Regulation of Angiogenesis by Vascular Endothelial Growth Factor and Angiopoietin-1 in the Rat Aorta Model

Distinct Temporal Patterns of Intracellular Signaling Correlate with Induction of Angiogenic Sprouting

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Vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) promote the spontaneous angiogenic response of freshly cut rat aortic rings. When VEGF and Ang-1 were tested in cultures of 14-day-old rings, which are quiescent and unable to spontaneously produce neovessels, only VEGF was capable of inducing an angiogenic response. Ang-1 failed to initiate angiogenesis in this system, but significantly potentiated VEGF-induced neovessel sprouting. Potential differences in cell signaling triggered by VEGF and Ang-1 were evaluated in cultures of quiescent rings. VEGF induced biphasic and prolonged (15 minutes and 4 to 24 hours) phosphorylation of p44/42 MAPK and Akt, while the effect of Ang-1 was transient and monophasic (15 minutes). Both VEGF and Ang-1 induced rapid, monophasic (15 minutes) phosphorylation of p38 MAPK. When VEGF and Ang-1 were administered together, the second peak of VEGF-induced p44/42 MAPK phosphorylation was markedly reduced. The effect of the VEGF/Ang-1 combination on AKT phosphorylation was, instead, additive over time, and sustained over a 24-hour period. The VEGF/Ang-1 combination caused an additive effect also on p38 MAPK phosphorylation at 1 hour. Confocal microscopy of VEGF-, Ang-1, or VEGF/Ang-1-stimulated aortic rings double stained at time points of maximal phosphorylation for cell markers and signal transduction proteins demonstrated phosphorylated p44/42 MAPK, p38 MAPK, and Akt predominantly in endothelial cells. Experiments with specific inhibitors demonstrated that p44/42 MAPK and Akt, but not

p38 MAPK, are necessary for neovessel sprouting. These results identify p44/42 MAPK and Akt as critical intracellular mediators of angiogenesis, whose transient phosphorylation is, however, not sufficient for the initiation of this process. The observation that sustained phosphorylation of these signaling pathways, particularly of Akt, correlates with induction of angiogenesis suggests that the duration of phosphorylation signals influences critical cellular events required for the induction of angiogenic sprouting. (*Am J Pathol* 2002, 161:823–830)

Angiogenesis, the formation of neovessels from the endothelium of pre-existing vessels, plays an essential role in embryonal development, tissue repair, and the progression of a variety of disease processes.^{1,2} Neovessels form in response to stimulation by soluble angiogenic factors, which regulate endothelial migration, proliferation, survival, and proteolytic activity. Among the factors that have been described to date, vascular endothelial growth factor (VEGF) and the angiopoietins have emerged as critical regulators of the angiogenic process.^{3–5} These molecules promote neovessel formation and morphogenesis by cooperating closely through a carefully orchestrated sequence of angioregulatory events.^{6,7}

VEGF, also designated as VEGF-A, belongs to a family of growth factors with predominant endothelial target-specificity.^{8,9} VEGF mainly binds to and activates two different receptor tyrosine kinases, designated Flt-1 (fms-like tyrosine kinase-1, VEGF receptor-1)¹⁰ and KDR (ki-

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nase-insert domain-containing receptor, VEGF receptor-2),¹¹ but it can activate other receptors such as neuropilin-1 and -2. VEGF promotes endothelial migration, proliferation, survival, and proteolytic activity.⁶

The angiopoietin family comprises at least four secreted proteins, angiopoietin (Ang)-1, -2, -3, and -4, all of which bind to the endothelial-specific receptor tyrosine kinase Tie-2. Ang-1 and Ang-4 have the ability to phosphorylate Tie-2,^{12,13} whereas Ang-2 and Ang-3 are believed to act as natural antagonists.^{5,13} Ang-2 may also function as an agonist when used at high concentration or for prolonged incubation times in the context of a three-dimensional matrix.^{14,15} Ang-1 promotes endothelial migration, survival, and proteolytic activity^{16–18} without significantly affecting endothelial proliferation.^{4,19}

VEGF and Ang-1 can both activate critical signaling pathways such as MAPK and Akt,^{20–23} but they differ significantly in their capacity to induce an angiogenic response and influence different steps of the angiogenic process.^{7,19} Although many studies have defined the angiogenic properties of VEGF and Ang-1, a gap remains in our understanding of how the unique biological signals elicited by these two growth factors are specifically transduced in the vessel wall during angiogenesis.

