Atherosclerosis-prone hemodynamics differentially regulates endothelial and smooth muscle cell phenotypes and promotes pro-inflammatory priming

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Hastings NE, Simmons MB, McDonald OG, Wamhoff BR, Blackman BR. Atherosclerosis-prone hemodynamics differentially regulates endothelial and smooth muscle cell phenotypes and promotes pro-inflammatory priming. Am J Physiol Cell Physiol 293: C1824–C1833, 2007. First published October 3, 2007; doi:10.1152/ajpcell.00385.2007.—Atherosclerosis is an inflammatory disease that preferentially forms at hemodynamically compromised regions of altered shear stress patterns. Endothelial cells (EC) and smooth muscle cells (SMC) undergo phenotypic modulation during atherosclerosis. An in vitro coculture model was developed to determine the role of hemodynamic regulation of EC and SMC phenotypes in coculture. Human ECs and SMCs were plated on a synthetic elastic lamina and human-derived atheroprone, and atheroprotective shear stresses were imposed on ECs. Atheroprone flow decreased genes associated with differentiated ECs (endothelial nitric oxide synthase, Tie2, and Kruppel-like factor 2) and SMCs (smooth muscle α-actin and myocardin) and induced a proinflammatory phenotype in ECs and SMCs (VCAM-1, IL-8, and monocyte chemotactant protein-1). Atheroprone flow-induced changes in SMC differentiation markers were regulated at the chromatin level, as indicated by decreased serum response factor (SRF) binding to the smooth muscle α-actin–CC/TTGG (CarG) promoter region and decreased histone H4 acetylation. Conversely, SRF and histone H4 acetylation were enriched at the c-fos promoter in SMCs. In the presence of atheroprotective shear stresses, ECs aligned with the direction of flow and SMCs aligned more perpendicular to flow, similar to in vivo vessel organization. These results provide a novel mechanism whereby modulation of the EC phenotype by hemodynamic shear stresses, atheroprone or atheroprotective, play a critical role in mechanical-transcriptional coupling and regulation of the SMC phenotype.

depotelial cells; smooth muscle cells; coculture; shear stress; phenotypic modulation

ATHEROSCLEROSIS is a vascular inflammatory disease characterized by lesion formation and luminal narrowing of the arteries. Endothelial cell (EC) and smooth muscle cell (SMC) regional phenotypes have significant implications in the progression of vascular disease. During early atherogenesis, the endothelium becomes activated, and SMCs undergo “phenotypic switching” (for reviews, see Refs. 14 and 33). Atherosclerosis is further characterized by its focal development in large arteries at hemodynamically defined regions, such as at bifurcations that produce complex flow patterns (14, 39). Atheroprone regions, susceptible to plaque formation, are subjected to low-time-averaged shear stress and “disturbed” oscillatory flow patterns. In contrast, atheroprotective regions, which are less susceptible to plaque formation, are exposed to relatively higher time-averaged shear stress and pulsatile laminar flow (13, 39).

To understand the role of shear stress on the endothelium in atherogenesis, in vitro models that expose ECs to a variety of shear stress conditions have been extensively studied (for reviews, see Refs. 14 and 11). Since ECs can discriminate variations in flow patterns and are sensitive to both shear stress magnitude and time-varying features of hemodynamics, emulating in vivo flow environments appears to have a greater impression on recapitulating the in vivo phenotype of the endothelium (2, 35). Additionally, few studies have shown the intricate interactions and cross-communications of ECs and SMCs in the presence of any type of flow, and no studies to date have examined how in vivo-derived human hemodynamic forces on the endothelium regulate SMC phenotypic switching, as it is classically defined by the literature.

In this study, we tested the hypothesis that exposure of hemodynamic forces from atheroprone and atheroprotective regions on the endothelium differentially regulates both EC and SMC phenotypes. We developed a novel in vitro coculture model using human ECs and SMCs to definitively show that human hemodynamic forces applied directly and only to the endothelium can modulate the SMC phenotype and influence SMC remodeling, a process we define as “mechanotranscriptional coupling.” The results reveal that both ECs and SMCs undergo differential phenotypic alterations in response to atheroprone compared with atheroprotective flow, indicating that vascular ECs and SMCs in an atheroprone environment are both susceptible to an early inflammatory response. Importantly, these results are consistent with classical studies in literature that defined EC and SMC phenotypic modulation in atherosclerosis (see Supplemental Table 1), thus validating the overall utility of this model for studying early atherosclerotic events.

