Cytoskeletal structure regulates endothelial cell immunogenicity independent of fluid shear stress

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Submitted 28 July 2009; accepted in final form 12 November 2009

Vartanian KB, Berny MA, McCarty OJ, Hanson SR, Hinds MT. Cytoskeletal structure regulates endothelial cell immunogenicity independent of fluid shear stress. Am J Physiol Cell Physiol 298: C333–C341, 2010. First published November 18, 2009; doi:10.1152/ajpcell.00340.2009.—The cardiovascular disease atherosclerosis is directly linked to the functions of endothelial cells (ECs), which are affected by fluid shear stress (FSS). High, unidirectional FSS causes EC elongation with aligned cytoskeletal components and nonimmunogenic EC functions that protect against atherosclerosis. In contrast, low, oscillatory FSS is associated with cobblestone-shaped ECs with randomly oriented cytoskeletons and proinflammatory EC functions that promote atherosclerosis. Whether EC shape plays a role in EC immunogenic functions, independent of FSS, has not been previously determined. The goal of this study was to determine the effect of EC elongation and cytoskeletal alignment on the expression of inflammatory genes and functions. With the use of micropatterned lanes, EC elongation and cytoskeletal alignment were achieved in the absence of FSS. EC gene expression of key inflammation markers determined that the elongation and cytoskeletal alignment of micropattern-elongated ECs (MPECs) alone significantly downregulated VCAM-1 while having no effect on E-selectin and ICAM-1. The positive control of FSS-elongated ECs promoted E-selectin and VCAM-1 downregulation and upregulation of ICAM-1. Functionally, monocytic U937 cells formed weaker interactions on the surface of MPECs compared with cobblestone ECs. Interestingly, MPEC expression of the known FSS-dependent transcription factor krüppel-like factor 2 (KLF2), which promotes a nonimmunogenic EC phenotype, was significantly upregulated in MPECs compared with cobblestone ECs. Cytoskeletal regulation of KLF2 expression was shown to be dependent on microtubules. Therefore, the cellular elongation and cytoskeletal alignment of MPECs regulated immunogenic gene expression and functions and may act synergistically with FSS to create an EC surface with reduced inflammatory capability.

ATHEROSEQUIS, a cardiovascular disease affecting millions of people each year (27), is characterized by the hardening of arteries and the buildup of fatty plaques within the arterial wall. Atherosclerotic fatty plaques develop in several stages over a long period of time. All stages of atherosclerosis are associated with inflammation; hence, it is often viewed as a chronic inflammatory disease. In the initial stage of atherogenesis, vascular inflammation is mediated by endothelial cells (ECs).

ECs mediate inflammation through the expression of the cell adhesion molecules E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). These molecules recruit leukocytes to the vessel wall for subsequent transmigration into the subendothelial space, ultimately leading to foam cell formation and plaque development. E-selectin promotes leukocyte tethering and rolling on the EC surface and is expressed in athero-prone vascular regions. The cell adhesion molecules VCAM-1 and ICAM-1 enable the firm adhesion of leukocytes to the EC surface (24). VCAM-1 is critical in early plaque development, whereas ICAM-1 has been attributed to the elaboration of late-stage atherosclerotic plaques (6). Interestingly, both EC expression of these pro-atherosclerotic molecules and the location of atherosclerotic plaques correlate to regions of the vasculature that bifurcate and curve (7). These bifurcated and curved vessels exhibit distinct hemodynamic forces and EC morphology.

Blood vessel geometry plays a critical role in the hemodynamic forces of blood flow. The hemodynamic force imposed by flowing blood that is tangent to the vessel wall, known as fluid shear stress (FSS), is the primary force acting on ECs. Low, oscillatory FSS is exerted on ECs in athero-prone bifurcated and curved vessels (1). In contrast, high, unidirectional FSS is exerted on ECs in the athero-protected straight vessels (1). ECs respond to these distinct FSS forces both morphologically and functionally.

