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Cyclic tensile strain triggers a sequence of autocrine and paracrine signaling to regulate angiogenic sprouting in human vascular cells

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Mechanical signals regulate blood vessel development in vivo, and have been demonstrated to regulate signal transduction of endothelial cell (EC) and smooth muscle cell (SMC) phenotype in vitro. However, it is unclear how the complex process of angiogenesis, which involves multiple cell types and growth factors that act in a spatiotemporally regulated manner, is triggered by a mechanical input. Here, we describe a mechanism for modulating vascular cells during sequential stages of an in vitro model of early angiogenesis by applying cyclic tensile strain. Cyclic strain of human umbilical vein (HUV)ECs up-regulated the secretion of angiopoietin (Ang)-2 and PDGF-ββ, and enhanced endothelial migration and sprout formation, whereas effects were eliminated with shRNA knockdown of endogenous Ang-2. Applying strain to colonies of HUVEC, cocultured on the same micropatterned substrate with nonstrained human aortic (HA)SMCs, led to a directed migration of the HASMC toward migrating HUVECs, with diminished recruitment when PDGF receptors were neutralized. These results demonstrate that a singular mechanical cue (cyclic tensile strain) can trigger a cascade of autocrine and paracrine signaling events between ECs and SMCs critical to the angiogenic process.

Angiogenesis requires an orchestrated series of cell activities in a specific spatial and temporal sequence (1). Significant research in this field has focused on documenting cell response to exogenous biochemical cues, and a variety of growth factors have been identified to have key roles in angiogenesis (2). Among these biochemical cues, VEGF has been identified as a potent factor during the early stages of angiogenesis, activating migration, and sprout formation (3). Angiopoietin (Ang)-1, a cytokine that mediates the interactions between endothelial cells (ECs) and smooth muscle cells (SMCs), and Ang-2, an early angiogenic factor that inactively acts to disrupt and dissociate these bonds, are ligands expressed by vascular cells that competitively bind to the membrane receptor Tie-2, and act synergistically with VEGF to regulate angiogenesis (4). PDGF-ββ, a chemotactant released by ECs, is a late stage cytokine that recruits SMCs to stabilize the nascent EC sprouts (5).

Understanding of the effects of these soluble factors alone is, however, likely insufficient to fully understand the angiogenic process. Physiologically, ECs and SMCs are exposed to cyclic tensile strain resulting from blood hemodynamic forces, a cue important to vessel adaptation (6). Mechanical signals regulate the function of both cell types in vitro, including the alteration of EC proliferation (7, 8), alignment (9, 10), migration (11, 12), and in vitro sprout formations (13, 14), likely through activating various intracellular signaling pathways (15–21). Similarly, physiologically relevant hemodynamic forces have demonstrated to modulate SM phenotype (22), migration (23), and intracellular signaling (24). Altogether, these past studies suggest that the angiogenic process is governed by an interplay between chemical and mechanical signals.

We hypothesized that cyclic tensile strain may provide a singular cue to regulate vascular cells by triggering EC secretion of angiogenic factors that mediate multiple stages in the angiogenic process. Human umbilical vein (HUV)ECs and human aortic (HA)SMCs were used here as model cell types representing the vascular endothelium and stabilizing supportive layer, respectively. Vascular cells were cultured in 2D directly on elastomeric poly(dimethylsiloxane) (PDMS) substrates and in fibrin 3D cultures. In vitro sprouting was used as a model for the initial stages of angiogenesis, as previously described (25–28). Vascular cell cultures were exposed to cyclic tensile strain with an amplitude of 7%, representative of that experienced by vascular cells in large vessels, and also in the same range as previous calculations of capillary wall strain (29, 30). Cyclic tensile strain was demonstrated to alter vascular cell phenotype and the secretion of Ang-2 and PDGF by EC. Altered Ang-2 secretion mediated changes in early angiogenic processes, EC migration, and in vitro capillary formation, whereas strain-enhanced secretion of PDGF provided a directional cue for SMC recruitment toward EC colonies.