In the present study we used a modification of the rat aorta model to investigate signal transduction events occurring in the native aortic wall during angiogenesis.²⁴ In this system, VEGF and Ang-1 generate unique temporal patterns of MAPK and AKT phosphorylation, which correlate with the presence or absence of an angiogenic response. Evaluation of these phosphorylation patterns and underlying regulatory mechanisms may provide novel insights into the early signaling events required for the initiation of the angiogenic process.

Materials and Methods

Materials

The recombinant Ang-1 used for this study (Ang-1*) was obtained from Regeneron Pharmaceuticals, Inc. (Tarrytown, NY). Ang-1* is a genetically engineered variant of Ang-1 of which the first 77 residues are replaced with the first 73 residues of Ang-2 while a nonconserved cysteine is mutated to the corresponding serine residue of Ang-2. Endothelial basal medium (EBM) was obtained from Clonetics Corporation (San Diego, CA). The PhosphoPlus antibodies for Akt (Ser473), p44/42 MAPK (Thr202/Tyr204), and p38 MAPK (Thr180/Tyr182) were purchased from New England Biolabs Inc. (Beverly, MA). Protease inhibitors (Complete Tablet) were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). The bicinchoninic acid (BCA) protein assay was from Pierce (Rockford, IL) and the enhanced chemiluminescence (ECL) system from Amersham Pharmacia Biotech (Piscataway, NJ). MAPK inhibitors U0126 (p44/42 MAPK pathway),²⁵ SB203580 (p38 MAPK pathway),²⁶ and the PI3 kinase inhibitor LY294002 (Akt pathway)²⁷ were purchased from Calbiochem (La Jolla, CA). Monoclonal mouse anti- α smooth muscle actin antibody was purchased from

Sigma (St. Louis, MO). Alexa Fluor 488 conjugated Griffonia isolectin B4 (I-B4), and Alexa Fluor 488-conjugated goat anti-mouse antibody or Alexa Fluor 568-conjugated goat anti-rabbit antibody were obtained from Molecular Probes (Eugene, OR).

Rat Aorta Model of Angiogenesis

Freshly cut aortic rings obtained from 5- to 10-week-old Fischer 344 male rats were embedded in collagen gels and transferred to 16-mm wells (4-well NUNC dishes) each containing 0.5 ml serum-free EBM (Clonetics Corporation), as described.^{24,28} The medium was changed three times a week starting from day 3. Collagen gel cultures were treated with increasing concentrations of VEGF or Ang-1. To evaluate the role of signaling pathways in this system, cultures were treated with specific p44/42 MAPK, p38 MAPK, or Akt inhibitors (see above). Inhibitors were added to the medium from the beginning of the experiment and with each feeding. Controls were treated with vehicle alone (0.2% dimethyl sulfoxide (DMSO)).

The Modified Rat Aorta Model of Angiogenesis with Quiescent Rings

Aortic rings lose their spontaneous angiogenic activity after a 14-day-long pre-incubation step in repeated changes of serum-free medium before collagen embedding. This is due to the gradual decrease in the system of endogenous angiogenic factors released from the rings.²⁴ The angiogenic activity of rings made quiescent with this treatment can be reactivated by exogenous angiogenic factors such as bFGF and VEGF. This modified rat aorta model can be used to evaluate the capacity of growth factors to induce angiogenesis. We used this approach to compare the effect on angiogenesis of VEGF and Ang-1, and to analyze the relationship between induction of angiogenesis and phosphorylation of signal transduction pathways. Collagen gel cultures of quiescent aortic rings were treated with VEGF, Ang-1, the VEGF/Ang-1 combination, or left untreated.

Measurement of Angiogenesis

The angiogenic response of aortic cultures was measured in the live cultures by counting the number of neovessels over time, according to published criteria.²⁸ Branching morphogenesis was evaluated by counting branch points at the peak of angiogenic growth.

