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Syndecan 4 is required for endothelial alignment in flow and atheroprotective signaling

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Atherosclerotic plaque localization correlates with regions of disturbed flow in which endothelial cells (ECs) align poorly, whereas sustained laminar flow correlates with cell alignment in the direction of flow and resistance to atherosclerosis. We now report that in hypercholesterolemic mice, deletion of syndecan 4 (S4\(^−/−\)) drastically increased atherosclerotic plaque burden with the appearance of plaque in normally resistant locations. Strikingly, ECs from the thoracic aortas of S4\(^−/−\) mice were poorly aligned in the direction of the flow. Depletion of S4 in human umbilical vein endothelial cells (HUVECs) using shRNA also inhibited flow-induced alignment in vitro, which was rescued by re-expression of S4. This effect was highly specific, as flow activation of VEGF receptor 2 and NF-κB was normal. S4-depleted ECs aligned in cyclic stretch and even elongated under flow, although nonirectionally. EC alignment was previously found to have a causal role in modulating activation of inflammatory versus antiinflammatory pathways by flow. Consistent with these results, S4-depleted HUVECs in long-term laminar flow showed increased activation of proinflammatory NF-κB and decreased induction of antiinflammatory Kruppel-like factor (KLF) 2 and KLF4. Thus, S4 plays a critical role in sensing flow direction to promote cell alignment and inhibit atherosclerosis.

Syndecan 4 (S4) is a transmembrane heparan sulfate proteoglycan that serves as a coreceptor for extracellular matrix proteins and growth factors (1–3). S4\(^−/−\) mice are viable and fertile (4, 5) but show defective wound healing consequent to impaired angiogenesis (6). They also have higher mortality after LPS injection (7) and exhibit defective muscle repair and myofiber organization as a result of inefficient differentiation and migration of muscle satellite cells (8). We and others have also demonstrated that S4 plays a critical role in the control of cell polarity, by controlling Rho GTPase activity (9–11), as well as in planar cell polarity (12). S4 has also been recently identified as a putative mechanosensor (13).

Atherosclerosis is an inflammatory disease of large to mid-sized arteries that is the major cause of illness and death in developed nations and is rapidly increasing in developing nations (14, 15). It is linked to a variety of risk factors including high LDL cholesterol level and triglycerides, diabetes, smoking, hypertension, sedentary lifestyle, and inflammatory mediators. However, atherosclerotic lesions occur selectively in regions of arteries that are subject to disturbances in fluid shear stress (FSS), the frictional force flowing blood exerts on the endothelium. Regions of arteries with lower flow magnitude, flow reversal, and other complex spatial/temporal flow patterns are predisposed to atherosclerosis. Systemic risk factors appear to synergize with local biomechanical factors in the initiation and progression of atherosclerotic lesions (16).

The importance of S4 in endothelial biology prompted us to test its role in atherogenesis. Surprisingly, S4 deletion not only drastically increased atherosclerotic plaque burden in hypercholesterolemic mice but also caused plaque to form in regions that are normally resistant to disease. These findings led us to investigate the role of S4 in flow signaling. Our results showed that S4 is specifically required in alignment of endothelial cells (ECs) in flow and suggest that loss of this atheroprotective mechanism leads to increased atherosclerosis in S4\(^−/−\) mice.

Results

Widespread Atherosclerotic Lesions in Hypercholesterolemic S4\(^−/−\) Mice. S4 KO mice were crossed into the hypercholesterolemic low-density lipoprotein receptor (LDLR)/apolipoprotein B (apoB)\(^100 \text{mg/dL}^{0.5}\) (DKO) background. DKO/S4\(^+/+\) and DKO/S4\(^−/−\) male mice at 12 wk were put on the lipid-enriched Paigen diet without cholate (PD) for 16–20 additional weeks to induce atherosclerotic lesions (17–20). In DKO/S4\(^+/+\) mice, small lesions in the descending aorta were visible after 16 wk of PD (Fig. 1 A and B), specifically localized near the branch points for small intercostal arteries, a known site of flow disturbance (21). These lesions covered, on average, 9% of the total aortic surface. In DKO/S4\(^−/−\) aortas, widespread lesions near the intercostal bifurcations were also visible after 16 wk of PD, covering up to 18% of the total area (P = 0.0028). Surprisingly, nascent lesions also appeared in other regions, away from intercostal branches, that are normally atheroresistant. After 20 wk of PD, DKO/S4\(^+/+\) mice showed larger lesions that were still highly localized to branch points, whereas DKO/S4\(^−/−\) mice showed massive appearance of lesions over much of the aorta (Fig. 1 A). Lesions were also visible inside the intercostal arteries, where the flow profile is laminar (Fig. 1 C). No significant difference in HDL and LDL levels was observed in the two strains, excluding a contribution of impaired lipoprotein metabolism: 239 ± 30 mg/dL for DKO/S4\(^−/−\) (n = 6) and 236 ± 6 mg/dL for DKO/S4\(^+/+\) (n = 4), and 46 ± 4 mg/dL. HDL