EXPERIMENTAL PROCEDURES

Human cell isolation and culture. Primary human ECs and SMCs were isolated from umbilical cords, expanded, and used as cell sources. Human SMCs were also purchased from Cell Applications at passage 2. All tissue procurement was approved by Human Investigation Committees of the University of Virginia and Martha Jefferson...
Hospital (no. 10486). Human ECs were isolated from the umbilical vein (human umbilical vein ECs) as previously described (2), followed by isolation of SMCs from the vein using a similar method as previously described (38).

ECs were used for experimentation at passage 2 and SMCs were for experimentation used up to passage 10, both of which have been established to retain the basal EC/SMC phenotype based on the retention of specific EC and SMC markers. Cell types were separately cultured and passaged using medium 199 (M199; BioWhitaker) supplemented with 10% FBS (GIBCO), 2 mM l-glutamine (BioWhitaker), growth factors [10 μg/ml heparin, Sigma], 5 μg/ml endothelial cell growth supplement (Sigma), and 100 U/ml penicillin-streptomycin (GIBCO).

Transwell coculture plating conditions. Porous Transwell membranes (polycarbonate, 10 μm thickness and 0.4 μm pore diameter, no. 3419, Corning) were initially coated with 0.1% gelatin on the top and bottom surfaces. The Transwell was inverted, and SMCs were plated at a density of 10,000 cells/cm² on the bottom surface for 2 h. The Transwell was then turned back over into the holding well for 48 h in reduced serum growth medium (M199 supplemented with 2% FBS, 2 mM l-glutamine, and 100 U/ml penicillin-streptomycin). ECs were then plated on the top surface of the membrane at a density of 80,000 cells/cm² under the same media conditions for an additional 24 h. For hemodynamic flow experiments, two dishes were prepared in parallel (Fig. 1A).

Coculture hemodynamic flow device and flow patterns. The novel coculture in vitro model used in this study was adapted from an existing version similar to that developed by Blackman et al. (2), where arterial flow patterns modeled from the human circulation were applied to human ECs. The newer version of the cone and plate device existing version similar to that developed by Blackman et al. (2), where arterial flow patterns modeled from the human circulation were applied to human ECs. The newer version of the cone and plate device was modified to incorporate a 75-mm-diameter Transwell model was modified to incorporate a 75-mm-diameter Transwell compartment, and special mounting brackets for in-flow and out-flow tubing for both the inner and outer chambers of the Transwell, which provides direct access to the culture fluid environment to continuously exchange media to both EC and SMC layers. Through the rotation of the cone, the system imposes hemodynamic shear stress on the EC layer of the EC/SMC coculture (Fig. 1B). The modified device operates as previously described (2).

Table 1. Real-time RT-PCR primers designed for gene and ChIP analyses

<table>
<thead>
<tr>
<th>Sense Primer</th>
<th>Antisense Primer</th>
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<tr>
<td><strong>Real-time RT-PCR primers</strong></td>
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<tr>
<td>β2-Microglobulin</td>
<td>5’-CTGCTGTGATACCTGTAACCTCT-3’</td>
</tr>
<tr>
<td>eNOS</td>
<td>5’-TTAAGCTGTTAGCTGGCTGCTG-3’</td>
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<tr>
<td>IL-6</td>
<td>5’-GGCCGCACCGCTGACACCTG-3’</td>
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<td>KLF2</td>
<td>5’-GCTGGTTCGTCGAGTGAAGT-3’</td>
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<td>KLF4</td>
<td>5’-TCAGTGAGGTTTGGAGAGAGT-3’</td>
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<tr>
<td>MCP-1</td>
<td>5’-CGCCGGGTTTGGAGAGAG-3’</td>
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<tr>
<td>Myocardin</td>
<td>5’-TCAGGGATCTGAGAGAAGAC-3’</td>
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<tr>
<td>SmxA</td>
<td>5’-AACACTCAGGATACCTGCTG-3’</td>
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<tr>
<td>SMMHC</td>
<td>5’-TGAGATTCTCTGAGAAAGACG-3’</td>
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<tr>
<td>Tau2</td>
<td>5’-CAGTTAAATTTCGGCCGGCC-3’</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>5’-GCGGTACGAGTTCACATTTGATGTA-3’</td>
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<tr>
<td>ChIP analysis primers</td>
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<tr>
<td>SmxA, 5′-CarG</td>
<td>5’-CTGGGGGCCGAGAACCAAAATCAGAGAG-3’</td>
</tr>
<tr>
<td>SMMHC, 5′-CarG</td>
<td>5’-CACGAGGAAAGCGGCGGCGGAC-3’</td>
</tr>
<tr>
<td>c-fos</td>
<td>5’-ACGAGGAAAGCGGCGGCGGAC-3’</td>
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ChIP, chromatin immunoprecipitation; eNOS, endothelial nitric oxide synthase; KLF, Kruppel-like factor; MCP, monocyte chemoattractant protein; SmxA, smooth muscle α-actin; SMMHC, smooth muscle myosin heavy chain; CarG, CC(At)6G.
flow experiment were collected on ice after 4, 8, 12, and 24 h for each chamber of the membrane (i.e., EC- and SMC-conditioned media from atheroprotone and atheroprotective flows). Samples were then stored at −80°C until they were assayed for IL-8 secreted protein via ELISA (GE Healthcare). The concentration of protein was determined using a spectrophotometer at 450 nm and normalized to the volume of media collected per hour.

