Endothelial cell traction and ECM density influence both capillary morphogenesis and maintenance in 3-D

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Kniazeva E, Putnam AJ. Endothelial cell traction and ECM density influence both capillary morphogenesis and maintenance in 3-D. Am J Physiol Cell Physiol 297: C179–C187, 2009. First published May 13, 2009; doi:10.1152/ajpcell.00018.2009.—Identifying the mechanisms regulating angiogenesis in pathological conditions such as cancer and heart disease is crucial to develop successful therapies. The dependence of angiogenesis on characteristic properties of these conditions, such as alterations in tissue stiffness due to changes in the composition of the extracellular matrix (ECM), may shed light on potential therapeutic strategies. Prior studies have suggested that ECM compliance regulates capillary morphogenesis, but the mechanisms remain unclear. In this study, we hypothesized that ECM density, which influences substrate mechanics, may regulate angiogenesis via a mechanism involving actin-mediated cell-generated forces. To investigate this hypothesis, we utilized an in vitro model of angiogenesis in which endothelial cells grown on microcarrier beads are distributed within a three-dimensional (3-D) fibrin ECM. A monolayer of fibroblasts, which provides pro-angiogenic factors, is cultured on top of the gel. Variations in fibrin gel density, along with a library of pharmacological agents that inhibit forces generated by the actin cytoskeleton, were used to probe the necessity of cell-generated tractional forces in blood vessel formation. Our data demonstrate that cell-generated forces not only play a crucial role in the early sprouting stages of capillary morphogenesis but are also required in the later maintenance stages, and thereby suggest a broader interdependence among tissue stiffness, cell contractile forces, and angiogenesis.

Angiogenesis refers to the process by which new blood vessels sprout from preexisting vessel networks. Whereas it is typically involved in normal physiological growth and development, angiogenesis is also a hallmark of numerous diseases when it is dysregulated. Excessive angiogenesis is characteristic of many forms of cancer, as well as psoriasis, blindness, and arthritis, whereas insufficient vessel growth and abnormal vessel regression can result in heart and brain ischemia, neurodegeneration, hypertension, osteoporosis, and preeclampsia (2). A feature common to all of these various pathologies is an alteration in the composition of the tissue, which affects the mechanical properties of the microenvironment and may in turn affect the formation of new blood vessels by up- or downregulating angiogenesis (28).

It has been previously demonstrated that extracellular matrix (ECM) compliance influences cell-generated contractile forces in two-dimensional (2-D) cell cultures (17, 23). A molecular pathway for actin-mediated contractility is of special interest as one of its upstream effectors RhoA, a small GTPase involved in the regulation of cell-generated tractional forces, has been implicated as an important link between ECM compliance and cell function in 2-D (27). RhoA and its associated signaling partners (1, 11) have also been implicated in capillary morphogenesis. This study therefore aims to determine whether cell-generated contractile forces play a significant role in the mediation of capillary morphogenesis in tissues of varied ECM density.

To study angiogenesis in vitro, we adopted a model first developed by Nehls and Drenckhahn (21) where endothelial cells (ECs) are grown on microcarrier beads within a fibrin matrix. Fibrin is one of the major structural proteins involved in the provisional matrix during wound healing and is formed by enzymatic polymerization of fibrinogen (29). As such, it can serve as a suitable substrate for an angiogenesis model and has been previously shown to be an excellent candidate for cardiovascular grafts due in part to its ability to be enzymatically degraded and to support new tissue development (33). Plating a monolayer of normal human lung fibroblasts (NHLFs) on the top surface of the gel provides a source of pro-angiogenic factors and allows the entrapped ECs to differentiate and begin a process similar to capillary morphogenesis in vivo. Using this model, here we have explored the impact of ECM density and mechanics on three-dimensional (3-D) morphogenesis through modulation of actin-mediated contractile forces on a molecular level. EC contractile forces were pharmacologically disrupted by applying various inhibitors to tissue constructs fabricated from hydrogels of varied fibrin density. The inhibitors were chosen to specifically bracket a pathway involving the regulation of cell-generated tractional forces to investigate a broad hypothesis that actin-mediated contractility serves a critical mechanosensory function translating changes in ECM mechanics to alterations in cell function (Fig. 1). The consequent impact of this pathway on angiogenesis was evaluated by observing phenotypic differences in capillary morphogenesis and by quantifying total vessel network length in gels of varied fibrin density.

