Angiopoietin-1 Induces Endothelial Cell Sprouting Through the Activation of Focal Adhesion Kinase and Plasmin Secretion

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Abstract—Angiopoietin-1 (Ang1) is a strong inducer of endothelial cell sprouting, which is a first step in both angiogenesis and neovascularization. We examined the mechanisms underlying Ang1-induced cell sprouting using porcine pulmonary artery endothelial cells. Ang1 induced the nondirectional and directional migration of endothelial cells mediated through the Tie2 but not the Tie1 receptor. Ang1 induced tyrosine phosphorylation of p125FAK, and this phosphorylation was dependent on phosphatidylinositol (PI) 3'-kinase activity. Ang1 induced the secretion of plasmin and matrix metalloproteinase-2 (MMP-2), which is inhibited by PI 3'-kinase inhibitors. Ang1 also induced the secretion of small amounts of proMMP-3 and proMMP-9 but not proMMP-1. Ang1 suppressed the secretion of tissue inhibitor of metalloproteinase-2 (TIMP-2), but not of TIMP-1. Addition of α2-antiplasmin, a combination of TIMP-1 and TIMP-2, or PI 3'-kinase inhibitors inhibited Ang1-induced sprouting activity. Therefore, Ang1-induced sprouting activity in endothelial cells may be accomplished by cytoskeletal changes and secretion of proteinases and may be largely mediated through intracellular PI 3'-kinase activation. (Circ Res. 2000;86:952-959.)

Key Words: angiopoietin-1 ▪ sprouting ▪ p125FAK ▪ plasmin

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Pharmaceuticals, Inc. Ang1* is a recombinant version of Ang1 with modified NH₂ terminus and mutated Cys²⁴⁵ that is easier to produce and purify. Mutation of Cys²⁴⁵ in Ang1, which is not shared between Ang1 and Ang2, does not alter its agonistic property. Recombinant human vascular endothelial growth factor₁₆₅ (VEGF₁₆₅) was purchased from R&D systems. Phosphatidylinositol (PI) 3'-kinase inhibitors wortmannin and LY294002 were purchased from RBI, Inc. Recombinant human TIMP-1 and TIMP-2 were purchased from Fuji Chemical Industries. Media and sera were obtained from Life Technologies, Inc. Most other biochemical reagents were purchased from Sigma, unless otherwise specified. All cells used in this study were porcine pulmonary artery endothelial cells (PPAECs). PPAECs were prepared from porcine pulmonary arteries by collagenase digestion and maintained as previously described.¹¹

Sprouting Assay
The sprouting assay in PPAECs was performed as previously described.¹¹ Briefly, PPAECs were grown to confluence on microcarrier beads (diameter 175 μm; Sigma) and placed in a 2.5 mg/mL fibrinogen gel containing 2.0% heat-inactivated FBS and the indicated recombinant protein. Fibrin gels were incubated in DMEM with a daily addition of the same amount of recombinant protein. After 3 days, 2 independent, blinded investigators counted the number of sprouts with the use of an inverted microscope.

Nondirectional and Directional Migration Assays
For the nondirectional migration assay, the microcarrier bead migration method was used.²³ For the directional migration assay, the method using a modified Boyden chamber (Neuroprobe, Inc) was used.

p125 FAK and Paxillin Tyrosine Phosphorylation Assay
PPAECs were incubated in serum-free DMEM for 24 hours. Then, Ang1* was added at the indicated amounts and incubated for the indicated times. The phosphorylation status of immunoprecipitated protein was detected by Western blot analysis using anti-phosphotyrosine, anti-p125 FAK, or anti-paxillin antibody as previously described.¹¹

Measurements of Plasmin and MMPs in Culture Medium
Confluent PPAECs were incubated in serum-free and phenol red-free DMEM for 24 hours. After the cells were washed with fresh medium, control buffer, Ang1*, or VEGF was applied for the indicated times. Plasmin activities were measured in the media by fibrin-zymography and by a colorimetric assay according to the manufacturer’s protocol (Chromozym PL, Boehringer Mannheim). The actual amounts of the proforms of the MMPs (proMMPs), TIMP-1, and TIMP-2 were assayed with enzyme immunoassays according to the manufacturer’s protocol (Fuji Chemical Industries). The hydrolytic activities of MMP-2 and MMP-9 were measured by gelatin-zymography as previously described.²⁴

Statistics
Data are expressed as mean±SD. Statistical significance was tested using 1-way ANOVA followed by the Student-Newman-Keuls test. Statistical significance was set at P<0.05.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results
Ang1*, but Not Ang2, Induces Sprouting in PPAECs
The placement of microcarrier beads onto a confluent monolayer of PPAECs for 2 to 3 days produces beads covered by a confluent monolayer of cells with ~25 to 30 cells per bead. These beads were embedded in 3-dimensional fibrin gels and cultured. Daily addition of Ang1* (50 to 400 ng/mL) increased sprout formation in a dose-dependent manner, whereas Ang2 (50 to 400 ng/mL) did not increase sprout formation (Figures 1A and 1B). Koblizek et al obtained similar results with adrenal cortex–derived microvascular endothelial cells, although the magnitude of sprout formation...
was somewhat lower than ours.\textsuperscript{6} As a positive control, VEGF (1 to 10 ng/mL) increased sprout formation in a dose-dependent manner (Figures 1A and 1B). In general, the relative potency in sprouting formation produced by VEGF was higher than that produced by Ang1*.