**Chromatin immunoprecipitation assay.** After the application of flow patterns, chromatin immunoprecipitation (ChiP) was performed as previously described with modifications allowing for a quantitative analysis of protein:DNA interactions (30). Outflow media from each experiment were supplemented with 1% formaldehyde and then incubated with cells for 10 min immediately following 24 h of flow. Antibodies included rabbit polyclonal anti-serum response factor (SRF; Santa Cruz Biotechnology, 5 μg/ml) and anti-histone H4 acetylation (Upstate Biotechnologies, 5 μg/ml). Recovered DNA was quantified by fluorescence with picogreen reagent (Molecular Probes) according to the manufacturer’s recommendations. Real-time PCR was performed on 1 ng genomic DNA from ChiP experiments with minor modifications as previously described (30). Real-time PCR primers were designed to flank the 5'-CC(a/T)GG(CAG) elements of SMMα, SMMHC, c-fos CArG. Table 1 shows the primers used for ChiP analysis. Quantification of protein:DNA interaction/enrichment was determined by the following equation: \(2^{C_{\text{Ref}} - C_{\text{IP}}} = 2^{C_{\text{Ref}} - C_{\text{No antibody control}}}\), where \(C_{\text{Ref}}\) is the reference threshold cycle (Ct) and \(C_{\text{IP}}\) is the Ct of the sample.

**Immunofluorescence.** For immunofluorescence (IF), Transwell membranes were fixed in 4% paraformaldehyde for both en face preparations and transverse sections. En face preparations were permeabilized in 0.2% Triton X-100. Primary antibody for SMCs was pipetted onto a piece of parafilm [Cy3-SM abilized in 0.2% Triton X-100. Primary antibody for SMCs was added directly to the inside of the well, and both antibodies were simultaneously incubated for 1 h. Similarly, secondary antibodies [Cy2 donkey anti-goat (Jackson ImmunoResearch, 4 μg/ml)] was then added directly to the inside of the well, and both antibodies were simultaneously incubated for 1 h. Similarly, secondary antibodies [Cy2 donkey anti-goat (Jackson ImmunoResearch, 4 μg/ml)] and Alexa 546 goat anti-rabbit (Molecular Probes, 6 μg/ml)] were added to samples as required and incubated for 1 h. Samples were mounted by adding Prolong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) to a large coverslip and dropping the well on top. Another drop of DAPI was added to the inside of the well, and a 22-mm-diameter coverslip was placed on top and allowed to solidify. The holding well was removed from the mounted samples using a scalpel to allow for imaging. Confocal microscopy was used to image en face samples through the z-axis from the EC to SMC layer (Nikon Eclipse Microscope TE2000-E2 and Melles Griot Argon Ion Laser System no. 35-IMA-840).

To prepare the transverse sections, EC/SMC cultures were stained with phalloidin-488 (Molecular Probes) or FM 4-64FX (Molecular Probes) using the methodology described above, immersed in 30% sucrose overnight, frozen in OCT compound, sliced into 5-μm-thick sections with a cryostat (University of Virginia Histology Core Facility), and then mounted for assessment by confocal microscopy. IF stained samples were analyzed using a confocal microscopy and differential interference contrast for cell-to-cell interactions within the pores of the Transwell membrane under static conditions, as previously described (22).

**EC/SMC orientation and morphometric measurements.** The orientation of ECs and SMCs relative to the direction of flow was quantified using confocal microscopy of IF stained samples. Following hemodynamic flow, the coculture was fixed as described above, and isoceles triangular samples from the 75-mm-diameter dishes were cut with the apex of the triangle pointing toward the center of the dish. This method established the correct orientation relative to the direction of flow. Samples were then stained as described above and mounted between two coverslips. For imaging, samples were oriented on the confocal stage with the triangle apex facing to the right, so that the direction of flow was consistent across all samples. Images were taken of ECs and SMCs in the same location, separated only by the membrane distance.