MATERIALS AND METHODS

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords and cultured in fully supplemented endothelial growth medium (EGM-2; Lonza Walkersville, Walkersville, MD) at 37°C, 5% CO2. Normal human lung fibroblasts (NHLFs; ATCC, Manassas, VA) were cultured in Media 199 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Mediatech, Herndon, VA) or in EGM-2 for conditioned media experiments, at 37°C, 5% CO2. Medium was changed three times per week, and cells were harvested at 80% confluency via trypsin-EDTA (Mediatech, Herndon, VA) treatment at passage three for HUVECs and passage eight for NHLFs.
Fibrin tissue assembly. Fibrin-based tissue constructs were assembled as previously described (6). Briefly, Cytodex-3 microcarrier beads (Sigma-Aldrich, St. Louis, MO) (diameter of 150 μm) were sterilized and prepared for seeding by autoclaving in a solution of Dulbecco’s phosphate-buffered saline (Invitrogen, Carlsbad, CA) followed by a series of washes in EGM-2. Four million HUVECs were then added to an inverted T-25 culture flask containing 5 ml of EGM-2 and 10,000 Cytodex beads. The inverted flask was then incubated (37°C, 5% CO2) for 4 h and was gently agitated every 30 min during that time period. After 4 h, the microcarrier beads were transferred to a new T-25 flask in solution along with 5 ml of fresh EGM-2. The flask was then incubated in the standard cell culture position for 24 h, allowing any suspended cells to attach to the beads at the bottom of the flask. After the 24-h period, fibrinogen solutions of varying concentrations (2.5, 5.0, and 10.0 mg/ml) were prepared in EGM-2 without FBS and sterile filtered. To assemble tissue constructs, HUVEC-coated beads (~25 beads/0.5 ml fibrinogen solution) and 5% FBS were added to the fibrinogen solution. The inverted flask was then incubated (37°C, 5% CO2) for 4 h and was gently agitated every 30 min during that time period. After 4 h, the microcarrier beads were transferred to a new T-25 flask in solution along with 5 ml of fresh EGM-2. The flask was then incubated in the standard cell culture position for 24 h, allowing any suspended cells to attach to the beads at the bottom of the flask. After the 24-h period, fibrinogen solutions of varying concentrations (2.5, 5.0, and 10.0 mg/ml) were prepared in EGM-2 without FBS and sterile filtered. To assemble tissue constructs, HUVEC-coated beads (~25 beads/0.5 ml fibrinogen solution) and 5% FBS were added to the fibrinogen solution. In a 24-well plate, 10 μl of a thrombin (Sigma-Aldrich) solution (50 U/ml) and 0.5 ml of the fibrinogen-bead solution were added to each well. The tissue was allowed to stand for 5 min before incubating for 20 min at the previously stated conditions. After the fibrin crosslinked to form a hydrogel, NHLFs were plated (25,000 cells/well) on top of the fibrin gel. Tissues were then cultured in fully supplemented EGM-2 with the media changed three times per week.