Results

Cyclic Tensile Strain Regulation of Vascular Cell Phenotype, Angiogenic Factor Secretion, and Migration. The previously reported effects of cyclic strain on EC migration and in vitro sprout formation were first confirmed. Cyclic tensile strain (7%, 1 Hz) enhanced, by 1.6-fold, the directional migration of HUVECs in 2D culture (Fig. S1A), as expected (31). Two days of cyclic tensile strain also enhanced sprout formation by HUVECs in fibrin gels by 4-fold, compared with static culture (Fig. S1B), again in agreement with earlier investigations (13). Addition of recombinant human VEGF-165, a known stimulant to capillary formation (3, 5), also increased sprout formation both under static and strained conditions (Fig. S1B), confirming the expected biological responsiveness of the cells used in these studies. The effect of varying the magnitude of cyclic tensile strain was first assessed by analyzing the fraction of cells that migrated out from the original cell culture region, the average cell velocity, and directionality of migratory cells. Cyclic tensile strain resulted in an increase in the fraction of HUVECs that migrated out of the original cell culture region by 2-fold when an amplitude of 6% cyclic strain

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was applied, whereas at an amplitude of 13% cyclic strain, the fraction of migrating cells was enhanced 4-fold (Fig. S1C). The average velocity of HUVEC migration at 13% cyclic strain was 15-fold higher, as compared with the static condition (Fig. S1D), resulting in an average velocity of 15 μm/h. Further, the migration of HUVECs exhibited a clear directionality, perpendicular to the direction of strain application (Fig. S1E). Similarly, 2 days of cyclic tensile strain led to a 3-fold enhancement in the number of migrating HASMCs at 13% strain, as compared with the nonstrained condition, but little effect was noted at 6% strain amplitude (Fig. S1F). The average velocity of migrating HASMCs, at 13% strain, was 6-fold higher than cells in static culture, but was notably slower than HUVEC migration rates (Fig. S1G). Strikingly, and in contrast to HUVECs, whereas HASMCs migration was increased with cyclic strain, the cells did not demonstrate directional migration under these conditions (Fig. S1H).

To examine whether cyclic strain up-regulated the expression of genes involved in angiogenesis, the levels of angiogenic proteins secreted by the vascular cells were quantified over a 5-day time-course of cyclic strain. Cyclic strain of HUVECs led to a 4.8-fold up-regulation of Ang-2 (Fig. 1A), and a 5-fold up-regulation in the secretion of PDGF-β (Fig. 1B). In contrast, cyclic strain of HASMCs resulted only in a slight enhancement...
of Ang-1 (Fig. 1C), whereas secretion of VEGF did not appear to be affected by strain (Fig. 1D). In both strained and nonstrained conditions, the secretion of PDGF-ββ and Ang-2 by HASMC, and Ang-1 and VEGF by HUVECs, respectively, was minimal. The time course of up-regulation of PDGF-ββ and Ang-2 secretion by HUVECs was next investigated. Ang-2 expression was increased 3-fold by strain at day 1, and then slowly subsided over the ongoing 13 days to control levels (Fig. 1E). PDGF-ββ secretion, in contrast, did not rise until 2 days of cyclic stretch, and then quickly returned to baseline control levels (Fig. 1F). Because minimal effects of cyclic strain on angiogenic factor secretion by HASMC were noted, all subsequent studies of factor secretion focused on HUVECs. The gene expression levels of Ang-2, and its receptor (Tie-2) in HUVECs, was analyzed using real-time RT-PCR, and application of cyclic strain resulted in 1.5- and 2-fold increases in mRNA levels for Ang-2 and Tie-2, respectively (Fig. 1G). Last, cyclic tensile strain was also found to up-regulate surface availability of PDGF-ββ receptors (R) on HASMCs (Fig. 1H).

**Role of Ang-2 in EC Migration and Sprout Formation.** To determine whether the levels of altered angiogenic factors resulting from cyclic strain were capable of altering EC phenotype, HUVECs in fibrin gels were exposed to exogenous recombinant human Ang-2, Ang-1, PDGF, and VEGF, at levels corresponding to those produced by cells under strained conditions. Ang-2 and VEGF enhanced the formation of sprouts (Fig. S2 A and B), whereas PDGF-ββ (Fig. S2C) and Ang-1 (Fig. S2D) had no discernible effects. The effects of these factors on HUVEC migration across porous transwell membranes was next examined, and VEGF was found to enhance HUVEC migration (Fig. S2E), concurring with previous descriptions of the effects of this cytokine (32). Ang-2 similarly enhanced HUVEC migration, whereas Ang-1 had no effect (Fig. S2E). Altogether, these data suggests new roles for Ang-2 in EC biology, and indicate the increase in Ang-2 expression in ECs that result from cyclic strain is sufficient to increase the angiogenic activity of these cells.

