Role of JNK in network formation of human lung microvascular endothelial cells

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THE FORMATION OF NEW blood vessels is a complex, multistep process. Vasculogenesis is the development of new vessels by proliferation, differentiation, and coalescence of endothelial cells into intact networks (43). This capillary plexus is then remodeled by angiogenesis or formation of new vessels from existing ones by sprouting, branching, and differential growth of blood vessels (43). In addition to this, angiogenesis involves further branching and recruitment of nonendothelial cells such as pericytes and vascular smooth muscle cells to develop more complex arterioles and venules (7). Whereas VEGF and other protein/peptide factors have received much attention in promoting growth and differentiation of new vasculature, our interest has been in the discovery of novel vascular functions of lipid metabolites of arachidonic acid to decipher their downstream signaling pathways in the lung (30, 31). Interestingly, enzymes such as human cytochrome P450 (CYP) 2C9 that metabolize arachidonic acid to the four regioisomeric epoxyeicosatrienoic acids (EETs) modulate the expression of a MAPK phosphatase, MKP-1 (35).

MKP-1 is a member of the family of dual specificity phosphatases, which functions promiscuously to regulate the activity of three MAPK cascades, ERK1/2, JNK, and p38 MAPK, via dephosphorylation of the TXY motif (6, 8, 16, 19). This enzyme is inducible with a fairly short half-life (45 min), making it a good candidate for the temporal regulation of MAPKs (5). Substitution of the catalytic active cysteine in MKP-1 to a serine residue results in inactivation, although the enzyme retains high substrate affinity (14). In contrast, MKP-3, another member of the dual specificity phosphatase, is present in the cytoplasm and preferentially inactivates phosphorylated ERK1/2 vs. JNK and p38 due to a high affinity binding domain located in the NH2 terminus of ERK (17).

The classical paradigm in vessel formation implicates endothelial proliferation, migration, tube assembly, and remodeling as sequential steps. Endothelial cells are regulated by host-specific matrices and factors to accomplish this (7). Tubulogenesis in a three-dimensional (3-D) matrix mimics some of the steps in angiogenic remodeling (7, 43) causing alignment of endothelial cells into networks. Proliferation is a less important aspect of this step where cords of assembled endothelial cells assemble to eventually form a lumen (7). Development of intracellular vacuoles has often been described to precede lumen formation. Reduced proliferation or controlled apoptosis may signal morphogenesis; in fact, tube formation appears to be initiated as a controlled stress (34, 44). Like most cellular reassembly, it is also dependent on the concentration and spatial organization of matrix proteins. Endothelial cells align in vitro in matrices that are generally composed of Matrigel, collagen I, laminin, or fibrin. Matrigel is derived from Engelbreth-Holm-Swarm mouse tumor (22) and is composed of laminin (56%), collagen IV (31%), and entactin (8%). Laminin promotes endothelial morphogenesis (11, 21, 26, 37) and is a component of vascular basement membranes (21). Mice deficient in the laminin-α5 chain (laminin is composed of 3 chains, α1-α3) have impaired microvascular maturation (40). Targeted disruption of laminin-α5 yielded aberrations in placental vessels (32). Laminin in synergy with FGF-2 has been reported to play an active role in angiogenesis possibly by stabilizing developing vessels via induction of the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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beyond The cells are primary cultures and remain differentiated for up to 15 as judged by acetylated LDL uptake, von Willebrand factor staining, by the manufacturer. Cells were cultured with the EGM-2-MV bullet Lonza, Rockland, ME, and maintained and passaged as recommended to a final protein concentration of Matrigel was applied after diluting the stock (1:1) with HBSS on ice Lab-Tek II chamber slides (Nalge Nunc, Naperville, IL) coated with from Cell Signaling Technology, Danvers, MA. Growth factors and anti-phospho-heat shock protein 27 (HSP27) antibodies were intensity of Dundee, Scotland, UNITED KINGDOM. Anti-phospho-ERK 184352 was provided by the laboratory of Dr. Philip Cohen, Univer-

Materials and Methods

Materials. The ERK1/2 inhibitors PD 98059, U0126, SB-203580, and SP-600125 and negative JNK control (N'-Methyl-1,3-pyrazoloanthrene, cat. no. 420123) were purchased from Calbiochem, La Jolla, CA. t-steroioisomer (t-JNK1-1) was purchased from Alexis Biochemicals, Lausen, Switzerland (cat. no. ALX-159-600). PD 184352 was provided by the laboratory of Dr. Philip Cohen, University of Dundee, Scotland, UNITED KINGDOM. Anti-phospho-ERK and anti-phospho-heat shock protein 27 (HSP27) antibodies were from Cell Signaling Technology, Danvers, MA. Growth factors VEGF and basic FGF (bFGF) were kindly provided by the Biological Resources Branch of the National Institutes of Health, Frederick, MD.