Contractility and proteolysis inhibition in regular and conditioned media. To study the effects of HUVEC-generated contractile forces, the following drugs were used and applied fresh with every media change: Y27632 (30 μM; Calbiochem, La Jolla, CA), which inhibits the Rho-associated protein kinase (ROCK) and thus the ROCK-mediated myosin light chain phosphorylation (13); 2,3-butanedione monoximine (BDM, 10 mM; Calbiochem), which inhibits the myosin ATPase downstream of MLC phosphorylation (22); ML-7 (10 μM; Sigma-Aldrich), a potent and selective inhibitor of myosin light chain kinase (MLCK) (26); and blebbistatin (50 μM; Calbiochem), which preferentially binds to the ATPase intermediate with ADP and phosphate bound at the active site and slows down phosphate release (16). Drug concentrations were based on previous literature, as well as dose-response studies performed in our lab. To inhibit proteolysis, either aprotinin (10 μg/ml; Sigma-Aldrich) or GM-6001 (10 μM; Calbiochem) was used and applied fresh with each media change. In conditioned media experiments, the drugs were applied in a 1:1 mixture of fresh EGM-2 and media collected from NHLFs cultured in EGM-2 in a regular T-75 tissue culture flask.

To confirm the general ability of the inhibitors to limit contractile forces at the chosen doses, HUVECs (200,000 cells/gel) were distributed within fibrin gel constructs (rather than coated on beads as for the angiogenesis assays), which were then overlaid with a NHLF (25,000 cells/gel) monolayer. After the cells were allowed to adhere to the constructs for 6 h, the gels were freed from the wells and remained floating for the duration of the study. The gels were subsequently treated with the inhibitors described above. The extent of gel contraction was assessed at every time point by first photographing the gels using a Canon SD450 digital camera (Canon, Lake Success, NY), saving the images as high-resolution files (*.tif), and then determining the diameters of the gels by using ImageJ software (National Institutes of Health, Bethesda, MD). Final plots and statistics were performed using KaleidaGraph software (Synergy Software, Reading, PA) as before. Data were represented as a mean diameter ± SD.

Cytotoxicity assay. At multiple time points, a LIVE/DEAD viability/cytotoxicity assay (Invitrogen) was performed on tissue constructs cultured with and without contractility inhibitors. The commercial kit consists of a two-color fluorescence cell viability assay with two probes that recognize intracellular esterase activity (calcein AM) and plasma membrane integrity (ethidium homodimer) and consequently produce green fluorescence in live cells and red in dead cells. Reagents were used according to manufacturer specifications. Fluorescent images were obtained using a Nikon E800 microscope (Nikon, Melville, NY) immediately upon completion of the assay.
Imaging and quantification of total vessel networks lengths. Tissue constructs were imaged at every medium change time point postassembly using brightfield phase-contrast microscopy (Nikon TE300 microscope; Nikon) and Metamorph software (Universal Imaging/ Molecular Devices, Union City, CA). At least 10 beads were randomly selected per condition and imaged at 4× power for all time points until the experiment was terminated, either due to tissue degradation (described in RESULTS) or the inability to capture an image of the entire network due to its extensive growth. Only isolated beads were imaged to avoid vessels anastomosing with neighboring networks. The images were then saved as high-resolution files (*.tif).

Real-time angiogenesis observation. For direct real-time monitoring of angiogenic progression, tissues were placed in an inverted Nikon TE300 microscope (Nikon) with an environmental chamber at 37°C and 5% CO2. Phase-contrast images were taken starting on day 8 of tissue culture and were acquired every 15 min for the course of up to 72 h with a digital CoolSNAP CCD camera (Roper Scientific, Tucson, AZ). Subsequent image stack assembly and movie compilation were performed with Metamorph software (Universal Imaging/ Molecular Devices).

Statistical analysis. Statistical analyses were performed using KaleidaGraph (Synergy Software). Data were represented as mean total network length ± SD. A one-way analysis of variance (ANOVA) was performed to obtain statistical significance comparisons among data sets. Statistical significance was assumed when \( P < 0.05 \).