The FSS-mediated EC response has been a source of intense interest for several decades. The dramatic effects of FSS on both EC shape and EC functions have been well established. The high, unidirectional FSS of long, straight vessels induces elongated EC shape and aligned cytoskeletal components, both parallel to the direction of flow, and decreased EC expression of immunogenic molecules (E-selectin and VCAM-1) (5, 8, 17, 20). The low, oscillatory FSS found in bifurcated and curved vessels induces cobblestone-like EC shape, randomly oriented cytoskeletal components, and promotes EC expression of immunogenic molecules (E-selectin, VCAM-1, and ICAM-1) (4, 8, 20, 31). Thus FSS-induced EC shape and function are inextricably linked, such that FSS-dependent EC shapes and functions happen concurrently both in vivo and in vitro. Whereas FSS may induce specific EC shapes, the direct influence of these drastically different EC shapes (elongated or cobblestone) and cytoskeletal organizations (aligned or random) on EC functions has not been investigated. However, decentralization theory suggests that the cytoskeleton is a critical mediator of the FSS-induced EC response, acting to distribute mechanical forces throughout the cells to elicit specific spatially localized mechanotransduction signaling pathways (13). Therefore, we hypothesize that EC shape and cytoskeletal structure are regulators of EC function, and investigation into these regulators may shed light on the molecular mechanisms behind EC athero-protective functions.

The molecular mechanisms for FSS-dependent EC athero-protective functions are controlled by transcription factors. Recently, krüppel-like factor 2 (KLF2) has been identified as a

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transcription factor that decreases EC expression of immunogenic molecules, including E-selectin and VCAM-1. Interestingly, KLF2 is the sole transcription factor that has been shown to be upregulated by long-term, high, unidirectional FSS and downregulated by cytokines (9). This indicates that the physical response of ECs due to FSS alone is an important regulator of KLF2 expression. However, whether the dramatic physical change in EC shape and cytoskeletal structure associated with high, unidirectional FSS affects KLF2 expression is unknown.

This study was designed to separate cytoskeletal alignment and FSS variables to determine whether EC shape and cytoskeletal structure contribute to EC immunogenic function independent of FSS. To control EC shape without applying FSS, surfaces containing micropatterned lanes were used. The shape, gene expression, and immunogenic functions of micropattern-elongated ECs (MPECs) were evaluated and compared with cobblestone-shaped ECs. Our results indicate that EC shape plays a role in regulating EC functions, whereby EC elongation and cytoskeletal alignment decreased EC immunogenic gene expression and function compared with cobblestone ECs. These results strongly suggest that cytoskeletal structure actively decreases EC immunogenic responses, indicating that the elongated shape of ECs may be a key contributor to EC-mediated atheroprotection.

MATERIALS AND METHODS

Cells and culture conditions. Primary ECs were isolated from baboon carotid arteries using 600 U/ml collagenase digestion and mechanical disruption (10). The ECs were plated on 50 μg/ml collagen I (BD Biosciences)-coated six-well plates in EGM-2 (Lonza) supplemented with 8% fetal bovine serum (FBS, Hyclone). ECs were used in these studies up to passage 5. ECs were supplemented with 8% fetal bovine serum (FBS, Hyclone). ECs were plated in EGM-2 (Lonza) DNA amplification. Quantitative results were obtained directly from agarose gels using ImageJ (NIH).

Flow studies. ECs were incubated on micropatterned or nonpatterned surfaces for 4 h before the application of FSS. At 4 h, the MPECs or nonpatterned ECs were placed in a parallel plate flow chamber (Glyotech) and attached to a flow loop. ECs were exposed to 20 h of 12.5 dyn/cm² FSS (19).