Significance

Atherosclerosis, the major cause of death and illness in industrialized nations, develops in regions of arteries in which fluid flow patterns are disturbed and endothelial cells fail to align in the direction of flow. In contrast, regions of laminar flow in which cells are aligned are protected. The current work shows that the transmembrane proteoglycan syndecan 4 is required for endothelial cell alignment in the direction of flow and for the protective effect of high laminar flow, yet other flow responses are intact. The data therefore identify a role for syndecan 4 in flow direction sensing, show that sensing flow direction is separable from sensing flow magnitude, and provide new support for the key role of cell alignment in atheroprotection.


The authors declare no conflict of interest.

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for DKO/S4−/− (n = 6) and 44 ± 6 for DKO/S4+/+ (n = 4; results are expressed as mean ± SD).

Deletion of S4 Inhibits Endothelial Alignment in Vivo. The presence of lesions in areas that are normally resistant suggested impairment of flow-dependent atheroprotective mechanisms by loss of S4. We first examined EC alignment, which is highly correlated with atheroresistant regions of arteries (22, 23) and plays an important role in the activation of antiinflammatory versus proinflammatory pathways by flow (24). Staining the descending thoracic aorta for β-catenin to mark EC boundaries in wild-type C57BL/6 mice revealed uniformly elongated ECs that were well aligned in the direction of blood flow. In contrast, the same region in S4 KO mice showed poor alignment in the direction of flow, with markedly less elongation and many misaligned cells (Fig. 2A). The average cell shape index (4π area/perimeter2) was 0.33 ± 0.01 in wild-type mice and 0.58 ± 0.01 in S4 KO mice (n > 300 cells, four arteries). ECs in wild-type mice had well-organized actin stress fibers that were highly oriented in the direction of the flow, whereas in S4 KO mice, actin stress fibers were present but were poorly organized and misaligned (Fig. 2B; n > 300 cells, four arteries). Together, these data demonstrate a drastic loss of alignment in the direction of flow in S4 KO mice.

S4 Knock-Down Inhibits Flow Alignment in Vitro. We then tested alignment under flow in vitro in ECs stably expressing S4 shRNA, which decreased S4 levels by 65–80% (Fig. 3C). These cells formed a confluent monolayer with normal actin cytoskeletal organization, similar to control cells with scrambled shRNA (Fig. 3A). Cells were subjected to steady laminar FSS (12 dynes·cm−2) for 16 h and then fixed, the nuclei stained, and alignment quantified by measuring the angle between the major axis of the nucleus and the flow direction. Nuclear orientation is highly correlated with stress fibers and cell orientation (25) (Fig. S1A) and is more accurately and easily measurable. S4-depleted cells showed a striking failure to align in the direction of flow (Figs. 3A and B). A second shRNA sequence gave similar effects, rescued by expression of shRNA-resistant murine S4 (Fig. 3 C and D). In fact, rescued cells aligned somewhat better than control, untreated cells, probably because of the moderately higher S4 levels (Fig. 3C). We also noted that in preparations in which S4 expression was 5–10-fold above endogenous levels, flow-induced alignment was inhibited (Fig. S1C), indicating that S4 must be present at physiological levels. We also tested the response of cells over a wide range of shear stress magnitudes: scrambled shRNA cells aligned between 10 and 20 dynes·cm−2, whereas cells depleted for S4 never aligned in the flow direction (Fig. S1B).