RESULTS

Effect of ECM density on capillary network length. Our lab has previously demonstrated that ECM density regulates capillary morphogenesis by significantly reducing the growth of capillary networks in 3-D fibrin gels (7). We have also shown that these changes in fibrin density affect both ECM compliance (6) and diffusive transport (7). The effects of ECM density on capillary morphogenesis were initially confirmed in this study. Time-lapse analysis of capillary morphogenesis in tissue constructs with 2.5, 5.0, and 10.0 mg/ml fibrin concentrations revealed significant inhibition of mean total capillary network lengths with increasing matrix density by day 11 (Fig. 2). These data were then used as a control case to compare the effects of matrix density alone on capillary networks before any modification of cell-generated contractile forces.

Limiting endothelial cell-contractile forces in tissues of varied ECM density. Once the baseline effects of matrix density on capillary network growth were confirmed, actomyosin-generated tractional forces were pharmacologically inhibited at multiple regulatory nodes using Y27632, BDM, blebbistatin, or ML-7 in gels of varied density. For every ECM density tested, mean total capillary network lengths were reduced in gels treated with each of these drugs compared with tissues without (Fig. 3). In 2.5 mg/ml fibrin gels, this inhibitory effect was more pronounced with blebbistatin, ML-7, and BDM, reducing the total network length nearly threefold by the last measured time point. For tissue constructs with 5.0 and 10.0 mg/ml fibrin, ML-7 appeared to have the least effect on total network length. As in the untreated conditions, the increase in ECM density had an inverse effect on total capillary network length in all drug conditions. However, in all gels treated with either Y27632, BDM, or blebbistatin, significant disruption of the fibroblast monolayer was observed at early time points, preventing reliable measurement at later times (as indicated by missing data points in Fig. 3) that eventually culminated in almost complete fibrin matrix degradation (Fig. 3D) and consequent termination of tissue culture. We hypothesized this unexpected effect could be due to upregulated proteolytic activity in either the fibroblast monolayer or the endothelial cells, possibilities of which were investigated further as described later.

Verifying the inhibition of tractional forces and the absence of cytotoxicity. To eliminate the possibility that the various inhibitors of tractional forces disrupt capillary morphogenesis through nonspecific cytotoxic effects, LIVE/DEAD assays were performed at all time points and drug conditions studied. As shown in a representative data set in 2.5 mg/ml fibrin (Fig. 4), these tests revealed that the various inhibitors of actomyosin-mediated tractional forces do not induce nonspecific cytotoxic effects. These data strongly suggest that the disruptions in

![Fig. 2. Effects of extracellular matrix (ECM) density on capillary growth in a fibrin-based model of capillary morphogenesis. A: schematic of bead angiogenesis tissue setup; NHLF, normal human lung fibroblasts. B: quantification of total capillary network length vs. time for fibrin gel densities of 2.5, 5.0, and 10.0 mg/ml. C: representative images showing the formation of capillary networks from human umbilical vein endothelial cells (HUVEC)-coated microcarrier beads embedded within 2.5 mg/ml fibrin tissue on day 1 (4X). D: day 6. E: day 11. *P < 0.05 relative to each other for last time point; error bars are equivalent to SD; scale bar is equivalent to 400 μm.](http://ajpcell.physiology.org/10.1152/ajpcell.00233.2009)
capillary morphogenesis observed in Fig. 3 are not due to cell death but instead are due to the intended alterations in contractile forces. Furthermore, total gel contraction assays confirmed that the inhibitory drugs utilized here do indeed alter cell-generated contractile forces. This type of contraction assay was previously used in a study investigating the role of fibronectin matrix assembly in capillary sprouting (34). Here, when tissue constructs with HUVECs distributed within the gels were treated with the various inhibitors of cell-generated tractional forces, the floating gels remained intact and their diameter did not significantly change over an 8-day time course. On the contrary, the untreated control tissues were significantly contracted and reduced in size over time (Fig. 5). These data verify that the inhibitory drugs used here are indeed effective at limiting contractile forces at the concentrations employed in this study.