Signal Transduction in Quiescent Rat Aortic Rings

Quiescent aortic rings were treated with VEGF, Ang-1, or a combination of VEGF and Ang-1 for 5 minutes, 15 minutes, 1 hour, 4 hours, and 24 hours. Each group consisted of 6 to 8 rings. Rings were cultured individually in agarose-coated 16-mm wells (4-well NUNC dishes),

each containing 0.5 ml serum-free medium. Mechanical disturbance of the system was minimized by carefully replacing existing medium with growth factor-supplemented fresh medium, without touching the explants. At the end of treatment, aortic rings of each group were pooled together in a 35-mm well of a 6-well plate filled with ice-cold phosphate-buffered saline (PBS). Microdissection forceps were used to move the rings from the NUNC plate to the 6-well plate. This was accomplished by gently holding each ring by its adventitial edge with the tips of the forceps during transfer. Without shaking, the rings were allowed to sink to the bottom of the well in the PBS solution. Each group of rings was then transferred to a liquid nitrogen-filled 18-mm well of a 24-well culture plate, pulverized with a pre-chilled pestle, and solubilized on ice with 100 μ l ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L EDTA, 2 mmol/L EGTA, 0.02% sodium azide, 50 mmol/L NaF, 2 mmol/L vanadate, supplemented with protease inhibitors). The resulting slurry was then transferred to a 1.5 ml microcentrifuge tube, sonicated for 10 second on ice, and centrifuged at $1000 \times g$ for 10 minutes at 4°C. The supernatant was used for Western blot studies. Protein concentration was measured with the BCA assay.

Western Blotting

Samples were boiled for 3 minutes in SDS-PAGE sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.65 mmol/L dithiothreitol, 0.06% bromophenol blue, and 5% 2-mercaptoethanol). 10- μ g protein was loaded in each lane. After electrophoresis on a 4 to 20% gradient polyacrylamide gel, proteins were transferred to a nylon membrane and probed first with antibodies against the phosphorylated protein. The blots were then stripped and reprobed with an antibody against the total protein. Specific antibody binding was detected with the ECL system. The intensity of the bands was measured with a GS-710 Calibrated Imaging Densitometer (BIO-RAD, Hercules, CA). Degree of phosphorylation was obtained by calculating from the densitometric measurements the percentage of kinase phosphorylation.

Confocal Microscopy: Double Fluorescent Labeling

Double fluorescent labeling followed by confocal microscopy was used to histologically localize the phosphorylation signal in the aortic wall. Quiescent aortic rings were treated with VEGF, Ang-1, or the VEGF/Ang-1 combination to induce phosphorylation signals in the aortic explant. The rings were then embedded in OCT compound, snap-frozen in isopentane at -100°C , cryosectioned at a thickness of 10 μ m, placed on Fischer plus histology slides, and fixed in acetone at -20°C . Frozen sections were rehydrated in PBS and double stained for 1 hour with a cocktail of cell-specific markers (1-B4 for endothelial cells or anti- α -SMA antibody for smooth muscle cells) and antibodies against phosphorylated signaling pro-

teins (phospho-p44/42 MAPK, phospho-p38 MAPK, or phospho-Akt). Cell markers and phosphorylated proteins were visualized in different colors by fluorescent dyes (Alexa Fluor 488 or 568) conjugated to the appropriate secondary antibodies. For the double staining with the Griffonia isolectin B4, the lectin, which is directly conjugated with Alexa Fluor 488, was added at the same time as the Alexa Fluor 568-conjugated (Leica Microsystem, Heidelberg, Germany) secondary antibody against the signaling protein of interest. Slides were washed for 5 minutes $\times$ 3 with PBS between staining steps and mounted in Gelvatol (Monsanto, St. Louis, MO). Confocal microscopy was performed using a Leica TCS-SP Laser Scanning microscope. Images were evaluated by Z-plane analysis followed by projection and overlay using Leica software.

Statistical Analysis

Experiments included three to four observations per data point and were repeated at least two times. Results were analyzed with GraphPad Prism statistics software (GraphPad Software, Inc., San Diego, CA). Student's *t*-test or one-way analysis of variance followed by Newman-Keuls multiple comparison tests were used to evaluate whether differences between groups were significant. Statistical significance was set at $P < 0.05$.