To test whether cyclic strain-induced up-regulation of Ang-2 was causative for the strain induced increase in EC migration and sprouting, RNAi was used to knockdown the endogenous expression of Ang-2 in HUVECs. HUVECs were transfected with a plasmid that was designed and constructed to release a 63-mer shRNA that binds specifically to the intracellular mRNA of Ang-2 and blocks the translation of this protein. Examination of Ang-2 secretion by cells positively transfected with shRNA (Ang-2) confirmed a dramatic inhibition of Ang-2 expression for 4 days after treatment (Fig. 2A). The baseline (no cyclic strain) migration of ECs with shRNA (Ang-2), was decreased by ~1.6-fold (Fig. 2B), whereas cells subjected to strain exhibited a 2-fold decrease in migration with shRNA treatment (Fig. 2C). Inhibiting Ang-2 also resulted in a 2.2-fold decrease in sprouting with exposure to cyclic strain, in both the absence and presence of exogenous VEGF in the culture medium (Fig. 2D).

**Cyclic Strain Directs SMC Recruitment Toward Migrating ECs.** The next series of studies examined whether strain induced change in EC cytokine expression could alter HASMC function, and in particular whether PDGF-ββ could serve to recruit SMCs to forming vessels. In the first experiment, HASMCs were exposed to increasing doses of exogenous recombinant human PDGF-ββ, and migration was found to increase with PDGF-ββ concentration (Fig. 3A). Next, conditioned media was taken from strained ECs and added to HASMC culture, and this enhanced HASMC migration by ~2-fold, as compared with conditioned media taken from cells under nonstrained cultures. To determine whether the enhanced SM migration was a direct result of the strain-mediated increased PDGF-ββ levels, we neutralized PDGF-R on HASMCs and reassessed their response to conditioned media taken from strained ECs. Neutralizing PDGF-Rs decreased migration by 45% (Fig. 3B). Next, to determine
whether PDGF-ββ has the ability to direct HASMC migration over significant distances, a depot of either recombinant human (rh)PDGF or a depot of conditioned media from strained cells was placed 2,000 μm away from patterned cultures of HASMC. The migration of HASMCs toward both depots increased by 2- and 2.25-fold, respectively (Fig. 3C), as compared a blank depot with no stimulants.

Finally, the ability of cyclic strain of ECs to induce not only directed EC migration, but also recruitment of nonstrained SMCs was assessed. Micropatterned cultures were developed (Fig. 4A), in which ECs were subjected to cyclic strain or maintained under static culture, whereas adjacent colonies of HASMC were subjected to minimal strain. Culture of SMCs adjacent to nonstrained ECs led to a 2-fold increase in SMC migration (Fig. 4B). Strikingly, when SMCs were cocultured adjacent to strained HUVECs, the migration of HASMCs was enhanced by 5-fold (Fig. 4B), as compared with a negative control (SMCs with no coculture, no strain). There was no notable directionality of HASMC migration when cultured adjacent to nonstrained HUVECs colonies. However, strained HUVECs not only increased HASMC migration, but also provided cues to direct the migration of HASMCs toward the migrating HUVEC colony (2-fold increase in directional migration) (Fig. 4 C and D).

Discussion

The results of these studies demonstrate that cyclic strain can activate endogenous biochemical cues to direct vascular cells through sequential stages important to the angiogenic process. The angiogenic phenotype of ECs, characterized here by directed cell migration and in vitro sprout formation, was enhanced by recombinant Ang-2 and in response to cyclic uniaxial strain. Cyclic strain increased the early expression of both Ang-2 and its receptor, Tie-2, in ECs, and this increased Ang-2 expression was found to mediate the cyclic strain induced alterations in EC angiogenic phenotype. Cyclic strain enhanced, at a later time point than Ang-2, the secretion of PDGF-ββ by ECs, and the PDGF-ββ modulated the directional migration of HASMC toward EC colonies (Fig. S3). Although past studies have largely focused on vascular remodeling of precapillary structures (e.g., arterioles) in response to altered load, these results suggest that the capillaries themselves may also undergo significant remodeling in response to mechanical cues. Breakdown or structural damage to capillary walls resulting from disease may provide one situation in which vascular cells experience altered loading (33); exercise may also provide this type of stimulus, and previous studies have noted alterations in capillary densities with exercise (34). Previously calculated strain levels in capillary walls during systolic contraction and stable diastolic arrest range between 0% to 22% (30, 35), supporting the physiologic relevance of the 7% strain amplitude used in our studies (29). Other investigations have used similar strain levels to study the effects of mechanical cue effects on ECs in vitro, specifically in the context of angiogenesis (36–38).