At least three microscopy fields were acquired over three independent experiments. MetaMorph software was used to determine the angle of orientation and shape factor (SF) for each cell analyzed relative to the direction of flow. To determine the elongation of cell types, borders stained for VE-cad (Fig. 2A) and β-catenin (not shown) of ECs (CCA: \(n = 111\) and ICS: \(n = 53\)) and SMMα (Fig. 2A), SMMHC, and β-catenin (not shown) of SMCs (CCA: \(n = 64\) and ICS: \(n = 25\)) were outlined, and measurements of the area and perimeter were outputted. SF was calculated using the following equation: \(SF = (4\pi A)/P^2\), where \(A\) is the cell area and \(P\) is the perimeter. For each SF bin in the histogram range, the number of cells per bin was normalized to the total number of cells analyzed over the whole range to yield a normalized frequency. Histograms were plotted to show the distribution of SFs for each condition (see Fig. 4B). For the angle of orientation, lines were drawn in both the direction of flow and along the long axis of the SMCs from both flow patterns (CCA: \(n = 119\) and ICS: \(n = 104\)) and ECs for atheroprotective flow only (CCA: \(n = 124\)). The angle between the two lines was measured as the orientation angle relative to the flow direction, and histograms were plotted so that the frequency of cells having the same orientation was represented as the bar length.

**Data analysis and statistics.** Real-time RT-PCR results are reported as the fold induction of cycle amplification times for atheroprotective flow samples compared with atheroprotective flow and normalized to endogenously expressed gene β2-microglobulin. Student’s t-test was conducted for mRNA, orientation, and elongation data to determine the significance in expression level or morphological changes as a function of hemodynamic flow pattern and time. Data from at least three independent experiments per condition were used for analysis and evaluated at \(P < 0.05\).

**RESULTS**

**Optimization of EC/SMC coculture plating and growth conditions.** Coculture conditions for human EC and SMC plating were optimized so that each cell type reached confluence prior to the application of hemodynamic flow (Fig. 1A). Figure 1D shows confluent layers of ECs and SMCs 24 h following EC seeding. ECs retained their classic polygonal morphology, forming adheren junctions, as demonstrated by the continuous seeding. ECs retained their classic polygonal morphology, forming adheren junctions, as demonstrated by the continuous peripheral staining of VE-cad, whereas SMCs were elongated and randomly oriented in the typical “hill and valley” formation. In SMCs plated alone, reduced serum media (2% FBS) increased the mRNA expression of SMC markers SMMα and myocardin, indicating a more differentiated SMC phenotype (normalized gene expression with 2% FBS: SMA, 2.51 ± 0.36 and myocardin, 0.23; with 10% FBS: SMA, 0.69 ± 0.23 and myocardin, 0.54 ± 0.14; see Supplemental Fig. 1).

Recent work by Isakson et al. (22) using a murine coculture model demonstrated that ECs and SMCs physically interact and communicate via gap junctions through linear pores of the Transwell membrane. This model emulates myoendothelial junctions present within the vascular wall in vivo, creating a means for ionic communication via gap junctions and physical heterocellular adhesion (17, 22). To determine whether EC/SMC physical interactions are formed in our human coculture model, transverse sections of the Transwell membrane were IF labeled for F-actin or FM 4-64FX and...
analyzed using confocal and phase contrast microscopy. The results shown in Fig. 1E demonstrate that cellular processes are present in the pores, establishing heterocellular interactions.

**EC/SMC morphological remodeling is altered in atheroprone flow.** The morphology of ECs and SMCs in vivo is highly ordered, with ECs being elongated and aligned with the direction of hemodynamic flow and SMCs oriented perpendicular to the long axis of the artery and direction of blood flow. However, the endothelium in regions of complex flow, such as in arterial bifurcations, is more polygonal and less aligned, and SMCs do not consistently align perpendicular to flow (14, 37, 39). To determine whether hemodynamic flow on the endothelium induces morphological changes to ECs and SMCs, the following SF measurements for both cell types were determined: 1) alterations in elongation and 2) orientation angle measurements relative to the direction of flow. Significant differences in both cell shape (SF) and cell orientation were observed after the application of atheroprone flow compared with atheroprotective flow (Fig. 2). SF indicates the extent of...
cellular elongation, where a value of 1 specifies a circle (i.e., no elongation) and a value closer to 0 specifies an elongated cell. Representative IF images are shown in Fig. 2A. As previously established (9, 10), ECs exposed to atheroprone flow maintained a more polygonal shape (SF_H11005/0.75/H11006/0.002), whereas ECs under atheroprotective conditions were more elongated (SF_H11005/0.64/H11006/0.015). Figure 2B shows the distribution of EC SF normalized to the number of cells analyzed. The alignment of ECs coincided with the direction of flow when exposed to atheroprotective flow (angle relative to flow_H11005/8.6/H11006/4.01°; Fig. 2C), whereas no preferential polarity of ECs under atheroprone flow could be measured due to the rounded morphology.