Results

Ang-1 Promotes the Angiogenic Response of Freshly Cut Aortic Rings

Aortic rings embedded in collagen gel immediately after excision from the animal produced a self-limited angiogenic response, which could be dose-dependently stimulated with VEGF, as reported.²⁴ Angiogenesis in this system was also promoted by Ang-1, which significantly increased the number of both neovessel sprouts (162.5 ± 15.3 SEM in 0.67 μ g/ml Ang-1-treated cultures versus 111.7 ± 6.8 in untreated control, $n = 4$, $P < 0.05$) and branch points (49.33 ± 6.9 SEM in 0.67 μ g/ml Ang-1-treated cultures versus 17.7 ± 1.2 in untreated control, $n = 4$, $P < 0.05$).

Ang-1 Is Unable to Induce Angiogenesis but Potentiates the Effect of VEGF in Cultures of Quiescent Aortic Rings

Aortic rings pre-incubated in serum-free medium for 2 weeks under floating conditions fail to generate a significant angiogenic response when embedded in collagen gels.^{24,29} We used this model to determine whether Ang-1, which is capable of promoting an existing angiogenic response, is also capable of initiating neovessel sprouting from quiescent aortic explants. Unstimulated control rings exhibited only rare and short endothelial sprouts (Figure 1, A and G). Stimulation with VEGF greatly enhanced the angiogenic response (Figure 1, C and G).

Figure 1. Effect of Ang-1 on the VEGF-induced angiogenic response of aortic rings made quiescent by a 14-day-long preincubation step in repeated changes of serum-free medium. Quiescent aortic rings were cultured in collagen gel without (A) or with Ang-1 (0.67 µg/ml, B), VEGF (10 ng/ml, C and E) or the Ang-1/VEGF combination (D and F). Note: Ang-1 was unable to induce angiogenesis but potentiated VEGF-induced neovessel sprouting and branching morphogenesis (arrows indicate branch points). The Ang-1 effect on VEGF-treated cultures was dose-dependent (G; Bars = SEM; $n = 3$; *, $P < 0.01$. **, $P < 0.001$ versus VEGF).

Conversely, Ang-1 was unable to switch on the angiogenic process (Figure 1, B and G). Ang-1, however, dose-dependently enhanced VEGF-induced microvessel sprouting (Figure 1, D and G) and branching (Figure 1, E and F; number of branch points: 49.3 ± 7.1 in VEGF/Ang-1-treated cultures versus 20.3 ± 4.5 in cultures treated with VEGF alone, $n = 6$, $P < 0.01$).

Distinct Temporal Patterns of Intracellular Signaling Correlate with the Angiogenic Response of the Aortic Rings to VEGF and Ang-1

To elucidate the regulatory mechanisms underlying the response of the aortic wall to VEGF and Ang-1, we studied the effect of these factors on the phosphorylation of p44/42 MAPK, p38 MAPK, and Akt. Initial studies performed after a 15-minute incubation period revealed that both VEGF and Ang-1 were capable of stimulating the phosphorylation of p44/42 MAPK, p38 MAPK, and Akt. Thus, the mere phosphorylation of p44/42 MAPK, p38 MAPK, or Akt signal pathways did not appear sufficient for the induction of angiogenesis. To evaluate whether the duration of intracellular signaling might affect the angiogenic response, quiescent aortic rings were incubated with VEGF, Ang-1, or the VEGF/Ang-1 combination for different times ranging from 5 minutes to 24 hours. VEGF induced biphasic phosphorylation of p44/42 MAPK, while the effect of Ang-1 on this pathway was monophasic (Figure 2, top panel). Phosphorylation of

Figure 2. Time course of p44/42 MAPK (top), p38 MAPK (middle), and Akt kinase (bottom) phosphorylation in quiescent rat aortic rings treated with VEGF (10 ng/ml), Ang-1 (0.67 µg/ml), or both factors (V+A1) for 5' to 24 hours in serum-free medium. Untreated quiescent rings were used as control (C). SDS polyacrylamide gels were blotted onto nitrocellulose paper and reacted with phosphospecific antibodies (left). Blots were then stripped and re-probed with non-phosphospecific antibodies to demonstrate equal loading per lane. Percentage change of phosphorylation over control values was measured by densitometry (right). Note: VEGF induced biphasic and prolonged (15 minutes and 4 to 24 hours) phosphorylation of p44/42 MAPK, whereas the Ang-1 effect was transient and monophasic (15 minutes). When VEGF and Ang-1 were administered together, the second peak of VEGF-induced p44/42 MAPK phosphorylation was markedly reduced. Both VEGF and Ang-1 induced rapid, monophasic (15 minutes) phosphorylation of p38 MAPK; the VEGF/Ang-1 combination had an additive effect on p38 MAPK phosphorylation at 1 hour. VEGF induced biphasic and prolonged (15 minutes and 4 to 24 hours) phosphorylation of Akt, while the effect of Ang-1 was monophasic and transient (15 minutes). The effect of combined VEGF/Ang-1 treatment on AKT phosphorylation was additive over time, and sustained over a 24-hour period.