Shape index and cytoskeletal structure. The shape indices and cytoskeletal components (actin and microtubules) were characterized as previously described (29). Briefly, with the use of Image J (NIH) on microscopy images of ECs, the surface areas and perimeters of ECs were measured for each condition. The shape index, a measure of cell shape, was calculated as 4π (surface area/perimeter). A shape index of 1 represents a circle, whereas a shape index of zero represents a line. The cytoskeletal components actin and microtubules were visualized using immunofluorescent staining with 1) mouse-anti-human β-tubulin (primary antibody and Alexa Fluor 488 secondary antibody), and 2) Alexa Fluor 568-conjugated Phalloidin (Invitrogen). The stained ECs were imaged at ×40 and a custom Matlab image analysis program was used to quantify the alignment of the stained cytoskeletal components as previously described (29). Briefly, the image was processed in two major steps: 1) Laplacian edge detection to highlight the cytoskeletal fibers and 2) radon transform to determine the distribution of fiber orientation angles. The variance about the peak angle was calculated and used as a measure of the degree of alignment.

RNA analysis. Total RNA samples were obtained from cell lysates using an RNA Mini Isolation Kit (Zymo Research) followed by RNA Clean Up (Zymo Research) to remove any remaining genomic DNA. Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen). PCR reactions were made using SYBR Green PCR Master Mix (Invitrogen). The primers for the genes of interest are listed in Table 1. PCR reactions in the 7500 Fast System thermocycler were run at 95°C for 2 min, for 40 cycles of the following sequence: 95°C for 25 s, T_m (melting point) of primer for 30 s, and 72°C for 30 s. Finally, the temperature was held at 60°C. Samples were run on 2% agarose gels at 100–150 V to confirm appropriate transcript size in the DNA amplification. Quantitative results were obtained directly from the ABI 7500 Fast System using the ddCT method and taking primer efficiencies into account.

E-selectin immunofluorescent staining. At 24 h after being plated, MPECs and nonpatterned cobblestone ECs were fixed in 3.7% paraformaldehyde and blocked for 30 min each with FX enhancer (Invitrogen) and 10% goat serum. Primary antibody mouse-anti-human E-selectin (R&D Systems) or negative control mouse anti-human IgG1 (Sigma) was incubated for 1 h followed by secondary antibody goat anti-mouse Alexa Fluor 488 (Invitrogen) for 30 and 5 min DAPI (Invitrogen) nuclear stain. Fluorescence was imaged in a Nikon Eclipse TE 2000-U microscope at ×40. Fluorescent intensity was quantified per cell using Image J (NIH).

Leukocyte rolling. For leukocyte adhesion assays, glass slides plated with ECs were assembled into the flow chamber (GlyoTech).

Table 1. Primers for RNA analysis

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer 5’-3’</th>
<th>Reverse Primer 5’-3’</th>
<th>Amplicon Length (bp)</th>
<th>Reference</th>
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<td>E-Selectin</td>
<td>gatgagggattcagacaa</td>
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<td>23</td>
</tr>
<tr>
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<td>21</td>
</tr>
<tr>
<td>VCAM-1</td>
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<td>126</td>
<td>22</td>
</tr>
<tr>
<td>KLF2</td>
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</tr>
<tr>
<td>GAPDH</td>
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<td>104</td>
<td>26</td>
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</tbody>
</table>

ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; KLF2, krüppel-like factor 2.
connected to a Harvard PHD22/200 syringe pump (Harvard Apparatus). Assembled flow chambers were mounted on the stage of an inverted microscope (Zeiss Axiovert 200M). U937 cells (1 × 10⁶/ml) suspended in Hanks’ balanced salt solution containing 2 mM Ca²⁺, 2 mM Mg²⁺, and 0.1% BSA were perfused over ECs at 150 s⁻¹ (corresponding to a FSS of 1 dyn/cm²) for 4 min. After 4 min, buffer was flowed over the EC surfaces, and the flow rate was doubled every 15 s for 1 min to achieve shear rates of 300, 600, 1,200, and 2,400 s⁻¹ (corresponding to 2, 4, 8, and 16 dyn/cm²) and then held for one additional minute at 4,800 s⁻¹ (32 dyn/cm²). U937 cell adhesion was imaged using brightfield microscopy with a Zeiss 10, 0.25 NA A-plan lens on a Zeiss Axiovert 200M microscope (Carl Zeiss), and recorded using Stallion 4.0 (Intelligent Imaging Innovations). The percentage of U937 cell detachment was determined from counts of the U937 cells attached to the EC surface after flowing the U937 cells for 4 min at 1 dyn/cm² and after flowing buffer at the highest shear stress (32 dyn/cm²).