Growth and culture of HLMVECs. Cells were purchased from Lonza, Rockland, ME, and maintained and passaged as recommended by the manufacturer. Cells were cultured with the EGM-2-MV bullet kit containing growth factors that maintain the endothelial phenotype as judged by acetylated LDL uptake, von Willebrand factor staining, and surface platelet endothelial cell adhesion molecule expression. The cells are primary cultures and remain differentiated for up to 15 passages if maintained as described above. We did not use cells beyond passage 13 and substituted the endothelial basal media (EBM; Cambrex, East Rutherford, NJ) as serum-free medium (SFM) in the final stages of selected experiments where described. The cells were grown in a tissue culture water-jacketed incubator under 95% air-5% CO2 following Biosafety 2 levels for human samples as well as recombinant adenosine.

Growth of adenosine virus. The Ad5-green fluorescent protein (GFP), Ad5-2C9, Ad5-dominant negative MEK (DN-MEK), Ad5-constitutively active MEK (CA-MEK), Ad5-MKP-3, Ad5-MKP-1, and Ad5-MKP-1C/S were grown, amplified (2), and purified on the adenosin core facility at the Medical College of Wisconsin. Each batch of virus was assayed for toxicity in a preliminary experiment and used at a multiplicity of infection of 50 plaque-forming units per endothelial cell. After infection in EBM supplemented with 2% fetal bovine serum, the cells were allowed to express the transgenes for 18–24 h. They were then lifted, counted, and used in the network formation in Matrigel.

Network formation in Matrigel. Cultured HLMVECs were suspended in SFM and seeded at 4 × 10^4 cells/well into four-well Lab-Tek II chamber slides (Nalge Nunc, Naperville, IL) coated with Matrigel (Becton Dickinson Labware, Bedford, MA). The coating of Matrigel was applied after diluting the stock (1:1) with HBSS on ice to a final protein concentration of ~5.5 mg/ml. Matrigel (250–300 μl) was applied per squared centimeter of each well, and the matrix was allowed to polymerize at 37°C before the addition of the cells, which were either infected or pretreated (as indicated in each experiment) with recombinant adenosine virus, vehicle, or corresponding inhibitors for 30 min. The inhibitors remained in the samples during the experiment. All the wells were examined after an 18-h incubation period in the tissue culture incubator after which cells were scanned under low power, and equal numbers (minimum of 3 per experimental condition) of fields with maximal tube formation were focused and captured using an Eclipse 600 (Nikon) microscope with attached digital camera and SPOT software. All cells in the field were not always in optimal focus due to the 3-D nature of the matrix. The images were viewed at a constant magnification (×300), and the length of the tubes in arbitrary units were measured and summated using MetaMorph (version 6.1; Universal Imaging, Molecular Devices, Downingtown, PA) to give the total length of tubes formed per image. The mean ± SE of relative tube lengths from 4 or more images per condition and at least three independent experiments are shown in the figures. The n for individual experiments appears in the figure legends. Groups of cells were compared by ANOVA followed by a post hoc (Tukey) test using SigmaStat 3.1, and those comparisons with P values <0.05 were considered significantly different from each other.

Network formation in collagen. HLMVECs were seeded at 2.5 × 10^5 cells/well into four-well Lab-Tek II chamber slides coated with a collagen matrix. The collagen matrix was prepared on ice by mixing 100 μl of 10× MEM ( Gibco, Carlsbad, CA), 800 μl of rat tail collagen type I (BD Biosciences, San Jose, CA), and 100 μl of 0.1 M NaOH (Sigma, St. Louis, MO) by pipetting up and down. Each well of the slide was coated with 250 μl of the collagen mixture and allowed to gel for 30 min at 37°C in a humidified incubator with 5% CO2 before cells were added. After the addition of cells, slides were incubated for 2 h to allow the cells to settle into the matrix. The media was aspirated from the well, and a fresh collagen matrix was prepared and applied to each well as described above to create a sandwich. This was allowed to gel for 1 h at 37°C in a humidified incubator. Endothelial growth media for microvascular cells (EGM-2M-V, 200 μl; Cambrex) or serum- and growth factor-depleted media with angiogenesis factors [VEGF (10 ng/ml), bFGF (10 ng/ml), or PMA (50 ng/ml)] were added to each well. All wells were examined at defined times for the next 18–72 h.