p44/42 MAPK in response to VEGF peaked at 15 minutes, decreased by 1 hour, increased a second time after 4 hours, and finally decreased close to baseline levels in 24 hours. Conversely, Ang-1 induced a single peak of phosphorylation at 15 minutes. When the quiescent rings were stimulated with the VEGF/Ang-1 combination, the Ang-1 pattern of p44/42 MAPK phosphorylation predominated, while the second peak of VEGF-induced phosphorylation was abrogated.

VEGF and Ang-1 both induced a monophasic phosphorylation of p38 MAPK, which was significantly elevated for the first hour of stimulation (Figure 2, middle panel). Phosphorylation of p38 MAPK increased significantly after 5 minutes, reached a peak at 15 minutes, then decreased gradually and remained around basal levels during the following 24 hours. VEGF and Ang-1 administered together had an additive effect on p38 MAPK phosphorylation at 1 hour.

VEGF induced biphasic phosphorylation of Akt kinase (Figure 2, bottom panel), with a small peak of phosphorylation at 15 minutes, followed by a decrease at 1 hour and a second peak at 4 hours. The second wave of VEGF-induced phosphorylation of Akt remained elevated during the following 24 hours. Ang-1-induced Akt phosphorylation was monophasic and peaked at 15 minutes

Figure 3. Confocal micrographs of cryosections from rat aortic rings treated with the VEGF/Ang-1 combination and double stained for endothelial cells (I-B4, green fluorescence) and phosphorylated p44/42 MAPK, p38 MAPK, or Akt kinase (red fluorescence). Shown are time points of maximal stimulation by Western analysis. Projection images showing both fluorescent signals demonstrate localization of phosphorylated signaling proteins predominantly in endothelial cells. Positive fluorescence in nonendothelial regions of the aortic wall corresponds to smooth muscle cell layers of the tunica media (confirmed by double staining with anti- α smooth muscle actin antibody; data not shown). Magnification, $\times 40$.

with a stronger signal than the one produced by VEGF during its first phase of Akt phosphorylation. Ang-1-induced Akt phosphorylation decreased gradually, reaching values close to basal level by 24 hours. In the presence of both VEGF and Ang-1, the phosphorylation pattern of Akt was characterized by a first strong peak of phosphorylation at 15 minutes, which was similar to that induced by Ang-1 and higher than the one generated by VEGF, and by a similarly robust second peak of phosphorylation at 24 hours indistinguishable from that obtained with VEGF alone. These two phases of Akt phosphorylation were separated by a slight decrease in Akt phosphorylation at 1 hour.

Localization of Phosphorylation Signals in the Aortic Wall

Confocal microscopy of VEGF-, Ang-1, or VEGF/Ang-1-stimulated aortic rings double stained at time points of maximal phosphorylation for cell markers and signal transduction proteins demonstrated phosphorylated p44/42 MAPK, p38 MAPK, and Akt predominantly in endothelial cells (Figure 3). This pattern was observed in rings treated with VEGF, Ang-1, or the VEGF/Ang-1 combination. Phosphorylation signals were demonstrated

also in some smooth muscle cells, with p44/42 MAPK showing the strongest staining reaction. Staining for phosphorylated p44/42 MAPK, p38 MAPK, or Akt was observed in the endothelium and smooth muscle cells of unstimulated controls. Differences in intensity of staining between treated rings and untreated controls were most noticeable in cultures reacted for phospho-p38 MAPK, which on Western analysis showed the greatest increase over control values (> 5 -fold).