Cytoskeletal inhibitors. Confluent nonpatterned cobblestone ECs were treated for 2 h with cytochalasin D (100 nM, Sigma), nocodazole (1 μM, Sigma), or DMSO vehicle. After treatment, ECs were trypsinized and RNA was isolated as described above.

Statistical analysis. Data are represented as means ± SD except when otherwise indicated. Each experimental condition was repeated at least three times. Statistical analysis was performed using SPSS version 15.0. Comparisons between three or more conditions employed ANOVA with Tukey post hoc test. For two conditions, all data were matched and compared using paired Student’s t-test. For the PCR results, statistical analysis of gene expression was performed on ΔCt values, calculated in reference to GAPDH, using paired Student’s t-test (33). Significance for all statistical tests required P ≤ 0.05.

RESULTS

Micropattern- and FSS-induced elongated shape and cytoskeletal alignment of ECs. Shape index and cytoskeletal alignment were quantified to confirm comparable cell shape and cytoskeletal structures between MPECs and FSS-elongated ECs. The shape index of MPECs at 24 h was 0.37 ± 0.01, which was equivalent to the shape index of FSS-elongated ECs 0.39 ± 0.06 (Fig. 1). At 24 h, both MPECs and FSS-elongated ECs had significantly different shape indices compared with nonpatterned cobblestone ECs, which had a shape index of 0.72 ± 0.12 (Fig. 1).

To characterize the cytoskeleton, the peak angles of orientation and variances of actin and microtubule fibers were quantified using immunofluorescent staining. The results showed that the peak angles of orientation for actin and microtubule fibers were not significantly different, ranging from 2° to 6° (horizontal defined as 0°). The variances in the actin orientation about the peak for MPECs and FSS-elongated ECs were 18.01 ± 0.92 and 19.82 ± 1.78, respectively (Fig. 2A). Variances in microtubule fiber orientation angle about the peak for MPECs and FSS-elongated ECs were 19.17 ± 0.75 and 18.8 ± 1.15, respectively (Fig. 2B). There was no significant difference in the variance of actin or microtubule fiber orientation for the ECs elongated by micropatterns or elongated by FSS. Both MPEC and FSS-elongated ECs had significantly less variance in their cytoskeletal structures compared with nonpatterned cobblestone ECs (Fig. 2), indicating
an increase in alignment. These results confirmed that actin and microtubules of both MPECs and FSS-elongated ECs were comparably elongated and aligned parallel to the direction of the long axis of the ECs (Fig. 2).

Cytoskeletal alignment-regulated gene expression. Fold changes in gene expression were calculated compared with nonpatterned cobblestone ECs to determine the effect of cellular elongation (induced either by micropatterned surfaces or by FSS) on the expression of inflammation genes. FSS-elongated ECs were used as positive control for atheroprotective EC levels of expression of immunogenic genes (Fig. 3). FSS-elongated ECs had significantly decreased E-selectin (0.19 ± 0.07) and VCAM-1 (0.17 ± 0.04) expression and significantly upregulated ICAM-1 (1.54 ± 0.38) (Fig. 3). Interestingly, MPECs also significantly downregulated VCAM-1 (0.67 ± 0.10) expression, whereas ICAM-1 expression was not upregulated (1.33 ± 0.47) (Fig. 3, B and C). MPECs did not have any effect on E-selectin expression (0.42 ± 0.58) (Fig. 3A). FSS was applied to elongating MPECs to determine how FSS would affect athero-protective gene expression. MPECs exposed to FSS had significantly less E-selectin (0.36 ± 0.14) and VCAM-1 (0.01 ± 0.005) expression compared with nonpatterned cobblestone ECs (Fig. 3, A and B). ICAM-1 expression (1.03 ± 0.92) was not significantly different (Fig. 3C). It should be noted that the shape index of MPECs exposed to FSS (0.33 ± 0.04) was comparable to MPECs and FSS-elongated ECs. The cytoskeletal alignment of MPECs exposed to FSS has been previously characterized in detail (29).