Network formation in fibrin. Human fibrinogen (Sigma) was prepared by dissolving 1.5 mg/ml fibrinogen in SFM containing 1% penicillin-streptomycin. The solution was filtered through a 0.4-μm sterile syringe filter to remove remaining clumps of fibrinogen that would interfere with the uniform polymerization of the gel. Thrombin (Sigma) was prepared by dissolving 50 U/ml thrombin in SFM containing 1% penicillin-streptomycin. The thrombin was then filtered as described above. Aliquots were frozen at −20°C. The fibrin matrix was prepared in 1.5-ml tubes on ice by mixing 1.5 mg/ml fibrinogen (300 μg, Sigma) and adding 50 μl of a suspension of HLMVECs containing 2.5 × 10^5 cells. Thrombin (20 μl) was then added to the fibrinogen one tube at a time. The mixture was applied to the four-well Lab-Tek II chamber slides immediately after the addition of thrombin and allowed to gel in a 37°C humidified incubator with 5% CO2 for 30 min. EGM-2-MV (250 μl) or serum- and growth factor-depleted media with angiogenesis factors were added to each well. All wells were examined at defined times for the next 18–72 h incubation.

Western Blots. HLMVECs were cultured in 100-mm plates at a density of 10^6 cells in 10 ml of EB-2 media containing 5% fetal bovine serum. For Western blots, the cells were treated as described in each experiment and then lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% SDS, 1% Nonidet P-40, sodium deoxy-

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proteins were transferred from SDS gels onto a nitrocellulose membrane for immunoblot analysis. Blocking was performed with 5% nonfat dry milk in 150 mM NaCl, 50 mM Tris, pH 7.2, 0.05% Tween 20 (TBST) buffer. The membrane was then incubated for 3 h at room temperature or overnight at 4°C using one of the following antibodies (diluted 1:1,000) as indicated in each experiment, MKP-1, phospho-ERK, ERK, phospho-c-Jun, phospho-HSP27, or HSP27 (Cell Signaling Technology), in 5% nonfat dry-TBST. The blots were then washed, incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG, diluted 1:2,000 in 5% nonfat dry-TBST, and developed using an ECL system (Amersham Biosciences, Piscataway, NJ). For each of the conditions tested for tubulogenesis, we performed parallel Western blots to evaluate functional endpoints (e.g., inhibition or activation of a pathway) with the same passage and origin of cells.

JNK activity. Activity in vitro was determined as specified by the manufacturer using a JNK assay kit (cat. no. 9810, Nonradioactive; Cell Signaling Technology) that included all the reagents necessary to measure JNK activity. A c-Jun fusion protein linked to agarose beads was used to immunoprecipitate SAPK from cell extracts. On addition of kinase buffer and ATP, the enzyme phosphorylated a c-Jun substrate. Phospho-c-Jun (Ser63) antibody was then used to measure enzyme activity by immunoblotting. The HLMVECs were treated with respective inhibitors for 15 min (vehicle DMSO, the JNK 1, 2, and 3 inhibitor SP-600125, and a negative control that inhibits JNK only at much higher concentrations than were used in this study), stimulated with anisomycin (200 μM), and washed and lysed in 1× cell lysis buffer. Proteins were estimated in the cell lysates, and 200 μg from each sample was taken for immunoprecipitation in an overall total volume of 200 μl. A slurry of immobilized c-Jun fusion protein beads (20 μl) was added, and the mixture was gently rocked overnight. The suspensions were microcentrifuged at 2,000 rpm, and the pellets were washed twice with cell lysis buffer and three times with kinase buffer, suspended in the same buffer supplemented with 200-μM ATP, and incubated for 30 min at 30°C. The reactions were terminated by adding an equal volume of Laemmli buffer, and the samples were vortexed and heated at 95°C for 5 min. Equal volumes of the reaction were loaded in SDS-PAGE gel, electrotransferred to a nitrocellulose membrane, and probed with the phospho-c-Jun antibody.