SMCs on the Transwell exposed to atheroprone flow showed a significant but small increase in elongation (SF_H11005/0.26/H11006/0.009) than those exposed to atheroprotective flow (SF_H11005/0.31/H11006/0.018; Fig. 2A and B). Interestingly, SMCs in atheroprotective flow consistently aligned more toward a perpendicular orientation relative to flow (Fig. 2C and D), whereas, in contrast, SMCs under atheroprone flow could be measured due to the rounded morphology.

Purity of RNA and protein isolation from ECs/SMCs following hemodynamic flow. The purity of collected RNA and protein from each cell layer following the flow experiment was assessed by real-time RT-PCR and Western blot analysis for the presence of EC- and SMC-specific proteins (eNOS and SMαA, respectively; Fig. 3). No cross-contamination at the mRNA or protein level was detectable.

Atheroprone flow differentially regulates EC and SMC phenotypes and promotes proinflammatory priming. The major goal of this study was to determine whether differential human-derived hemodynamic flow patterns applied to ECs influence SMC phenotypic modulation. Given this objective, changes in established markers indicating EC and SMC phenotypic modulation were examined 24 h after the application of atheroprone or atheroprotective flow. Genes of interest were classified as EC- or SMC-specific cell markers (EC: eNOS, Tie2, and KLF2/KLF4; SMC: SMαA, SMMHC, and myocardin) or inflammatory markers (VCAM-1, IL-8, and MCP-1). Additionally, protein analysis was performed on a subset of markers (eNOS, SMαA, VCAM-1, and PCNA). Modulation of genes and proteins was determined by the relative change in atheroprone compared with atheroprotective flow.

Consistently, significant reductions in mRNA levels of EC quiescent markers eNOS, Tie2, and KLF2/KLF4; SMC: SMαA, SMMHC, and myocardin) or inflammatory markers (VCAM-1, IL-8, and MCP-1). Additionally, protein analysis was performed on a subset of markers (eNOS, SMαA, VCAM-1, and PCNA). Modulation of genes and proteins was determined by the relative change in atheroprone compared with atheroprotective flow.

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Protein analysis further confirmed this observation for SmoA (Fig. 4C). Although the transcription factor KLF4, which was recently discovered to be important in suppressing myocardin-dependent transcription, was not significantly induced \( (P = 0.10) \) for atheroprotective relative to atheroprotective flow, this trend may still point toward a mechanism of regulating SMC phenotypic switching (30, 33). Since vascular injury maximally induced KLF4 after just 4 h, it is possible that at 24 h of flow, the maximal response of KLF4 was missed (27). Notably, SMMHC was not significantly modulated \( (P = 0.62) \).

Most interesting was that the reduction in EC quiescent markers and SMC contractile markers corresponded with the upregulation of several proinflammatory genes. VCAM-1 was significantly upregulated in both ECs and SMCs at both the mRNA and protein level (Fig. 4, B and C) (12). A significant increase in IL-8, a proinflammatory gene downstream of the NF-κB activation (13), was also observed in ECs at the mRNA level. Secretion of IL-8 from EC and SMC layers was further measured as a function of time during the application of both flow patterns and was only significantly augmented in ECs during later time points of atheroprone flow (Fig. 4D). In contrast, decreases in IL-8 and MCP-1 were concurrently observed in SMCs (Fig. 4B). Finally, analysis of the proliferative marker PCNA showed increased protein levels in ECs exposed to atheroprotective flow but no change for SMCs (Fig. 4C).