E-selectin protein expression. MPEC and nonpatterned cobblestone EC expression of E-selectin protein was evaluated using immunofluorescent staining. MPEC and nonpatterned EC staining patterns were comparable, with patches of bright staining on the cell borders and diffuse staining of the cell surface (Fig. 4A). Qualitative measurements of fluorescent intensity per cell indicated variable E-selectin protein levels for MPECs and nonpatterned cobblestone ECs (Fig. 4B), which is consistent with gene expression data.

MPECs decreased U937 cell attachment strength. EC inflammatory function was evaluated by quantifying the attachment of perfused monocytic U937 cells. The surface of MPECs and nonpatterned cobblestone ECs both facilitated U937 rolling and attachment. U937 attachment strength was determined...
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Fig. 3. MPEC-induced expression of inflammatory genes. Fold changes in gene expression represented with respect to nonpatterned cobblestone ECs (horizontal line). Expression of E-selectin (A), vascular cell adhesion molecule-1 (VCAM-1) (B), and intercellular adhesion molecule-1 (ICAM-1) (C), for MPECs, FSS-elongated ECs, and MPECs with FSS. *P < 0.05, Paired Student’s t-test compared with nonpatterned cobblestone ECs. #P < 0.05, ANOVA, Tukey’s post hoc.

Qualitatively by ramping shear stress after U937 attachment. The highest measured shear stress (32 dyn/cm²) promoted U937 detachment from MPECs and nonpatterned cobblestone ECs. A significantly larger percentage of U937 cells detached from MPECs (42.63 ± 7.43%) compared with nonpatterned ECs (18.76 ± 2.55%; Fig. 5).

KLF2 expression was dependent on state of cytoskeletal alignment. EC elongation and cytoskeletal alignment were investigated as a possible mechanism behind FSS-induced KLF2 expression. The expression of KLF2 by MPECs was determined and compared with cobblestone ECs at 1, 4, 6, and 24 h (Fig. 6A). Early stages of micropattern-induced EC elongation had a variable effect on KLF2 expression, yet a significant increase in KLF2 expression was observed at 24 h (Fig. 6A). These data indicate that an aligning cytoskeleton has differing KLF2 expression compared with a randomly orienting cytoskeleton. Furthermore, expression of KLF2 in micropattern-induced and FSS-induced elongated ECs was compared at 24 h. Fold changes in MPEC, MPECs exposed to FSS, and FSS-elongated EC expression of KLF2 were determined with respect to nonpatterned cobblestone ECs (Fig. 6B). At 24 h, fold changes in KLF2 expression for MPECs, MPECs exposed to FSS, and FSS-elongated ECs were 1.71 ± 0.14, 3.4 ± 1.72, and 4.05 ± 0.84, respectively (Fig. 6B). Therefore, MPECs and FSS-elongated ECs both significantly induced KLF2 gene expression, but FSS-elongated ECs upregulated KLF2 approximately twice as much as MPECs.

Disruption of microtubules decreased KLF2 expression. Nonpatterned cobblestone ECs were treated with cytochalasin D or nocodazole to disrupt actin or microtubules, respectively. KLF2 gene expression was determined in each group compared with DMSO treatment (vehicle control). The results show a significant decrease in KLF2 expression in ECs treated with nocodazole (0.67 ± 0.21) but no significant difference with cytochalasin D (0.87 ± 0.35) (Fig. 7).