CAM model to measure angiogenesis in vivo. Chick chorioallantoic membranes (CAMs) were developed in embryos grown in shell-less culture as previously described (12). Fertilized eggs of White Leghorn chickens were purchased from Sunnyside, Beaver Dam, WI, and incubated for 3 days at 37.5°C with 62–72% humidity in a commercial incubator equipped with automatic turning trays that changed the angle of orientation every 3 h. After 3 days, the eggs were dipped briefly in 70% ethanol, dried, and carefully opened with a razor blade (12). The contents were poured into clear plastic wrap hampocks (Roundy’s Quality; Roundy’s, Milwaukee, WI) suspended in custom-designed tripods with holders made from 3-in.-diameter plastic drainpipe and covered with the top half of a sterile petri dish (12). Tripods were sterilized by soaking overnight in 10% bleach before use. The developing embryos were visible at this time on the uppermost portion of the yolk. Cultures were incubated in a water-jacketed tissue culture incubator at 37.5°C, 99% humidity, and 1.5% CO2 mixed with room air. Angiogenesis was assayed in the presence of vehicle (DMSO) or the JNK inhibitor SP-600125 (Calbiochem, San Diego, CA). Some CAMs were treated with VEGF as a positive control. The reagents were immobilized on sterile Thermonox plastic coverslips (cut into quarters; Nagle Nunc, Rochester, NY) by drying DMSO (6.6 μl of 0.004%), SP-600125 (6.6 μl at a concentration of 1 μM), or VEGF (1.6 μg) for 1 h. Each of these was gently placed on the corresponding vessels of a CAM (9–12 days old, when maximal angiogenesis occurs). The eggs were imaged immediately after application of the coverslips (day 0) with an Insight Firewire Color Mosaic Camera (Fryer, Huntley, IL) fitted with a Nikon SMZ-1000 stereo microscope and then incubated for 72 more hours in a water-jacketed tissue culture incubator at 37.5°C, 99% humidity, and 1.5% CO2 mixed with room air. Final images of the vasculature were captured (on day 3) after underlaying the CAM with contrasting agent made up of warm emulsified milk (heavy whipping cream, Roundy’s; Ref. 12). Images were analyzed by counting the number of secondary, tertiary, and quaternary branches of a primary vessel underneath the coverslip using MetaMorph (Universal Imaging, Molecular Devices). The number of quaternary vessels was counted under coverslips containing vehicle or JNK inhibitor groups according to the day the experiment started. Each group had between 2–4 samples of each treatment. Average values from each group were normalized to percent control using the mean values of the vehicle. An unpaired t-test was performed with SigmaPlot version 9.0 (Systat Software, San Jose, CA) to determine statistical difference (P < 0.05) between the JNK-inhibited CAMs and vehicle control.

RESULTS

MKP-1, a dual specificity phosphatase, blocks HLMVEC network formation in Matrigel. We (30) have previously demonstrated that HLMVECs elongate to form tube-like networks in Matrigel. Since this process may be driven by different integrin-mediated signaling pathways that depend on the engagement of receptors with the extracellular matrix, we embedded the cells in a “sandwich collagen” matrix composed of rat tail collagen I or a fibrin gel (prepared from human fibrinogen) to bind other integrins that may be present on these cells. HLMVECs did not readily form tube-like structures in collagen or fibrin even after addition of up to three angiogenic factors (VEGF, FGF, and PMA) unlike rat or bovine pulmonary artery endothelial cells, which, in our hands, consistently assembled robust networks in these matrices in <48 h (results not shown). We therefore continued further experimentation to investigate tubular morphogenesis of HLMVECs in Matrigel.

We (30) have also previously demonstrated high efficiency gene transfer (>90% delivery) to HLMVECs using replication-deficient adenoviral vectors. Infection of the cells with wild-type recombinant MKP-1 (Ad5-MKP-1) demonstrated this phosphatase was able to completely block the formation of endothelial networks in Matrigel compared with GFP-expressing cells (Fig. 1A). Importantly, infection of HLMVECs with an adenovirus expressing a catalytically inactive mutant of MKP-1 (MKP-1/CS) did not inhibit tube formation. Figure 1B is a representative Western blot confirming overexpression of virally delivered MKP-1 (lane 4) and MKP-1/CS (lane 5) compared with GFP-expressing cells (lane 2) or those expressing an unrelated recombinant protein CYP 2C9 (lane 3). Since wild-type MKP-1 is effective at dephosphorylating ERK, JNK, and p38, we sought to examine these pathways separately to determine which one(s) plays a dominant role in network formation.