To determine whether S4 is generally required for alignment in response to mechanical stimulation, cells were subjected to cyclic uniaxial stretch. S4 knock-down cells aligned perpendicularly to the direction of the force, similar to control cells (Fig. 3A and B). Thus, the failure to align in flow does not reflect a general defect
in mechanical or cytoskeletal responses. In addition, we did not detect any difference in the endothelial glyocalyx after S4 knockdown in human umbilical vein endothelial cells (HUVECs; Fig. S2A). Heparan sulfate, the major proteoglycan component of S4, mostly localized on the basal side of scrambled shRNA cells, and its organization and localization were not affected by S4 depletion (Fig. S2B). These observations argue against direct roles for the endothelial glyocalyx or heparan sulfate organization in sensing flow direction.

**S4 Knock-Down Cells Still Respond to Flow.** We have reported that multiple EC responses to flow, including cell alignment, require a complex of proteins at cell–cell junctions consisting of platelet endothelial cell adhesion molecule 1 (PECAM-1), vascular endothelial (VE)-cadherin, and VEGF receptor 2 (VEGFR2) (26). Flow triggers activation of the VEGFR2 tyrosine kinase, which mediates downstream events, including activation of NF-κB. We therefore examined activation of these events by flow. Phosphorylation at VEGFR2 tyrosine 1054 at 45 s after flow was increased similarly in S4 shRNA and control shRNA cells (Fig. 4A). Activation of NF-κB by flow was modestly, but significantly, higher in S4 knock-down cells compared with controls (Fig. 4C; P < 0.05). Thus, signaling through the junctional complex does not require S4.

Fluid shear stress also induces elongation of the cell body and nucleus (27, 28). Remarkably, after flow for 16 h, nuclear eccentricity increased in S4 knock-down cells to a slightly greater extent than in control cells (Fig. 4B), despite their random orientation (Fig. 3). Together, these results underscore the highly specific defect in sensing flow direction after S4 depletion.

**S4 Promotes Flow-Dependent Atheroprotective Pathways.** Alignment of ECs in the direction of flow is an important adaptive mechanism by which inflammatory pathways are down-regulated and antiinflammatory pathways are activated (24). This point is evident in Fig. 4C, where laminar flow at later times reduces p65 nuclear translocation below the levels seen in unstimulated conditions in wild-type cells. In contrast, in S4 knock-down cells, p65 translocation remained above the no-flow baseline and was strikingly higher than for control cells in flow for 16 h. To further assess proinflammatory versus antiinflammatory mechanisms, we measured levels of the antiinflammatory transcription factors kruppel-like factor (KLF) 2 and KLF4, which are induced by sustained laminar flow (29, 30). Induction by flow was substantially less in S4 shRNA cells compared with control cells (Fig. 4D; P < 0.05). Taken together, these results show that ECs lacking S4 fail to align in flow and have higher NF-κB activity and lower antiinflammatory KLF2 and KLF4 expression. These effects thereby may provide a mechanism for increased atherosclerosis in S4−/− mice, especially at normally atheroresistant regions of the vasculature.

**Discussion**

Although atherosclerosis is strongly associated with systemic risk factors such as high LDL cholesterol or diabetes, the localization of atherosclerotic lesions within arteries is highly correlated with areas of disturbed blood flow, characterized by low-magnitude FSS and directional changes during the cardiac cycle (31, 32). In contrast, high laminar shear inhibits the inflammatory, oxidative, and thrombotic pathways that promote atherosclerosis. The transcription factors Klf2 and Klf4 are major mediators of the atheroprotective phenotype in high laminar flow (29, 30), whereas NF-κB is a major proinflammatory transcription factor that promotes atherosclerosis (33). In vitro, onset of high-laminar FSS applied to ECs transiently activates the inflammatory transcription factor NF-κB; however, over several hours, cells align in the direction of flow and NF-κB declines to levels below baseline (34). Cell alignment in the direction of flow has therefore been proposed to be an adaptive mechanism that alters the way forces act on the cells (35). In contrast, cells in disturbed flow do not align, Klf2 and Klf4 remain low (36, 37), and NF-κB and other
inflammatory pathways remain high (22, 23, 34). Poor endothelial alignment is also a marker for susceptibility to atherosclerosis in vivo (38).

