice were irradiated with a single 9.6-Gy dose from a cesium-137 γ source. 4 hours later, mice were injected via tail vein with 2 × 10<sup>6</sup> femur-derived ApoE<sup>−/−</sup> BM cells. Atherogenic diets were started 4 weeks after transplantation.

**Atherosclerosis studies.** Mice were fed ad libitum with either standard rodent chow or Clinton/Cybulsky High Fat Rodent Diet (D12108C; Research Diets Inc.) starting at 6 weeks of age for 16–20 weeks. See Supplemental Methods for tissue processing.

**Thrombosis studies.** In vitro clotting assays were performed using mouse cardiac ECs. See Supplemental Methods for details. In vivo studies were performed using the Rose Bengal carotid artery thrombosis model, as
previously described (9). BM transplantation studies were performed 8 weeks after transplant. Complete blood counts were obtained using a HEMAVET 950FS (Drew Scientific).

**Isolation of mouse cells.** See Supplemental Methods for murine cardiac EC isolation. Cells were pooled from 5 mice and used for experiments at passages 2–3. Peritoneal macrophages were isolated after intraperitoneal injection of thioglycollate. Lymphocytes were isolated as the nonadherent cells in culture after plating PBMCs obtained from whole blood and separated by centrifugation in Histopaque (Sigma-Aldrich).

**Virus infection and siRNA knockdown.** For overexpression, HUVECs at passage 3–6 were infected with control or KLF4 adenovirus at 10 MOI. Loss-of-function experiments were performed with HUVECs transfected with nonspecific or KLF4-specific siRNA (50 nM; Dharmacon) using Lipofectamine RNAiMAX (Invitrogen). Experiments were performed 24–48 hours after infection/transfection.

**In vitro hemodynamic flow model.** After adenoviral overexpression or siRNA knockdown of KLF4, HUVECs were exposed to an atheroprotective or atheroprone waveform created with a dynamic flow system as described previously (10). RNA was isolated after 24 hours of flow, at which time a steady-state flow-dependent phenotype emerges. For oxidized LDL experiments, data from primary human aortic ECs are shown; their response was identical to that of HUVECs.

**RT-PCR and immunoprecipitation.** See Supplemental Methods for RNA isolation and quantitative real-time RT-PCR analysis. Gene expression was normalized to GAPDH, β2-microglobulin, or β-actin using the ΔΔCt method and expressed as fold change relative to respective control samples. Immunoprecipitation experiments were performed in cultured 293 cells (see Supplemental Methods).

**Promoter assays.** Transient transfection in COS-7 cells was performed using Xtremegene 9 (Roche) according to the manufacturer’s instructions. Cells were harvested 24 hours after transfection and assayed for luciferase activity, which was expressed normalized to total protein (BCA assay; Pierce Biotechnology). The KLF4AP3ID expression plasmid has a deletion of the sequence ETTEEFNDLDDLDFILS (amino acids 86–101, accession no. BC030811.1).

**Statistics.** All data are presented as mean ± SEM. Statistical analyses were done using 2-tailed Student’s t test. A P value of 0.05 or less was considered significant.

**Study approval.** All animal protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

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