The MAPK ERK1/2 is not required for HLMVEC tubular assembly in Matrigel. In an effort to determine the role of ERK in HLMVEC networks, we used two specific molecular inhibitors (MKP-3, a dual specificity phosphatase, and DN-MEK; Fig. 2A), one activator of ERK (CA-MEK; Fig. 2A), and three pharmacological inhibitors of ERK (PD 98059, U0126, and PD 184352; Fig. 2B). The results demonstrated that PD 98059 and U0126, but not PD 184352, DN-MEK, or MKP-3, reduced HLMVEC network formation (Fig. 2, A and B). We interpreted these results as suggesting that ERK may not be necessary for
alignment of networks since the more specific molecular inhibitors (DN-MEK and MKP-3) did not prevent this cellular remodeling as did two (PD-98059 and U0126) of three phosphatase inhibitors (DN-MEK and MKP-3) did not prevent this cellular alignment of networks since the more specific molecular inhibitors that did not show this effect (PD 184352, DN-MEK) demonstrated effective ERK phosphorylation (Fig. 2 lane 3). PD 98059 (Fig. 2D, lane 2), U0126 (lane 3), and PD 184352 (lane 4) all attenuated this reaction (Fig. 2D).

The MAPK p38 is not required for HLMVEC tubulogenesis in Matrigel. We used pharmacological inhibitors of p38 (SB-203580) to determine their effect on network formation. Inhibition of p38 MAPK failed to block this process and, in fact, tended to enhance it (Fig. 3A). Experiments were performed to demonstrate that the concentration of SB-203580 used in this study was effective at blocking p38 MAPK activity. This was done by evaluating the phosphorylation of a downstream in vivo substrate, HSP27, as an indirect measure of p38 MAPK activity (33). As shown in Fig. 3B, anisomycin, a potent activator of p38 MAPK, produced a robust increase in HSP27 phosphorylation in HLMVECs. Pretreatment with SB-203580 blocked the anisomycin-induced phosphorylation of HSP27 (lane 4). These data demonstrate that SB-203580 was effective in blocking p38 MAPK activity in these cells but also that this inhibition did not decrease network formation of HLMVECs.

The JNK pathway is required for HLMVEC tubulogenesis in Matrigel. Similar experiments were performed to determine the potential role of JNK in network formation. As shown in Fig. 4A, treatment with the JNK inhibitor SP-600125 (5 μM) resulted in inhibition of network formation in the Matrigel assay compared with the vehicle, DMSO. Furthermore, low concentrations of SP-600125 (0.5 or 2.5 μM) blocked anisomycin-induced activation of JNK as measured by a JNK activity assay (see MATERIALS AND METHODS and Fig. 4B). Negative controls consisting of similar concentrations of a compound related to SP-600125 that did not inhibit JNK activity demonstrate specificity of the assay (Fig. 4B, lanes 4 and 6).

To test more rigorously the contribution of JNK to tubulogenesis in endothelial cells, we examined the impact of a second inhibitor that is known to block specifically JNK-1 (l-JNKI-1). Like SP-600125, l-JNKI-1 decreased tube assembly in Matrigel relative to vehicle controls (Fig. 4C).