To control for a flow-induced EC influence on the SMC response, SMCs were plated under two conditions in monolayer: 1) on the bottom of the Transwell holding dish in the presence of a Transwell membrane (SMC D) or 2) on the bottom of the Transwell membrane (SMC T), as shown in Fig. 5A. For each condition, flow was applied to the top of the Transwell membrane without ECs. Real-time RT-PCR analysis of samples showed that significant differences existed between each condition for SmoA and VCAM-1 but not for myocardin (Fig. 5A). VCAM-1 was the only gene appreciably induced by atheroprotective flow for both conditions. Potential confounding factors introduced for the SMC T condition were smooth muscle cellular processes that extruded through the porous membrane to the top of the Transwell where flow was being applied (Fig. 5B), which was not observed in the experiments with ECs present. The significant changes between each condition (SMC D vs. SMC T) indicate the sensitivity of SMCs to their local environment. Thus, for this study, comparison between the two distinct flow patterns applied in the presence of both cell types was the most robust method to control for all features (e.g., media exchange, experimental setup, time in culture, and heterocellular presence) of the hemodynamic coculture environment.

Articular hemodynamics control epigenic regulation of SMC gene expression. Many of the promoter regions of genes that encode SMC-selective contractile proteins contain CArG cis-regulatory elements that bind SRF, including SmoA and SMMHC (33). ChIP experiments were conducted to determine whether SRF binding and histone H4 acetylation in 5′-CArG promoter regions of the SmoA, SMMHC, and c-fos promoters were regulated at the epigenic level by hemodynamic flow. The results indicated a reduction of histone H4 acetylation and SRF binding in response to atheroprotective flow relative to atheroprotective flow for SmoA and SMMHC (Fig. 6). Conversely, histone H4 acetylation and SRF binding to the c-fos CarG region was not statistically different among flow conditions (Fig. 6). This epigenetic fingerprint was identical to in vitro experiments in SMCs in response to PDGF-BB and in vivo in response to acute vascular injury (30).
DISCUSSION

Atherosclerosis preferentially develops at arterial regions, such as bifurcations and regions of high curvature, characterized by disturbed, low time averaged and oscillatory wall shear stress. Atheroprone regions in vivo and atheroprone shear stress on the endothelium in vitro can induce proinflammatory priming indicated by the activation and regulation of downstream inflammatory targets (13, 18). Although ECs and SMCs

Fig. 4. Atheroprone and atheroprotective hemodynamics regulate EC and SMC phenotypes. Real-time RT-PCR was performed to determine the relative changes in the expression of genes associated with the EC quiescent/anti-inflammatory state and SMC differentiation (A) and the early inflammatory response (B). KLF, Kruppel-like factor; SMMHC, smooth muscle myosin heavy chain; Myoc, myocardin; MCP, monocyte chemoattractant protein. Expression was normalized to β2-microglobulin. Values are means ± SE; n = 6. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. C, top: Western blot analysis on EC and SMC samples showed changes in protein levels. Bottom, integrated density values were computed and reported as atheroprone relative to atheroprotective flow. Protein was loaded by equivalent mass. Values are means ± SE; n = 4. *P < 0.05; **P < 0.01. D: ELISA analysis for IL-8 was performed on atheroprone and atheroprotective flow-conditioned media. Left, EC layer; right, SMC layer. Values are means ± SE; n = 3. *P < 0.05.
are two major cell types known to undergo phenotypic modulation, or “switching,” during initiating atherosclerotic events. These results are consistent with previously published atherosclerosis-related in vivo and in vitro flow studies (Supplemental Table 1). Moreover, previous Transwell cocultured models of ECs and SMCs have been restricted to static-type experiments, with the exception of a few flow studies (9, 10, 21), and no studies have employed physiologically relevant, human-derived hemodynamic flow patterns. The present study overcomes these limitations of previous studies by directly comparing two hemodynamic flow patterns, yielding a more physiologically relevant model for accurately comparing in vivo regions in the vasculature, and focused on classic SMC differentiation markers.

A hallmark of SMC phenotypic modulation in vascular disease is altered expression of genes that define the contractile phenotype (33). Here, we show that SMC differentiation markers and transcription factors that are delineators of a differentiated SMC are affected by atheroprone flow (Fig. 4). The loss of expression of differentiation markers (SMαA and myocardin) and induction of the inflammatory marker VCAM-1 at both mRNA and protein levels confirmed that ECs exposed to atheroprone flow differentially regulate the SMC phenotype compared with atheroprotective flow. ChIP analysis revealed that the mechanism initiating atheroprone-induced loss of CArG-dependent SMC gene expression involved reduction of SRF binding to CArG box regions of SMαA and SMMHC and deacetylation of histone H4 compared with atheroprotective flow. This was not the case for the early growth response gene c-fos. These results are consistent with a monoculture SMC study (30) in response to PDGF-BB treatment and, most importantly, the epigenetic fingerprint for SMαA, SMMHC, and c-fos in intact blood vessels in response to acute vascular injury. Thus, the general paradigm that histone H4 acetylation is critical for maintaining CArG chromatin promoter regions in a SRF-accessible state is differentially regulated by two distinct hemodynamic flow patterns exposed to ECs. The SRF coactivator myocardin plays a critical role in forming a higher-