DISCUSSION

To study cytoskeletal regulation of EC functions, a FSS condition was selected to ensure that both MPECs and FSS-elongated ECs would undergo the elongation process in a similar timeframe. As expected (29), 4 h of adhesion followed by 20 h of 12.5 dyn/cm² FSS induced EC shape indices and cytoskeletal alignments that were comparable to MPECs at 24 h. Therefore, these two conditions were used to delineate the effects of EC shape and cytoskeletal structure, induced by either micropatterned surfaces or by FSS, on EC immunogenicity.

High, unidirectional FSS, that induces EC elongation and cytoskeletal alignment, generally promotes a nonimmunogenic gene expression profile of decreased expression of E-selectin and VCAM-1 (5, 17), whereas expression of ICAM-1 is often shown to be increased (2). In this study, gene expression by MPECs and FSS-elongated ECs was determined with respect to cobblestone ECs. Micropattern-induced EC elongation and cytoskeletal alignment alone did not influence E-selectin expression, whereas FSS-induced EC elongation downregulated E-selectin expression. The application of FSS to MPECs decreased the variability in E-selectin expression resulting in a significant downregulation compared with nonpatterned cobblestone ECs. This suggests a FSS-dependent rather than shape-dependent mechanism for E-selectin regulation. Previous research on the influence of steady FSS, in the presence of tumor necrosis factor-α, on E-selectin demonstrated regulation of E-selectin at the translational level, even when mRNA levels were unaffected (17). Therefore, E-selectin protein levels in MPECs compared with nonpatterned cobblestone ECs were evaluated and found to be comparable. This indicates that previously reported FSS-dependent protein translation effects are not dependent on cytoskeletal alignment. In MPECs, VCAM-1 expression was significantly downregulated and ICAM-1 expression was unchanged compared with cobblestone ECs. This indicates a role for EC shape and cytoskeletal alignment in the regulation of VCAM-1 but not ICAM-1. In agreement with previous studies, FSS-elongated ECs also downregulated VCAM-1 (3, 5, 28) and did so to a greater degree than MPECs. The application of FSS to MPECs reduced VCAM-1 gene expression to a level that was comparable to FSS-elongated ECs. Thus FSS must also play a role in VCAM-1 regulation that is synergistic with cytoskeletal alignment. Interestingly, MPECs had no effect on ICAM-1 while FSS-elongated ECs had a significant increase in ICAM-1 expression compared with cobblestone ECs. This regulation of ICAM-1 indicates that cytoskeletal alignment, independent of FSS, does not play a role in regulation of ICAM-1. However, the addition of FSS to MPECs also did not significantly alter ICAM-1 expression, which may be due to either differences in cytoskeletal remodeling in prealigned cells or differences in the number of cell-cell junctions. Overall, gene expression of
VCAM-1 and ICAM-1 by MPECs favored a reduced inflammatory profile, whereas FSS-elongated ECs had conflicting anti- and pro-inflammatory gene expression of cell adhesion molecules corresponding to decreased VCAM-1 and increased ICAM-1, respectively.