Cyclic strain up-regulated EC secretion of angiogenic cytokines, specifically, PDGF-ββ and Ang-2. Previous studies reported that cyclic strain enhanced the expression of PDGF-R (12, 39), and shear stress enhanced gene expression of PDGF-ββ (40, 41) and Tie-2 (42). However, up-regulation of PDGF-ββ and Ang-2 in response to cyclic strain has, to our knowledge, not been previously documented. Interestingly, VEGF, a potent factor in angiogenic activation, was not affected by strain, although regulation of this cytokine is governed by other local cues (43). The temporal profile of increased angiogenic cytokine secretion by ECs in response to cyclic strain was striking, because Ang-2, a factor important to the initiation of angiogenesis (44), was up-regulated early, followed by later expression of PDGF-ββ, which plays important roles in later stages of angiogenesis (44, 45). These data suggest that cyclic strain modulates angiogenesis by altering the balance of angiogenic factors, and by temporally mediating the up-regulation of factors driving activation versus those promoting subsequent vessel stabilization.

Ang-2 was found in this study, even in the absence of mechanical stimulation, to enhance sprout formation and migration of ECs. The role of angiopoietins in vascular development has been the subject of active investigation, and until

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)
recently, it was believed that Ang-1 had solely a stabilizing role via activation of the tyrosine kinase receptor Tie-2 (46), whereas Ang-2, the antagonist to Ang-1, was believed to have more of a facilitative role (47). For example, expression of Ang-2 was identified primarily at sites of active vessel remodeling (47–50). However, recent studies demonstrate that there may exist a contextual role to the functions of Ang-2, because it serves in some instances to inhibit vascular leakage (51), whereas in other situations, it may function as a proinflammatory cytokine (52). Increased secretion of Ang-2 in response to biochemical stimuli has also been documented, supporting the suggestion (53) that Ang-2 function is more complex than initially identified (54). Although VEGF-A and angiopoietins have distinct roles in vascular development, they also have complementary and coordinated roles. VEGF-A has been shown to modulate migration (32) and in vitro capillary formation (55) of ECs, and Ang-2, at levels secreted in response to cyclic strain signals, appears to have similar effects on ECs. Although the molecular mechanisms linking cyclic strain to Ang-2 expression are not clear, they likely involve the various intracellular signaling pathways previously documented to mediate mechanical effects on ECs (16, 56) that induce local differentiation and the formation of nascent blood vessels.

Cyclic tensile strain was found in this study and by others (57) to enhance the expression of PDGF-Rβ on HASMCs. A resulting enhanced responsiveness to PDGF signaling via the tyrosine kinase pathway (activated by ligand binding to PDGF-R) is likely involved in the HASMC migration in response to cyclic strain. Cyclic strain has been previously demonstrated to modulate ERK1/2 (58, 59), PI3K (60), p21 (19, 61), tyrosine kinase (59, 62), and RhoA signaling. Cyclic strain also likely enhances the expression of other receptors as well, all which may cooperatively regulate the HASMC responses to cyclic strain, including direct and indirect responses (e.g., secondary to EC-induced alterations in gene expression resulting from cyclic strain). The finding that HASMCs cultured under static conditions can be actively and directionally recruited to strain-stimulated ECs supports the potential physiologic relevance of these findings.

The sequential regulation by cyclic tensile strain of early and late stages of vascular remodeling represents a previously unrecognized and potentially critical role for mechanical signaling in angiogenesis. Reciprocal signaling of EC-induced SMC recruitment via PDGF has been examined in vivo (63, 64), but not with events activated by external mechanical stimuli. More broadly, the findings of this study provide a specific example of how localized mechanical signals can be translated into biochemical cues (65) capable of signaling over physiologic relevant distances. This coupled mechanism may provide multiple points to intervene and regulate the angiogenic process, and may also improve the current understanding of various vascular diseases.

**Materials and Methods**

For cell culture, HUVECs (Cambrex) and human HASMCs (Cambrex) were cultured at 37 °C, 5% CO2 in endothelial growth medium (EGM)-2 and smooth muscle cell growth medium (SmGm)-2, respectively (Cambrex), containing 2% FBS. HUVECs were used between passages 3 and 6, and HASMCs were used between passages 3 and 7. Cocultures of HUVEGs and HASMCs were maintained in culture medium constituted of 1:1, EGM-2 and SmGm-2.

Detailed information on quantification of migration in response to chemotactic gradients, quantification of migration in response to cyclic strain, in vitro angiogenesis, sprouting assay, quantification of angiogenic protein secretion, real-time RT-PCR, vector construction and synthesis, plasmid transfection and FACS, creation of an array of isolated cell cultures, and quantification of Vascular Cell Migration in response to cyclic tensile strain are included in the SI Materials and Methods section.

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