Lastly, in an attempt to resolve the ambiguity observed with two compounds that inhibit phosphorylation of ERK1/2, U0126 (10 μM) and PD 98059 (20 μM), that also blocked network formation vs. three other more specific inhibitors that did not show this effect (PD 184352, DN-MEK, and MKP-3), we tested the pharmacological reagents for cross-reactivity with JNK activity (Fig. 4D). Our data demonstrate that these reagents substantially attenuated JNK function (phosphorylation of the c-Jun substrate; see MATERIALS AND METHODS). Therefore, these agents may have imparted their effect on network formation by blocking the JNK pathway along with ERK.
Attenuation of physiological angiogenesis in vivo by inhibition of JNK. To determine if JNK is required for angiogenesis in vivo, we used the shell-less CAM as a model that could be studied and imaged in real-time in situ. Figure 5A depicts untreated CAM at day 0, whereas Fig. 5B represents the CAM after 72 h of treatment with VEGF, which induces growth of new vessels. This serves as a positive control to illustrate angiogenesis by VEGF (that has been immobilized on a cov-
erslip as described in MATERIALS AND METHODS). Developing embryos were also treated with a JNK inhibitor (Fig. 5C) or vehicle (Fig. 5D) for 3 days, after which the vessels were scored as demonstrated in the enlarged section of Fig. 5D (see MATERIALS AND METHODS and Fig. 5E). The number of quaternary branches (marked 4 in Fig. 5E) was counted in at least four vehicle- and inhibitor-treated CAMs. This number was significantly reduced by the JNK inhibitor, indicating that JNK enhances development of new vessel branches in vivo.

DISCUSSION

The present study was performed in an attempt to determine the role of phosphatases and their substrates, MAP kinases, in network formation of HLMVECs. Our results indicate that HLMVECs form tube-like networks in Matrigel but not collagen I or human fibrin gels. We found, as summarized in Fig. 6: 1) pharmacological inhibitors of MEK, PD 98059 (20 μM) and U0126 (10 μM), effectively blocked network formation and ERK phosphorylation, whereas a more potent and specific inhibitor, PD 184352 (1 μM), blocked ERK phosphorylation but failed to alter network formation; 2) a promiscuous molecular inhibitor of MAPK, MKP-1, blocked network formation, whereas inhibitors that reduce specifically the phosphorylated state of ERK1/2 (MKP-3 or DN-MEK) failed to block tubular morphogenesis; 3) pharmacological inhibition of the p38 MAPK resulted in a slight enhancement of network formation; 4) the JNK inhibitor, SP-600125 (5 μM), blocked network formation as well as JNK activity, and a second, structurally different JNK-1 inhibitor also blocked network formation; 5) pharmacological inhibitors of ERK phosphorylation, PD 98059 (20 μM) and U0126 (10 μM), partially attenuated JNK activity; and 6) a pharmacological inhibitor of JNK attenuated formation of small vessel branches during physiological angiogenesis in vivo in the CAM. Taken together, these results suggest, that in HLMVECs, the JNK pathway is important for functional network formation in 3-D Matrigel, whereas the ERK and p38 pathways play less significant roles. In support of these results, Bein et al.(2) also demonstrated a significant role for JNK in TGF-β-mediated capillary tube formation in bovine aortic endothelial cells in a fibrin sandwich 3-D assay. In addition, we believe that HLMVECs in culture may express only low levels of MKP-1 to permit the tubular assembly we observed when the cells were embedded in Matrigel.

It is not clear why HLMVECs did not align into networks in collagen I or fibrin gels. The most likely reason for this could be the lack of expression of relevant cell surface integrins that engage these proteins, a condition that is known to result in integrin-mediated cell death (IMD; Refs. 38, 39). Both proteins are known to promote tube formation of other endothelial cells and stabilize blood vessels, although the most significant phenotype of animals that have had either of these proteins knocked out is spontaneous hemorrhage (37). The human endothelial cells maintain a number of markers in culture under conditions that we carefully adhered to (specified by the manufacturer; see MATERIALS AND METHODS), which makes it unlikely, although not impossible, for surface integrin receptors to be significantly altered. We observed that Matrigel, a combination of laminin and collagen IV, supports morphogenesis of these HLMVECs that was inhibited by overexpression of MKP-1.

MKP-1 is a nuclear localized enzyme that is upregulated in response to stimuli such as endothelin-1 (15), hypoxia (23), and serum growth factors (3). Stress-responsive stimuli also activate the MAPKs p38 and JNK (9, 33) and often induce