**Ang1*, but Not Ang2, Induces Nondirectional and Directional Migration for PPAECs Through Tie-2 Receptor Binding**

When PPAEC-bearing microcarrier beads were placed onto gelatinized plastic dishes with control buffer for 20 hours, they yielded a basal level of nondirectional migration (\(\approx 40\) to \(\approx 45\) cells per 10 beads; Figures 2A and 2B). The number of migrating cells increased with Ang1* stimulation in a dose-dependent manner. In contrast, Ang2 (50 to 400 ng/mL) did not produce any increase in nondirectional migration. Consistent with a previous report,\textsuperscript{7} Ang1*, but not Ang2, also induced directional (chemotactic) migration in a dose-dependent manner (Figure 2B). As a positive control, VEGF induced nondirectional and directional migration in a dose-dependent manner (Figure 2B). A 5-fold molar excess of rTie2-Fc, but not rTie1-Fc, almost completely blocked the nondirectional and directional migratory effects of Ang1* (Figure 2B).

**Ang1* Induces p125 FAK and Paxillin Phosphorylation**

Because Ang1* has a migratory effect in endothelial cells, we examined whether Ang1* could stimulate tyrosine phosphorylation of p125FAK and paxillin. Ang1* (200 ng/mL) induced p125 FAK and paxillin phosphorylation as early as 5 minutes and produced a maximal effect at 10 minutes (Figures 3A and 3B). These effects declined but continued to be higher than control levels at up to 30 to 60 minutes. The maximum mean increases in p125 FAK and paxillin phosphorylation were 3.6- and 2.6-fold, respectively. Ang1* induced p125 FAK and paxillin phosphorylation at 10 minutes in a dose-dependent manner (Figures 3A and 3B). Thus, the Ang1*-induced phosphorylation of p125 FAK and of paxillin occurred in the same time frame, and to a similar extent. In contrast, Ang2 (200 and 400 ng/mL) made no change in either p125 FAK or paxillin phosphorylation at 10 minutes after stimulation (data not shown).

**PI 3’-Kinase Inhibitors Suppress Ang1*-Induced Tyrosine Phosphorylation of p125FAK and Migration**

To examine the involvement of PI 3’-kinase in Ang1*-induced tyrosine phosphorylation of p125 FAK and migration in directional migratory activity. Control buffer, Ang1* (200 ng/mL), Ang2 (200 ng/mL), or VEGF\textsubscript{165} (10 ng/mL) was added. The beads were stained with Giemsa solution. More migrating cells can be seen around the surface of well in Ang1*- or VEGF-treated beads. B, Quantification of nondirectional (a and b) and directional (c and d) migratory activities. Various amounts of recombinant protein were added to the beads (a and c). Ang1* (200 ng/mL) and control buffer (C) or a 5-fold molar excess (2 \(\mu\)g/mL) of rTie2-Fc or rTie1-Fc was added to the beads (b and d). HPF indicates high-power field (\(\times 100\)). Data are mean\(\pm\)SD from 5 experiments. *\(P\leq 0.05\) vs control.
endothelial cells, we examined the effects of PI 3'-kinase inhibitors on Ang1*-induced p125FAK phosphorylation at the peak time point (10 minutes) and migration. PPAECs were preincubated with wortmannin or the structurally unrelated synthetic PI 3'-kinase–specific inhibitor, LY294002. Wortmannin or LY294002 almost completely inhibited the Ang1*-induced p125FAK tyrosine phosphorylation and nondirectional and directional migration (Figures 4 and 5).

**Figure 3.** Effect of Ang1* on p125FAK and paxillin phosphorylation in PPAECs. A, Top, Tyrosine-phosphorylated p125FAK (a and b) and paxillin (c and d). Bottom, Total amount of p125FAK (a and b) and paxillin (c and d). Cells were exposed to Ang1* for the indicated times and amounts and Western blotted. B, Densitometric analyses of the blots are presented as relative ratios of phosphoprotein/total protein. Ratio in the control is arbitrarily presented as 1. Data are mean±SD from 3 experiments. *P<0.05 vs control.