The Angiogenic Response of the Rat Aorta Is Blocked by p44/42 MAPK and Akt Inhibitors

To evaluate the functional role of p44/42 MAPK, p38 MAPK, and Akt signal transduction pathways in the angiogenic response of the rat aorta, collagen gel cultures of freshly cut aortic rings were treated with specific inhibitors: U0126 (p44/42 MAPK pathway), SB203580 (p38 MAPK), and LY294002 (PI 3-kinase/Akt pathway) (Figure 4). U0126 blocked microvessel sprouting with only minor inhibitory effects on fibroblast growth. Angiogenesis was also blocked by LY294002, which partially inhibited fibroblast outgrowth. SB203580 did not affect microvessel sprouting, but it significantly reduced the number of non-endothelial cells in the cultures. The anti-angiogenic ef-

Figure 4. Effect of specific inhibitors of p38 MAPK, p44/42 MAPK, and Akt signaling pathways on rat aortic angiogenesis. Freshly cut rat aortic rings were embedded in collagen gel and cultured without (control) or with specific inhibitors of p38 MAPK (SB203580, 20 μ M), p44/42 MAPK (U0126, 10 μ M), or Akt (LY294002, 10 μ M) pathways. Microvessels were counted on day 6. Note: angiogenesis was blocked by inhibiting p44/42 MAPK or Akt. Inhibition of p38 MAPK had no effect on microvessel sprouting. The anti-angiogenic effect of U0126 and LY294002 was not due to toxicity because aortic rings initially treated with these inhibitors responded to VEGF stimulation after drug withdrawal (inset). Bars = SEM; $n = 4$.

fect of p44/42 MAPK and Akt pathway inhibitors was not due to toxicity, because aortic rings previously treated with U0126 or LY294002 produced a florid angiogenic response when re-embedded in new gels and cultured in inhibitor-free medium containing VEGF (Figure 4, inset).

Discussion

In this study we evaluated signal transduction events occurring in the rat aorta model of angiogenesis in response to two critical regulators of the angiogenic process: VEGF and Ang-1. Both VEGF and Ang-1 promoted the spontaneous angiogenic response of freshly cut rat aortic rings. To define potential differences in cell signaling between VEGF and Ang-1, we repeated these experiments with quiescent aortic rings, which release non-stimulatory doses of endogenous angiogenic factors, and are therefore more suitable than freshly cut rings for evaluating effects of exogenous VEGF and Ang-1. Quiescence was obtained by pre-incubating the explants in repeated changes of serum-free medium for 14 days.²⁴ Aortic rings treated in this manner exhibited lower baseline levels of p44/42 MAPK, p38 MAPK, and Akt activity. VEGF fully reactivated the angiogenic response of the rings, whereas Ang-1 was unable to initiate neovessel formation in this system. Maximal stimulatory effects were obtained by treating the cultures with the VEGF/Ang-1 combination. Surprisingly, there were no significant differences in p44/42 MAPK, p38 MAPK, and Akt kinase phosphorylation between VEGF- and Ang-1-treated cultures after 15 minutes. However, when VEGF and Ang-1 were evaluated for their capacity to phosphorylate these pathways over a 24-hour time period, it became apparent

Figure 5. Schematic drawing showing relationship between induction of angiogenesis and temporal phosphorylation patterns of p44/42 MAPK, p38 MAPK, and Akt in quiescent rat aortic rings treated with VEGF (10 ng/ml), Ang-1 (0.67 μ g/ml), or the VEGF/Ang-1 combination.

that each factor generated distinct signaling patterns of p44/42 MAPK and Akt phosphorylation, and combined treatment with both factors induced p44/42 MAPK, p38 MAPK, and Akt phosphorylation responses that were quantitatively and/or qualitatively different over time from responses to individual factors (Figure 5).

The observation that Ang-1, like VEGF, activates p44/42 MAPK, p38 MAPK, and Akt, taken together with the biological response of the aortic rings to these factors, indicates that the mere phosphorylation of these pathways is not sufficient for triggering angiogenic sprouting. Experiments with specific inhibitors, however, suggest that both p44/42 MAPK and Akt kinase are required for angiogenesis. These blocking studies are consistent with previous reports implicating the p44/42 MAPK and Akt pathways as critical intracellular regulators of the angiogenic process,^{21–23,30,31} but do not fully explain the difference in angiogenic capacity between VEGF and Ang-1. A possible explanation may be found in the temporal patterns of intracellular signaling elicited by these growth factors.