![Graphs and images illustrating capillary morphogenesis and cell death](image_url)
Effects of limiting contractile forces on mature capillary networks. Another important aspect of limiting contraction was observed in mature capillary networks. The tissues were cultured without the various contractile force inhibitors for 6 days until mature vessels with well-defined lumens were established. The inhibitors were then applied for the remainder of the experiment. Although the tests were performed in all fibrin densities, a representation of the observed phenomenon is shown in a 2.5 mg/ml fibrin gel (Fig. 6). The application of the drugs had a significant effect on total network length even after the vessels were sufficiently established. As before, in conditions with ML-7, the network lengths were shorter compared with controls a few days past the first drug application, and the tissues remained intact. For all other drugs, the reduction in network length was accompanied by degradation of the tissue constructs, which again prevented quantification of total vessel network lengths at later time points (indicated by missing points in Fig. 6). In addition, ML-7 and BDM conditions both displayed notable phenotypic changes on the mature capillary networks. In the case of BDM, a thinning of the vessels occurred, which could possibly be explained by degradation or collapse of the lumen. In response to ML-7, some blood vessels exhibited cell dissociation and a general “patchy” vessel morphology. To investigate this phenomenon further, we performed real-time imaging of the constructs treated with ML-7 and BDM from days 8 through 11, a time range when the most significant morphological changes seemed to be occurring based on static phase-contrast images. Based on the video data (supplemental video 1 and 2), it is evident that inhibition of actomyosin-mediated contractile forces in both cases essentially destroyed the vessel structure, leaving behind healthy individual HUVECs as confirmed by LIVE/DEAD assays (data

Fig. 5. Global gel contraction verifying the functionality of the various contractile force inhibitors. A: average diameter (cm) of the gels treated with inhibitors were measured and compared with control conditions without inhibitors at days 4, 6, 8. B: representative images showing the actual gel data set at day 8; scale bar is equivalent to 1 cm; *P < 0.05 for all drug conditions relative to control for day 8; error bars are equivalent to SD.

Fig. 6. Effects of contractile force inhibition on the stability of mature vessel networks. A: mean total vessel network length in 2.5 mg/ml fibrin with the various contractile force inhibitors applied at day 6 as marked by a star. B: representative image of a control tissue without drug application in 2.5 mg/ml at day 11. C: representative image showing vessel deterioration by cell dissociation cells in networks treated with ML-7 at day 11 (arrow). D: thinning vessels were observed in tissues treated with BDM at day 11 (arrow). P < 0.05 relative to control for the latest measurable point; error bars are equivalent to SD, star indicates drug application time point; scale bar is equivalent to 200 μm.
not shown). With BDM, thinning of the vessel occurred first, followed by cell dissociation; with ML-7, the vessels readily collapsed without thinning first. These differences may be attributable to the inhibitors’ different targets, and thus proximity to the final step, in the cascade regulating tractional forces (Fig. 1). In higher density tissues at the same time point, the less developed vessels tend to retract back to the bead rather than collapse completely (data not shown). Thus the inability to support the vessel structure without contractile forces in these smaller vessels results in the cells’ return back to the bead origin, where they can exist in a monolayer.

Inhibition of EC-mediated tractional forces in cultures treated with fibroblast-conditioned media. As noted earlier, some of the various force inhibitors led to an enhanced degradation of the tissue constructs. We hypothesized that the enhanced proteolysis was due to the response of the fibroblasts to these contractility inhibitors, and we sought to verify that the reductions in capillary network length were not due merely to this enhanced degradation of the gels. To do so, additional experiments involving fibroblast-conditioned media supplemented with these traction force inhibitors were performed. Again, reduced network lengths were observed in all drug-treated constructs compared with controls without the inhibitors, and a similar decrease in network length with increasing ECM density was seen (Fig. 7). In these tissues, the multicellular structures sprouting from the beads did not exhibit a well-developed lumen but were more appropriately characterized as tubular structures instead (Fig. 7, F–J). However,