Fig. 3. Effect of inhibition of p38 MAPK on HLMVEC network formation in Matrigel. A: HLMVECs were plated on Matrigel in the presence of vehicle (DMSO, control) or SB-203580 (10 μM). Representative bright field images indicating active network formation were quantified as relative tube length in the bar graph on the right. Twelve fields of cells under each condition from 5 separate experiments were studied. There was a modest but significant increase in the relative length of tubes formed after inhibition of p38 MAPK with SB-203580. B: the effect of SB-203580 on p38 MAPK activity was verified by determining the phosphorylation of heat shock protein 27 (HSP27), a downstream target of the p38 MAPK pathway, in HLMVECs: lane 1 = untreated cells, lane 2 = after stimulation of stress pathway p38 MAPK with anisomycin (200 nM for 30 min), lane 3 = after pretreatment with inhibitor SB-203580 (without anisomycin), and lane 4 = after stimulation with anisomycin in the presence of SB-203580. The bottom represents the same samples developed with antibody for HSP27 to demonstrate protein loading. The result demonstrates that SB-203580 was able to block activation of p38 MAPK that was induced by anisomycin.
Fig. 4. Effect of inhibition of JNK on HLMVEC network formation in Matrigel. A: representative bright field images of HLMVECs plated on Matrigel as described in MATERIALS AND METHODS in the presence of vehicle (DMSO), SP-600125 (5 μM), or the negative control supplied with SP-600125 are shown. The graph (bottom right) represents relative tube length in each sample. Ten to seventeen fields of cells for each condition and 3 separate experiments were completed for this set of investigations. The JNK inhibitor SP-600125, but not the same concentration of the structurally related negative control, significantly reduced tube length. B: the effect of SP-600125 on JNK activity was verified by treating HLMVECs with anisomycin to activate the JNK pathway followed by determining in vitro phosphorylation of the c-Jun substrate using immunoprecipitated JNK from top: lane 1 = untreated cells, lane 2 = after stimulation with anisomycin (200 nM for 30 min), lane 3 = anisomycin after pretreatment with JNK inhibitor SP-600125 (0.5 μM), lane 4 = anisomycin after pretreatment with JNK inhibitor-related compound (negative control, 0.5 μM), lane 5 = anisomycin after pretreatment with JNK inhibitor SP-600125 (2.5 μM), and lane 6 = anisomycin after pretreatment with JNK inhibitor-related compound (negative control, 2.5 μM). The Western blots were performed on the same passage of cells used for network formation. C: a second JNK inhibitor, which is active only against JNK-1 [L-stereoisomer (L-JNKI-1), 0.5 μM] but not the negative control, significantly reduced tube length. Eight fields of cells under each of these conditions were evaluated from these investigations. Representative images are observed in 3 labeled frames, and averaged results appear in the bottom right graph. D: cells were processed as described in B after treatments as follows: lane 1 = untreated cells, lane 2 = anisomycin (200 nM for 30 min), lane 3 = anisomycin after pretreatment with U0126 (10 μM), lane 4 = anisomycin after pretreatment with PD 98059 (20 μM), and lane 5 = anisomycin after pretreatment with PD 184352 (1 μM). SP-600125 (0.5 and 2.5 μM), U0126, and PD 98059 attenuated the anisomycin-induced phosphorylation of c-Jun substrate, whereas PD 184352 was not effective.
growth of new vessels. Specific induction of the kinases are balanced or modulated by expression of phosphatases that eventually inactivate them.

The stress-activated p38 MAPK pathway appears to be critical for cytokine production (24, 36). Specifically, activation of p38 MAPK stabilizes mRNA of critical inflammatory targets including TNF-α, COX-2, interleukins, etc. (24). Our data indicate that blockade of the p38 MAPK pathway with SB-203580 results in enhanced network formation. Bovine capillary endothelial cells embedded in collagen also exhibited increased tubulogenesis after inhibition of p38 MAPK (28). Combined with the observations that the SB compounds available are restricted to inhibition of the α- and β- forms of p38, the results indicate that these isoforms may be involved in attenuation of network formation. This idea is again supported by results in human umbilical vein endothelial cells (HUVECs) where SB-203580 blocked the time-dependent increase in apoptotic cells in a 3-D collagen matrix (44). Moreover, these authors demonstrated that the p38 MAPK pathway is involved in the activation of caspase-3. Whereas cell viability and morphology were retained in these experiments, HUVECs failed to finally coalesce, although they did exhibit unusually elongated cells bodies. One major difference between this study and ours was the use of a collagen matrix after the addition of angiogenic factors (bFGF, VEGF, and PMA), conditions that did not support network formation of HLMVECs. A separate study using bovine aortic endothelial cell aggregates in a fibrin sandwich 3-D assay demonstrated that inhibition of p38 MAPK reduced tube formation, although blockade of p38 MAPK failed to alter this process when elicited by the addition of TGF-β, the latter activity being dependent on the JNK signaling pathway (2). Taken together, these data suggest that p38 MAPK activity is not always essential for network formation and that the matrix, cell type, and stimuli may be critical factors in determining pathways of morphogenesis.