The results presented here identify S4 as a potent anti-atherosclerotic molecule. In particular, the appearance of plaques in normally atheroresistant regions of arteries was striking. We cannot completely exclude that lesions may propagate into atheroresistant areas because of the effects of lesions on downstream flow. However, the observed phenotype is not observed in other mouse models of severe atherosclerosis, even after a prolonged high-fat diet (39–42). Moreover, mislocalized plaque was evident even at earlier times. The increase and broad distribution of plaque correlated with loss of EC alignment in the direction of flow. S4 suppression also inhibited flow-dependent alignment in vitro, accompanied by elevated NF-κB activity and decreased Klf2 and Klf4 expression at longer times in high laminar shear. This effect was highly specific, as loss of S4 inhibited neither signals through the PECAM-1/VE-cadherin/VEGFR2 complex involved in shear stress sensing (26) nor alignment of ECs in cyclic stretch. Remarkably, nuclei in S4-depleted ECs elongated in flow, although without any preferred direction. These results lead to the conclusion that S4 is specifically required for sensing flow direction, which is independent of other aspects of flow mechanotransduction. Although ECs are generally thought to have multiple flow sensors (16), to our knowledge, this work provides the first evidence that flow direction sensing is separate from sensing flow magnitude.

The alignment defect in vivo, together with recent results showing that alignment is critical for the switch from proinflammatory to antiinflammatory signaling (24), suggest the hypothesis that loss of alignment leads to an activated endothelium, which increases susceptibility to atherosclerotic risk factors. It is tempting to speculate further that these findings may relate to the minority of cases in which atherosclerotic plaque occurs in regions of coronary arteries where flow patterns are expected to be laminar (43). Examining EC alignment and S4 expression in human specimens could test whether loss of this mechanism is a factor in the diffuse atherosclerosis seen in some patients (44–47).

In summary, these data reveal a highly specific role for S4 in sensing flow direction. Its loss, in vitro, leads to misaligned cells that show proinflammatory “priming,” which we hypothesize is similar to what is normally seen in atheroprone regions of disturbed flow (33). These cells are then susceptible to further activation by global risk factors, leading to formation of atherosclerotic lesions. How S4 mediates shear stress direction sensing is presently unknown. Indeed, the highly specific role of S4 in direction sensing underscores our ignorance about the mechanisms of this process. S4 can cooperate with integrins in adhesion to extracellular matrix and subsequent signaling (48); however, in the context of flow signaling, our data show they clearly act on distinct pathways. S4 has been reported to interact with polarity proteins (12), to control

Fig. 4. Effect of S4 knock-down on fluid shear-stress responses. (A) Western blot of phosphorylated and total VEGFR2, with actin as a loading control (n = 4, mean ± SEM). (B) Average nuclear eccentricity for all the nuclei in each experiment was quantified using the Matlab Eccentricity function (n ≥ 4 individual experiments, n = 3,000 nuclei per experiment; NF, no flow). (C) To assess NF-κB translocation, cells were stained for p65, imaged, and analyzed as described in Methods. The translocation factor is the total nuclear p65 fluorescence relative to the total cytosolic p65 fluorescence. Results for each experiment were then normalized to the static scramble cells. At least 2,000 cells were analyzed per condition, in at least three independent experiments (*P < 0.05, values are mean ± SEM). (D) S4, KLF2, and KLF4 message levels, relative to the static scramble mRNA for each individual experiment. mRNA levels were normalized to GAPDH (n = 4; ***P < 0.001 versus static scramble; ###P < 0.001 versus flow scrambled). SCR, scrambled shRNA; S4 #1, S4 shRNA #119; S4 #2, S4 shRNA #121. Values are mean ± SEM.
polarized recycling of integrins (49, 50), and to control polarized activation of Rac1 (9). It is attractive to speculate that the interac-
tions between S4 and integrins might therefore be involved in the inte-
gration of adhesion/signaling pathways and polarity pathways in
responses to flow. These questions await further research. The
identification of S4 effector pathways is therefore an important
question for future work that may provide a means for un-
derstanding more generally how ECs sense flow direction.

Methods

Cell Culture. HUVECs, in which each batch was pooled from three different
donors, were obtained from the Yale Vascular Biology and Therapeutics
program. Cells were cultured in M199 medium supplemented with 20% FBS, 50 μg mL−1 endothelial cell growth supplement prepared from bovine hy-
pothalamus, 100 μg mL−1 heparin, 100 μM penicillin, and 100 μg mL−1 streptomycin. They were used between passages 3 and 5.