Fig. 7. Inhibition of endothelial cell tractional forces in baseline 2.5 mg/ml fibrin cultures compared with those treated with fibroblast-conditioned media (CM). Measurements of total capillary network lengths show that cultures fed with CM generate tubular structures that are significantly shorter than the capillaries that form in the control cultures with an intact fibroblast monolayer. Treatment of these cultures with Y27632 (A), BDM (B), ML-7 (C), or blebbistatin (D) show further reduction in network lengths in CM-fed cultures, which indicates that tractional forces generated by endothelial cells are required for sprouting. Shown are representative images of sprouting structures in control tissues at day 11 (E); control tissue supplemented with CM (F); tissues treated with CM and Y27632 (G); tissues treated with CM and BDM (H); tissues treated with CM and ML-7 (I); and tissues treated with CM and blebbistatin (J). *P < 0.05 relative to control for the latest measurable time point, error bars are equivalent to SD; scale bar is equivalent to 200 μm.
significant effects on network length as a function of ECM density and traction force inhibition were still observed, even though the absolute length values were smaller than in conditions with regular media. More importantly, the tissue degradation apparent in previous experiments was eliminated by use of conditioned media, suggesting that fibroblasts indeed proteolytically degraded the gels when the contractility inhibitors were applied to the integrated culture system. Moreover, this proteolytic effect did not appear to account for the reduction in total capillary vessel lengths induced by contractility inhibition.

Proteolytic activity inhibition and tissue degradation. To further investigate the effect of proteolytic degradation observed in the presence of some contractility inhibitors and to completely eliminate the possibility of its effect on capillary growth, tissues treated with the various contractile force inhibitors were also treated with either apronit, which inhibits plasmin-mediated proteolysis, or GM-6001, a broad-spectrum matrix metalloproteinase (MMP) inhibitor. In these experiments, tissue degradation was prevented only in conditions where apronit was applied, either alone with the various contractility inhibitors or when combined with GM-6001. This implies that the fibroblast-mediated degradation of our fibrin constructs was most likely due to plasmin proteolysis. Gel degradation still occurred when constructs treated with contractility inhibitors were also treated with GM-6001, suggesting that MMP-induced proteolysis did not play a role in fibroblast-mediated fibrin degradation in this system. When actual mean total network lengths were compared in the baseline drug applications to those also treated with apronit, no significant differences were observed (Fig. 8). These data confirm that inhibition of EC-mediated tractional forces disrupts capillary morphogenesis independent of fibroblast-mediated fibrinolysis.

DISCUSSION

Capillary morphogenesis is a complex and dynamic process that depends on the highly localized degradation of the surrounding ECM, subsequent EC migration, proliferation, and eventual differentiation into functional tubular networks (14). In this study, we attempted to mechanistically address the hypothesis that changes in ECM density, which in turn alter substrate mechanics, might influence capillary morphogenesis by affecting the ability of ECs to generate traction while invading through a 3-D fibrin matrix. With the use of a battery of pharmacological agents to inhibit contractile forces in tissue constructs of varied ECM density, our data support this hypothesis and demonstrate that the interdependence of ECM mechanics and cell-generated contractile forces applies not only to the initiation of this complex morphogenetic process but also to the homeostasis of existing vessels as well.

There is compelling evidence that ECM mechanics influence individual cells in 2-D cultures by modulating actin-mediated contractile forces (25). Much less is known about how ECM mechanics influence coordinated multicellular differentiation processes, such as the capillary morphogenesis studied here. Pioneering work by Ingber and Folkman (12) demonstrated that changing ECM ligand density as a way to control endothelial cell shape influenced their growth and the morphological changes associated with the formation of capillary-like tubes. More recent evidence corroborates the idea that substrate mechanical cues directly impact tubulogenesis (4, 28, 31). Vailhe et al. (31) demonstrated that HUVECs seeded on top of 2-D fibrin gels varying in concentration from 0.5 to 8 mg/ml only formed capillary-like structures on the softest of the gels. They concluded that the ECs do not form capillary-like structures on the more rigid gels because the cells are unable to generate the necessary contractile forces to remodel the substrate (12). Likewise, Deroanne et al. (4) showed that ECs seeded on collagen-functionalized polyacrylamide gels of different stiffness change morphologies from a monolayer to a tube-like phenotype as substrate rigidity decreases.