High, unidirectional FSS promotes an EC surface with reduced inflammatory capability and decreased leukocyte adhesion (3, 28). Similarities in MPEC and FSS-elongated EC gene expression suggest that the MPEC surface may also be innately nonimmunogenic. To functionally evaluate the adhesiveness of leukocytes to EC surfaces, MPEC and nonpatterned cobblestone EC surfaces were exposed to flowing U937 cells, which express integrins LFA-1 (CD11a/CD18), VLA-4, and SLe\(^{\alpha}\), the ligands for ICAM-1, VCAM-1, and E-selectin, respectively (32). Both MPECs and cobblestone ECs surfaces promoted U937 rolling and adhesion under shear. The U937 cell attachment to the surface of MPECs was significantly weaker than to cobblestone ECs, demonstrated by the percentage of U937 cell detachment at higher shear stresses. This decrease in firm adhesion may be due to changes in leukocyte affinity, the strength of adhesion to a single EC surface ligand, or avidity, the clustering of EC surface ligands that would increase binding strength. It is difficult to determine whether affinity or avidity had a more prominent role in firm adhesion of U937 cells to MPECs and nonpatterned cobblestone ECs since the spatial geometry of these receptors (VCAM-1 and ICAM-1) is unknown. However, these data are consistent with MPEC gene expression data where MPECs had less VCAM-1 and comparable levels of ICAM-1, both of which mediate irreversible firm leukocyte adhesion, compared with nonpatterned cobblestone ECs. Under these basal conditions, surface expression of VCAM-1 was not detectable using immunofluorescence (data not shown). Although already well established in ECs, blocking studies are necessary to elucidate the individual contribution of each cell adhesion molecule in MPECs on leukocyte rolling and attachment.
KLF2 is a potential mechanism for cytoskeletal-dependent regulation of gene expression because 1) KLF2 is a known regulator of EC athero-protective immunogenic phenotype, and 2) KLF2 expression is induced by high, unidirectional FSS. KLF2 gene expression was evaluated at 1, 4, 6, and 24 h for MPECs and cobblestone ECs. MPECs had cytoskeletal structures that were aligning over these 24 h (29), while the cobblestone EC’s cytoskeleton were undergoing random spreading. Whereas both the MPECs and nonpatterned cobblestone ECs were undergoing attachment and spreading in this same timeframe, the MPECs with an aligning cytoskeleton demonstrated increased KLF2 expression compared with the ECs with a randomly orienting cytoskeleton. Interestingly, Dekker et al. (9) also showed an increase in KLF2 expression over 24 h in human umbilical vein endothelial cells exposed to 25 dyn/cm² steady FSS. This similar trend in ECs that are aligning, induced either by micropatterned surfaces or FSS or both in combination, further suggests a possible cytoskeletal-dependent regulation of KLF2. The guided alignment on micropatterned lanes versus the random spreading on nonpatterned surfaces can increase cytoskeletal stability, which may also affect KLF2 expression. Since microtubules are the first cytoskeletal structures to align both in MPECs (29) and FSS-elongated ECs (18), microtubules may play an important role in KLF2 expression.

To delineate the effects of cytoskeletal alignment and FSS, MPECs’ and FSS-elongated ECs’ expression of KLF2 at 24 h were determined relative to cobblestone ECs. Both MPECs and FSS-elongated ECs upregulated KLF2 gene expression. Similar to the cytoskeletal alignment regulation of VCAM-1, where FSS-induced EC elongation had a greater effect on gene expression, FSS-induced elongation upregulated KLF2 to a greater extent than micropattern-induced EC elongation. The application of FSS to MPECs restored KLF2 expression to levels that were comparable to FSS-elongated ECs (ANOVA, Tukey’s post hoc). This validates that cytoskeletal alignment, whether it is induced by micropatterned surfaces or FSS, has a regulatory role in KLF2 expression, and FSS effects are synergistic with cytoskeletal alignment.

Isolation of the individual roles of actin and microtubules contributes to the understanding of how the cytoskeleton may regulate KLF2 expression. Disrupting either actin or microtubules, using cytochalasin D or nocodazole, in MPECs led to a loss of EC elongation (29). Therefore, nonpatterned cobblestone ECs were used to determine the importance of the integrity of the cytoskeleton in the regulation of KLF2. Results indicated that disrupting microtubules downregulated KLF2, whereas disrupting actin had no effect. Interestingly, the known transcription factor NF-κB, which promotes an athero-prone EC phenotype, is also regulated by the cytoskeleton. Opposite of KLF2, disruption of microtubules in HeLa S3 cells significantly upregulate NFκB expression (25). KLF2 and NFκB have been shown to share a transcriptional cofactor p300/CPB. Thus, dependent on the state of the cytoskeleton, a balance may be maintained between KLF2 and NFκB activity through binding to p300/CPB. This has interesting implications in vivo, where KLF2 is found in regions of high, unidirectional FSS, where the cytoskeleton is elongated and possibly more stable compared with the low, oscillatory FSS regions, where NFκB is predominantly found and the cytoskeleton may be more unstable.