Our results as well as those of Bein et al. (2) point to participation of the JNK pathway in tubular assembly of HLMVECs and bovine aortic endothelial cells. Whereas we used Matrigel, the aortic cells were activated by TGF-β1 in a fibrin gel. HUVECs undergo vacuolation and lumen formation in fibrin gels, but unfortunately we were not able to examine the role of MAPKs in this matrix (1). The JNK pathway has also been described in other models of differentiation such as...
regulated dorsal closure, cell polarity, and convergent extension in genetic and overexpression in *Drosophila* and *Xenopus* (4, 29, 41). Whereas it is not possible to analyze network formation in isolation of other steps in angiogenesis in vivo, we established that JNK enhanced vascular branching in a developing model of physiologic angiogenesis, the CAM. This branching in the CAM may or may not engage the same receptor-ligand interaction as HLMVECs in Matrigel due to presence of different matrix, cell surface integrins, and source of endothelial cells.

The role of the ERK pathway in tube formation has been tested in HUVECs in 3-D collagen gels. Only after stimulation with angiogenic factors (bFGF, VEGF, and PMA) did these cells differentiate via activation of ERK1/2, Akt, and PKC (20). In a similar model, the MEK inhibitor PD 98059 (20 µM) attenuated tube formation by inducing apoptosis in collagen gels (44). Our data demonstrate that PD 98059 used at the same concentration also partially blocked JNK activity. A different model of proliferation and remodeling, VEGF-induced neovessel sprouting from a freshly cut rat aortic ring, described prolonged activation of ERK1/2 and a transient activation of p38 MAPK. In a separate report, VEGF-induced migration of endothelial cells was diminished by the p38 MAPK inhibitor SB-203580, whereas PD 98059 had little effect (27). Migration induced by the lipid sphingosine-1 phosphate was inhibited by the MEK1/2 inhibitor U0126 but not SB-203580 (13, 25), whereas other investigators demonstrated activation of both ERK and p38 MAPK (13) in stimulated migration. Thus there is a lack of consensus on the roles of MAPKs in the regulation of network formation, which once again suggests that these pathways may differ depending on the cell type, matrix, or angiogenic reagent being studied.

In summary (see Fig. 6), unlike previous reports of the role of the ERK pathway in endothelial tubulogenesis (20, 44), our results using multiple pharmacological blockers PD 98059 (20 µM), U0126 (10 µM), and PD 184352 (1 µM) as well as molecular inhibitors MKP-1, MKP-3, and DN-MEK suggest that the ERK pathway may not play a role in network formation of HLMVECs in Matrigel. However, some less specific ERK1/2 inhibitors do block network formation, making it difficult to entirely rule out the contribution of this pathway. We obtained similar results as other investigators regarding the role of p38 MAPK (28, 44) where inhibition of this pathway may promote assembly of endothelial networks in a 3-D matrix. There are few reports of the participation of JNK in endothelial tubulogenesis (20, 44), our results demonstrate that this stress-activated signaling system is essential for network formation of HLMVECs in a laminin/collagen IV matrix.

Although we observed inhibition of morphogenesis using the pharmacological inhibitors of ERK1/2 phosphorylation, U0126 and PD 98059, we believe this effect is due to their nonspecific actions on other MAPK pathways including signaling by JNK. Our observations highlight the importance of the use of multiple approaches, especially including molecular inhibitors, toward the study of signaling pathways. These and other studies to define the signaling pathways underlying organ-specific endothelial cell-mediated angiogenesis are essential steps to predict or intervene in conditions characterized by uncoordinated proliferation, network formation, apoptosis, or culture of capillaries in vitro. Our experiments using microvascular endothelial cells from human lung may be applicable to disorders of the pulmonary vasculature including pulmonary hypertension, emphsema, or revascularization after extensive lung injury that is caused by unchecked inflammation.

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**REFERENCES**

MAPK SIGNALING IN NETWORK FORMATION OF HUMAN LUNG ENDOTHELIAL CELLS