In 3-D culture, Urech et al. (30) investigated angiogenic process extension in fibrin gels and manipulated their mechanical properties by adding exogenous factor XIII to form additional crosslinks. Consistent with our observations, this work showed that fewer and shorter capillary-like processes formed as cross-link density increased, although their cultures were limited to 24 h and lacked demonstration of lumens. Sieminski et al. (28) also studied the 3-D formation of capillary-like structures by ECs in freely floating versus mechanically constrained collagen gels. They concluded that changing the col-

Fig. 8. The combined effects of inhibiting contractile force generation and proteolytic degradation. Total network length plots in 5.0 mg/ml tissues treated with blebbistatin (A), Y27632 (B), or BDM (C), and inhibitors of ECM proteolysis are shown. All conditions were treated with the contractile force inhibitors except those noted as control; As shown in Fig. 3, treatment of 5 mg/ml fibrin cultures (with an intact fibroblast monolayer) with these three traction force inhibitors led to global gel degradation mediated by the fibroblasts, leading to the inability to measure total network lengths beyond certain time points. By contrast, these data show that treatment with apronit blocked the global gel degradation induced by the contractile force inhibitors. *P < 0.05 relative to control for the latest measurable time point, error bars are equivalent to SD.
lagen concentration modulates the formation of structures by regulating the amount of traction force exerted by the cells (28). Our results are consistent with this conclusion but were obtained via a completely different approach through direct traction manipulation at multiple points in a putative molecular pathway via a battery of pharmacological agents (Fig. 1).

Different agents exhibited varying degrees of inhibition and statistical significance in our studies. These differences may be due to varied drug potency, different inhibition mechanisms, and the consequent variability in cellular contractility. For example, Y27632 exhibited the least inhibition compared with other drugs perhaps due to the dual action of ROCK, which directly promotes activation of myosin light chain together with MLCK and indirectly via blocking myosin phosphatase (15, 32). When ROCK is inhibited, MLCK may still be capable of inducing sufficient contractility to counteract Y27632 inhibition. Similarly, ML-7 is second in terms of the magnitude of inhibition. Its ability to inhibit tractional forces through MLCK may be partially balanced by the ability of ROCK to promote basal levels of contractile force to support some capillary growth. Consistent with these arguments, since BDM and blebbistatin affect myosin ATPase far downstream of the cascade, they are able to block tractional forces most significantly without competing with other pathway participants or even alternative pathways (6, 22). In addition, blebbistatin has been previously shown to inhibit HUVEC migration on materials with a variety of coatings, including Matrigel, type I collagen, and fibronectin (20), as well as prevent aggregation of cells to form coherent structures (9). Similar mechanisms likely account for the most profound influence of blebbistatin on vessel growth in our assay. Despite these subtle differences in drug action and degree of inhibition, blocking any of the components of the putative molecular pathway (Fig. 1) results in marked decrease in capillary vessel growth, thus confirming that actin-mediated tractional forces are required for early stages of vessel development.

It is perhaps not surprising that these cell-generated forces are necessary for the early stages of vessel formation, which involve both proliferation and migration of ECs. The role of tractional forces in 2-D migration has been widely studied, and thus it has been assumed that traction is equally as critical for coordinated cell movements in 3-D. However, a unique contribution of our study here is the finding that these cell-generated tractional forces play a homeostatic role in maintaining vessel lumens and stabilizing the overall network structure. When contractile force inhibitors were applied 6 days after the onset of tissue culture, the continued growth of mature vessel structures was disrupted (Fig. 6). In addition to growth cessation, ML-7 and BDM, in particular, affected the morphology of the lumens in the capillaries, resulting in their dissociation in the former and thinning of the lumens by about half (12 to 6 μm) in the latter. These effects were also confirmed by direct real-time observation of vessel structure collapse. A previous study showed that these inhibitors act by reducing focal adhesions and stress fibers and may rapidly block DNA synthesis leading to growth arrest in muscle myoblasts (5). Similar effects may be involved here in which the ECs are partially unable to sustain growth and result in compromised vessel networks. Alternatively, relaxing cytoskeleton tension in the ECs may disrupt a preexisting balance of forces between the cells and the surrounding ECM and thereby cause a mechanical collapse of the established vessel structures.