Lentivirus Generation. Lentiviruses for stable shRNA expression was generated as
described (51). Briefly, packaging plasmids (Addgene) were mixed with shRNA plasmid (Mission shRNA, Sigma-Aldrich) in Opti
tem medium (Invitrogen) and Lipofectamine 2000 (Invitrogen) with the following ratios: 5 μg pMDu/pKRE, 2.5 μg pRSV-Rev, 2.5 μg pCMV- VSVG, and 10 μg shRNA. The mixture was transferred to 90% confluent 293T cells in 10-cm dishes for 6 h. The medium was replaced with regular DMEM 10% FBS and collected after 48 h. Medium containing virus was filtered through a 0.45-μm filter and used immediately for HUVEC transduction. The target sequences for human
ShRNA were fed normal chow diet for 12 wk, followed by 14
weeks of treatment with puromycin (1 μg/mL) and used for no more than two more passages.

Endogenous Adenovirus. Rat S4 [previously known as ruydocon (52)] with a
hagemammatin tag after the signal peptide was cloned into the Ad-Cla
(vector provided by the Harvard Gene Therapy Initiative (hgti.med.harvard.edu), virus core laboratory, which subsequently used the construct for the production of replication-deficient adenovirus.

Shear Stress. Cells were seeded on fibronetin-coated (20 μg mL−1) slides. After
reaching confluency, cells were starved with M199 medium containing 5% FBS with 100 U mL−1 penicillin and 100 μg mL−1 streptomycin for 3 min, followed by 45 cycles that used 95 °C for 10 s and 60 °C for 30 s. Gene expression was normalized with the housekeeping gene (GAPDH), and relative expression was calculated using the ΔΔCt method. Primers sequences: GAPDH: 5′-GGTCATACCTGTGGTCTCAG-3′ and 5′-GACCACTGCTCCGCTCCTAG-3′ (antisense); S4: 5′-TTGTTCCTGTAGGGAGATCTGGAGATCT-3′ and 5′-CCACACTACCTCTCATGCTG-3′ (antisense); KL2: 5′-GACACAGGCTGGCTCCTG-3′ (sense) and 5′-GGCGAGGCTTCCTG-3′ (antisense); and KL4: 5′-ATCTGGCC-CAAATGGGTTT-3′ (sense) and 5′-TTGAGAGCCTGTTCCCTC-3′ (antisense).

Mouse Strains and Diet. S4+/− and LDLR+/− KO/Apo 100/100 54−/− mice were generated by heterozygous crossing and then nine backcrosses, including daughter−father crosses on a C57BL/6 background. B6;129-Apob tm1Her/S 4−/−; DKO (Jackson Laboratories) were crossed with S4−/− mice. LDLR−/− and LDLR−/− mice were fed normal chow diet for 12 wk, followed by 14−20 wk PDK (Research Diets), as previously described (17−19). PD consists of 20% protein, 45% carbohydrate, 35% Fat, and no cholate (54).

Statistical Analysis. At least three independent experiments were performed for each condition. Statistical differences were tested by using either analysis
developmental deficit. of variance tests or nonparamed Student t tests, as indicated.

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Immunofluorescence. Cells were fixed for 10 min with 3.7% PFA, permeabilized
10 min with 1% Triton X-100 in PBS, blocked for 30 min with StartingBlock buffer (Thermo Scientific), and probed with primary antibodies overnight at 4 °C. Cells were then incubated with Alexa Fluor 488 (Molecular Probes) and DAPI. Cells were mounted in Fluoromount G. Aortas were perfusion-fixed with 3.7% PFA, excised, and adventitial tissue was removed. The vessels were opened longitudi-

Image Analysis. Masks of the images were made using a combination of an
adaptive histogram equalization algorithm and size intensity and thres-
holding. Cell orientation was calculated by taking the masks of the cell nuclei (determined from DAPI images), fitting them to an ellipse, and determining the angle between the flow direction and the major axis of the ellipse. Nuclei eccentricity was measured on the basis of the eccentricity of the fitted ellipse.

Nuclear translocation was computed by taking the mask of the transcrip-
tion factor stain (p65) and calculating the product of both the area and the

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