VEGF, which is able to initiate angiogenesis, elicits a biphasic and sustained phosphorylation of p44/42 MAPK and Akt kinase, whereas Ang-1 has a monophasic and transient effect on both pathways (Figure 5). The mechanisms and related mediators responsible for the second wave of p44/42 MAPK and Akt phosphorylation in the rat aorta model are unclear because VEGF is known to induce only transient phosphorylation of these pathways in isolated endothelial cells.^{20,32,33} The observation by confocal microscopy that phosphorylated p44/42 MAPK and Akt predominate in endothelial cells but can be demonstrated also in some smooth muscle cells raises the possibility that smooth muscle cells contribute to this process. More studies are, however, needed to define the significance of these observations, and to clarify the nature of the cross talk between endothelial cells and smooth muscle cells in the early stages of angiogenesis.

By comparing the biological effects of VEGF and Ang-1 and the temporal patterns of p44/42 MAPK phosphorylation by these factors, one may expect a causative link between the second peak of VEGF-mediated p44/42 MAPK phosphorylation and the induction of angiogenesis (Figure 5). This would also be consistent with previous

reports indicating that mitogenicity correlates with growth factor ability to induce sustained phosphorylation of p44/42 MAPK.³⁴ However, Ang-1, which potentiates the angiogenic effect of VEGF in the rat aorta model, almost completely abrogates the VEGF-induced late p44/42 MAPK phosphorylation when administered together with this factor, suggesting that the second peak of p44/42 MAPK phosphorylation is probably not required for VEGF-induced angiogenesis. The Ang-1-mediated abrogation of the second peak of p44/42 MAPK phosphorylation in cultures treated with the VEGF/Ang-1 combination may be due to the reported ability of Ang-1 to activate intracellular phosphatases.^{35,36}

Of the signaling pathways evaluated in this study the one whose phosphorylation best correlates with induction of angiogenesis is the Akt pathway. Several points support this interpretation. First, inhibition of the Akt pathway with a specific PI-3 kinase inhibitor blocks angiogenesis. Second, induction of angiogenesis is observed only when the Akt pathway phosphorylation is sustained over a 24-hour period. Third, the potentiation of VEGF-induced angiogenesis by Ang-1 correlates with a marked enhancement of Akt phosphorylation over time. The finding of a strong link between sustained phosphorylation of the Akt pathway and induction of endothelial sprouting is consistent with previous studies indicating that genetic and pharmacological modulation of PI-3 kinase function can significantly influence angiogenesis. Akt may regulate initiation of angiogenic sprouting through its stimulatory effects on endothelial cell migration and actin reorganization, proliferation, and survival.^{21,22,37,38} Akt has also the capacity to mediate MMP production.³⁹

Experiments with the p38 MAPK inhibitor SB203580 indicate that the p38 MAPK pathway, which is activated by both VEGF and Ang-1, is not required for the induction of endothelial sprouting in the rat aorta model. This interpretation is corroborated by a previous report that p38 MAPK inhibition does not block but actually enhances endothelial cell proliferation.³³ Genetic ablation studies in mice, however, indicate that p38 α and its upstream activator Mekk3 are required for adequate blood vessel development.^{40,41} A possible explanation of these results may be that the p38 MAPK pathway affects angiogenesis indirectly by influencing the behavior of nonendothelial cells critical for the successful outcome of the angiogenic process.⁴² In our system, we found that aortic outgrowths treated with SB203580 have a markedly reduced number of nonendothelial cells. These cells may secrete proangiogenic cytokines, whose production is regulated by the p38 MAPK pathway.⁴³ Activation of p38 MAPK by this mechanism may regulate angiogenesis not only during development but also in reactive processes and pathological conditions.

In conclusion, this study demonstrates that VEGF and Ang-1 engage signal transduction pathways in temporally distinct manners that correlate with the biological response of the vessel wall. Our results indicate that p44/42 MAPK and Akt represent important intracellular regulators of angiogenesis, whose transient phosphorylation is, however, not sufficient for the initiation of the angiogenic process. The observation that prolonged

phosphorylation of these pathways, particularly of Akt, correlates with induction of angiogenic sprouting and potentiation of the angiogenic response suggests that critical angiogenic signals are generated only under conditions of sustained phosphorylation of a selected group of signaling molecules. Identification of these mediators and their downstream targets may define key molecular checkpoints in the intracellular cascade of signaling events required for the formation of new blood vessels.

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