Another distinction between our model and findings relative to those of others is the presence of a monolayer of fibroblasts. These cells have been shown to act as a required source of pro-angiogenic factors that promote the formation and stability of capillary networks with well-defined lumens (8, 19). Co-transplantation of interstitial fibroblasts and EC-coated Dextran beads within a 3-D fibrin matrix has also been shown to yield capillary networks that form functional anastomoses with host vasculature in immunocompromised mice (3). However, the presence of NHLFs made it essential for us to distinguish the effects of the various contractile force inhibitors on the ECs from the fibroblasts. One of the prominent effects of contractility inhibition in our integrated culture model appeared to be tissue degradation or proteolysis, which we attributed to the fibroblasts. To further investigate this phenomenon, we replaced the fibroblast monolayer with fibroblast-conditioned media to pinpoint whether NHLFs were responsible for the global degradation of the tissue in the presence of contractility inhibitors. With application of fibroblast-conditioned media, fibrinolysis no longer occurred and network length data exhibited vessel growth inhibition similar to previous studies with the monolayer. Whereas the multicellular structures that formed in the presence of conditioned media lacked some of the structural characteristics of the vessels that sprout in the presence of NHLFs, application of contractile force inhibitors in both cases attenuated network formation. From these data we conclude that the inhibitory effects on capillary morphogenesis in the presence of the contractile force inhibitors were due, at least in part, to their direct effects on EC-generated tractional forces as opposed to fibroblast-mediated proteolysis. Moreover, since both MMPs and plasmin systems were previously shown to be responsible for proteolytic activity (24), we further demonstrated that global tissue degradation was not prevented by blocking MMP activity, whereas plasmin inhibitor effectively eliminated it without adversely affecting vessel sprouting (Fig. 8). Previous studies have also shown that plasmin null mice display no dysfunctional angiogenesis (18) and that ECs invade the matrix in plasmin-independent fashion (10). This portrays that plasmin-induced fibrinolysis initiated by NHLFs is responsible for the global tissue degradation in our assays, whereas MMPs are likely responsible for the focalized, EC-mediated fibrinolysis at the tips of sprouting capillaries.

Overall, our study was aimed at elucidating a mechanism involving cell-generated forces, ECM mechanics, and angiogenesis. Our findings reveal that actomyosin contractile forces are necessary not only for the initiation of blood vessel formation during the early stages of angiogenesis but also for the maintenance of stable vessels during the later stages of angiogenesis. We also show that the inhibition of endothelial cell contractility with pharmacological inhibitors is proportional to ECM density, consistent with the idea that ECM mechanics regulate contractile forces generated by the endothelial cells. Finally, the enhanced proteolytic activity of fibroblasts induced by the various inhibitors of tractional forces was demonstrated to depend primarily on plasmin-mediated fibrinolysis. Further studies addressing the links between cell-generated forces and angiogenesis might involve upregulating the basal levels of EC contractility in tissues of higher stiffness to determine whether
this can overcome the inhibitory effects of higher ECM density on angiogenesis. This strategy might be potentially applied in conditions where upregulation of angiogenesis is necessary for recovery, such as ischemia or myocardial infarction. Alternatively, downregulation of EC-mediated trational forces might limit hyperactive angiogenesis and provide a therapeutic strategy for tumor growth control.

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