![chart]

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Fig. 5. The monoculture SMC response to hemodynamics is position dependent. A: SMCs were plated under two separate conditions in the absence of ECs: SMC D, where cells are only on the bottom of the dish, and SMC T, where cells are only on the bottom of the Transwell, as indicated. For each condition, atheroprone and atheroprotective flow patterns were applied to the inner surface of the Transwell membrane and mRNA analysis examined changes in gene expression. Values are means ± SE; n = 4. *P < 0.05. B: SMCs were plated on the bottom of a Transwell membrane in the absence of ECs. Scanning electron microscopy was used to analyze both surfaces of the membrane for the presence of SMCs. Images show SMCs on the top and bottom of the Transwell at low (left) and high magnification (right). Note cellular processes on the inner surface of the membrane where no cells were plated (top). n = 2.

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are two major cell types known to undergo phenotypic modulation, or “switching,” during initiating atherosclerotic events, until this study it was unknown whether hemodynamic forces on ECs regulated or contributed to this process in SMCs. Here, we show that human-derived atheroprone shear stresses applied to ECs modulate a proinflammatory phenotype in ECs and SMCs and proatherogenic phenotypic switching in SMCs via epigenetic modifications at the chromatin level. This is a process we refer to as mechanotranscriptional coupling.

Results from the present coculture model support the hypothesis that hemodynamics induce vascular EC and SMC priming toward a proatherogenic response, thus validating the use of the coculture system as a new physiologically relevant biomimetic vascular model for the study of early atherosclerotic events. These results are consistent with previously published atherosclerosis-related in vivo and in vitro flow studies (Supplemental Table 1). Moreover, previous Transwell cocultured models of ECs and SMCs have been restricted to static-type experiments, with the exception of a few flow studies (9, 10, 21), and no studies have employed physiologically relevant, human-derived hemodynamic flow patterns. The present study overcomes these limitations of previous studies by directly comparing two hemodynamic flow patterns, yielding a more physiologically relevant model for accurately comparing in vivo regions in the vasculature, and focused on classic SMC differentiation markers.

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Fig. 6. Atheroprone flow exhibited decreased chromatin binding of serum response factor (SRF) and histone H4 acetylation (H4Ac) in SMC genes. Chromatin immunoprecipitation analysis was performed on six pooled SMC samples following 24 h of flow experiments. SRF enrichment and H4 acetylation to 5'-CC(T/A)6GG boxes for SMαA, SMMHC, and c-fos are shown as atheroprone relative to atheroprotective and were normalized to fold enrichment over equivalent amounts of input DNA. Values are means ± SE; n = 2. +P < 0.10; *P < 0.05.
order complex with SRF for the positive regulation SMC-selective CarG-dependent genes (7). In contrast, KLF4 can abrogate myocardin-dependent regulation of CarG-dependent SMC differentiation genes (23, 27). Myocardin expression was significantly reduced in response to atheroprone flow, whereas KLF4 tended to have increased expression. Since KLF4 gene expression can be rapidly and transiently induced in response to PDGF-BB in cultured SMCs and transiently induced in intact vessels following acute vascular injury up to 6 h and returning to baseline by 24 h (28), it is possible that the maximal and most significant changes in KLF4 expression were not captured at this time point. Nevertheless, gene profiles generated in this study correlate with existing data from the literature, and, taken together, the results suggest that phenotypic modulation of SMCs exposed to atheroprone flow occurs at the transcriptional level and involves the well-characterized SRF/myocardin and KLF4 signaling axis (30, 33).

Of interest, ECs exhibited reduced KLF4 expression in atheroprone flow. KLF4 has been shown to be regulated by flow in ECs in monoculture (19, 29); however, until this study, it was not known that KLF4 is differentially expressed by atheroprone flow compared with atheroprotective flow. The functional significance of KLF4 in ECs has recently been shown to be similar to that of KLF2 (i.e., anti-inflammatory, atheroprotective, and hemostasis control) (19). Moreover, KLF4 has been implicated in cell cycle regulation (40), and greater cell cycle activity has been reported for atheroprone relevant flow in vitro and regions in vivo (2, 31). Thus, the regulation of KLF4 transcription may serve an equally vital role in regulating vascular EC and SMC proliferation. Furthermore, while myocardin has been shown to decrease with acute, mechanical vascular injury (20) and KLF4 increases (27), this is the first evidence that these transcription factors are differentially regulated in a model that mimics early atherogenic events. Regulation in vivo in atherosclerosis is currently unknown.