Taken together, these data suggest cytoskeletal-dependent regulation of EC immunogenicity. Mechanistically, a role of

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**Fig. 6.** MPEC expression of krüppel-like factor 2 (KLF2). A: time course of KLF2 expression in aligning ECs (MPECs) versus randomly orienting ECs (cobblestone ECs). B: MPEC, FSS-elongated ECs, and MPEC + FSS expression of KLF2 compared with nonpatterned cobblestone ECs. Horizontal line represents KLF2 expression level for nonpatterned cobblestone ECs. MPEC, FSS-elongated ECs, and MPEC + FSS all significantly upregulated KLF2 expression. *P < 0.05, paired Student’s t-test.

**Fig. 7.** Isolated roles of actin and microtubules in KLF2 expression. KLF2 expression in nonpatterned ECs with disrupted actin or microtubule using cytochalasin D or nocodazole, respectively. Fold change represented compared with DMSO vehicle control (horizontal line). Disrupting microtubules significantly downregulated KLF2 expression. *P < 0.05, paired Student’s t-test.
the cytoskeleton is to maintain a cell’s internal tension, which is altered by changes in cytoskeletal structure and by exposure to external forces (e.g., FSS) (14). Cytoskeletal changes induced by micropatterned lanes or by FSS result in an increase in cellular internal tension and stiffness (15, 16). Because the distribution of mechanical forces is critical to eliciting specific mechanotransduction pathways, the internal tension induced by the aligned cytoskeletal structure, with or without FSS, may play an important role in regulating EC functions. Previous research has shown that cytoskeletal alignment can also independently affect the composition of extracellular matrix (ECM) deposited by ECs (30), which could alter the type of focal adhesions formed at EC attachment points to the ECM, ultimately influencing cell signaling. Gene expression results from this study revealed that certain genes are regulated by cytoskeletal alignment, whereas others required stimulation with high, unidirectional FSS. These results suggest the following: 1) the sensitivity of a tension-induced response varies such that internal tension due to cytoskeletal alignment alone is sufficient to initiate the expression of certain genes, e.g., VCAM-1, whereas others must overcome larger mechanical thresholds, possibly through the application of FSS, to induce expression; 2) the signaling response to tension is dependent on the strength of the tensional force, where internal tension induced by cytoskeletal alignment induces less signaling activity than the larger force of FSS; and 3) both major source points of tension, focal adhesions, and cell-cell junctions may be required to promote cytoskeletal-dependent signaling that is athero-protective. The cell-cell junctions, which MPECs are lacking on the cell edges, may be required to increase internal tension or may promote specific signaling cascades that support athero-protective EC functions.

Conclusion. EC regulation of inflammation is critical to the maintenance of vascular health. ECs exposed to high, unidirectional FSS promote a reduced EC inflammatory state. The results from this study suggest that cytoskeletal alignment alone can also promote reduced EC inflammatory functions. In particular, VCAM-1 was significantly downregulated by cytoskeletal alignment. ICAM-1 was not changed in MPECs but it was upregulated in ECs exposed to FSS. Functional evaluation of EC-mediated inflammatory responses showed that MPECs formed weaker interactions with flowing leukocytes compared with nonpatterned cobblestone ECs, which supports the decreased VCAM-1 gene expression data. KLF2, the transcriptional regulator of athero-protective EC inflammatory function, was also shown to be upregulated by EC elongation and cytoskeletal alignment compared with cobblestone ECs. This is the first demonstration of a cytoskeletal structure-dependent upregulation of KLF2 expression without the application of high, unidirectional FSS. The microtubule cytoskeleton was a regulator of KLF2 expression. Collectively, this study presents evidence that the cytoskeleton is not simply a passive internal cellular support, but rather, serves as an interactive organelle involved in the regulation of important EC functions that contribute to EC inflammatory function.

ACKNOWLEDGMENTS
The authors thank Achievement Rewards for College Scientists for their generosity.

GRANTS
This work was supported by the American Heart Association Grants 081017ZZ and 09BGLIA2260384.

DISCLOSURES
No conflicts of interest are declared by the author(s).

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