Surprisingly, SMMHC was the only SMC marker that did not follow the expected modulation trends. This may be due to RT-PCR primer recognition of both SMMHC isoforms (SM-1 and SM-2). Analysis of each isoform separately may elucidate a response consistent with the other SMC markers. Analysis at later time points (i.e., 48 h) may resolve this. The combined phenotypic responses of both ECs and SMCs in the presence of atheroprone flow are strikingly similar to historical EC and SMC phenotype profiles defined in human and experimental models of atherosclerosis (Supplemental Table 1).

Evaluation of EC gene expression in response to atheroprone relative to atheroprotective flow is consistent with the only EC monoculture study using similar flow profiles as well as studies using similar magnitudes of steady shear stress and in vivo models of atherosclerosis, emphasizing that hemodynamics more robustly regulate the EC phenotype than the presence of SMCs (13, 34, 36). ECs exposed to 24 h of atheroprone flow induced higher levels of proatherogenic and proliferative genes and proteins for IL-8, VCAM-1, and PCNA commensurate with reductions in eNOS, Tie2, and KLF2. The expression loss of eNOS and Tie2 suggests higher rates of remodeling and increased permeability, characteristic features of atherosusceptible regions in vivo. Evidence has established the role of KLF2, and possibly KLF4, as an upstream transcriptional regulator of atheroprotection (19, 26). Atheroprotective hemo-

dynamics in vitro and regions in vivo appear to be a key modulator of KLF2 expression and transcriptional control (15, 34, 36). SMCs also exhibited an early inflammatory response to atheroprone flow, as indicated by increased VCAM-1 mRNA levels. VCAM-1 modulation has been observed in SMCs of human atherosclerotic plaques (3, 4) and has been linked to proliferation during early atherogenesis in vitro and in vivo (5, 25). However, since the proliferative marker PCNA showed no change in SMCs for atheroprone flow, it is possible that a more migratory SMC phenotype is present in this system.

The EC-secreted cytokine(s)/mitogen(s) that regulates SMC phenotypic modulation during early atherogenesis has yet to be elucidated and includes candidates such as PDGF-BB, IL-1, and IL-8. Here, we show ECs increase IL-8 mRNA production and IL-8 secretion following atheroprotection flow (13). Indeed, IL-8 can stimulate the induction of a migratory phenotype in SMCs (41). Therefore, IL-8 secretion by ECs may be one mechanism by which SMCs regulate a more synthetic phenotype. Of interest, a recent study (8) in apolipoprotein E−/− mice showed that experimentally induced low shear stress resulted in an increase in growth-related protein (Gro)-α mRNA. However, given the in vivo nature of this study, it was not determined whether changes in Gro-α mRNA were in ECs, SMCs, or both. Although Gro-α binds the same receptors as IL-8, no murine homolog of IL-8 exists (32). The human coculture model is therefore ideal for examining the role of EC-derived IL-8 on SMCs, and future studies are ongoing to establish the relative contributions of such cross-communication mechanisms.

Cell morphology changes observed in atheroprone versus atheroprotective flow were also signs of early remodeling that could lead to localized downstream atherogenic responses. ECs are known to reorient in the direction of flow under pulsatile physiologic conditions and maintain a more polygonal shape after exposure to disturbed flow (14), as observed in our system. However, our understanding of SMC reorientation due to shear stress sensed by the endothelium is in its earliest stages. SMCs orient more perpendicular to hemodynamic flow under the atheroprotective waveform, whereas SMCs exposed to atheroprone flow resulted in more random alignment. Importantly, this SMC orientation is nearly identical to the spatial patterning of SMCs in an intact blood vessel at bifurcating regions (37), regions highly susceptible to atherosclerosis. Together, this suggests that hemodynamic flow can regulate both EC and SMC orientation by unique control mechanisms inherent to distinct atheroprone or atheroprotective flow patterns.

In conclusion, we present a novel in vitro coculture model using human ECs and SMCs that shows that human hemodynamic forces, atheroprotective or atheroprole, applied directly to the endothelium can modulate the SMC phenotype and influence SMC remodeling, a process we defined as mechano-transcriptional coupling. Moreover, the snapshot of phenotypic and morphologic alterations in ECs and SMCs indicates that hemodynamic forces on the endothelium are an important modulator of atherogenesis.

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