**Ang1* Induces Plasmin and MMP Secretion but Suppresses TIMP-2 Secretion**

Because of the ability of Ang1 to induce cells to sprout in fibrin gels, we examined whether Ang1* causes plasmin secretion from PPAECs. Addition of Ang1* (200 ng/mL) or VEGF (10 ng/mL) produced ~3.5- or 4.8-fold increases, respectively, in plasmin secretion for 3 hours compared with addition of control buffer (Figure 6A). The plasmin secretion was confirmed by
fibrin zymography (Figures 7A and 7B). Culture medium from Ang1*-or VEGF-treated cells clearly had increased 85-kDa fibrinolytic bands compared with the cells treated with buffer alone (Figure 7A). This effect was still observed in cell media collected 24 hours after treatment (Figure 7A).

Preliminary enzyme immunoassay showed that the culture media from Ang1*- or VEGF-treated cells clearly had increased ~85-kDa fibrinolytic bands compared with the cells treated with buffer alone (Figure 7A). This effect was still observed in cell media collected 24 hours after treatment (Figure 7A).

Figure 4. Effect of PI 3'-kinase inhibitors on Ang1*-induced p125FAK tyrosine phosphorylation in PPAECs. Cells were pretreated for 30 minutes with wortmannin (WT, 30 nmol/L) or LY294002 (LY, 100 nmol/L). Then, cells were incubated with Ang1* (200 ng/mL) for another 10 minutes. Top, Tyrosine-phosphorylated p125FAK. Middle, Total amount of p125FAK. Bottom, Fold induction of phosphorylated p125FAK/total p125FAK, comparing Ang1* treatment with control. Data are mean±SD from 3 experiments.

Figure 5. Effect of PI 3'-kinase inhibitors on Ang1*-induced nondirectional (A) and directional (B) migration activity. PPAECs were grown to confluence on microcarrier beads, which were then placed in gelatinized 24-well plates in medium containing 2.0% FBS with addition of buffer, Ang1* (200 ng/mL), or VEGF (10 ng/mL) was added to 0.5 mL of the same culture medium, cells were incubated for 3 hours, and media were assayed. Control buffer (C), wortmannin (W, 10 nmol/L) or LY294002 (L, 30 nmol/L) was pretreated 30 minutes before Ang1* or VEGF addition. Data are mean±SD from 5 or 6 experiments. *P<0.05 vs control; †P<0.05 vs Ang1*; ‡P<0.05 vs VEGF only.

Figure 6. Effect of Ang1* and VEGF on secretion of plasmin (A), proMMPs (B), and TIMPs (C) in the absence and presence of PI 3'-kinase inhibitors in PPAECs. Cells were incubated in serum-free and phenol red-free DMEM for 24 hours. Control buffer (Co or Cont), Ang1* (200 ng/mL), or VEGF (10 ng/mL) was added to proMMP-2, whereas proMMP-1, proMMP-3, and proMMP-9 levels were low or undetectable (data not shown). Addition of Ang1* (200 ng/mL) or VEGF (10 ng/mL) for 3 hours produced ~2.2- or 3.3-fold increases, respectively, in proMMP-2 secretion, compared with control buffer (Figure 6B). Although Ang1* or VEGF produced a significant induction of proMMP-3 and proMMP-9 secretion, their increased amounts were low (Figure 6B). Neither Ang1* nor VEGF induced proMMP-1 secretion (Figure 6B). The profiles of MMP-2 and MMP-9 in the media were semiquantiatively assayed by gelatin zymography. Consistent with the results obtained from the enzyme immunoassays, gelatin zymography revealed that proMMP-2 secretion (~68 kDa) was
dominant (Figure 7A). Ang1* or VEGF produced 1.6- or 1.8-fold induction, respectively, of proMMP-2 secretion in the media at 3 hours (Figures 7A and 7B). At 24 hours after Ang1* or VEGF addition, the similar pattern of proMMP-2 secretion was observed (Figures 7A and 7B). Higher size (∼74 kDa) of gelatinolytic bands were observed in the media at 3 hours and were increased at 24 hours. Addition of Ang1* or VEGF induced these gelatinolytic activities similarly with patterns that resembled proMMP-2. In addition, increased proMMP-9 (∼92 kDa) and active MMP-2 (∼62 kDa) secretions were detected, although their amounts were low (Figures 7A and 7B). Addition of Ang1* or VEGF for 3 hours produced ∼45% or 60% suppression in the basal secretion of TIMP-2 (Figure 6C). However, the basal secretion of TIMP-1 was low, and the level was not changed by treatment with either Ang1* or VEGF.

We examined the effect of PI 3'-kinase inhibitors on secretion of plasmin, MMP-2, and TIMP-2 from PPAECs. Preincubation with wortmannin (10 nmol/L) or LY294002 (30 nmol/L) produced ∼50% to 60% suppression in Ang1*- and VEGF-induced plasmin secretion and ∼60% to 65% suppression in the Ang1*- and VEGF-induced MMP-2 secretion (Figures 6A and 6B). These results were confirmed by gelatin and fibrin zymography (Figures 7C and 7D). Interestingly, addition of wortmannin (10 nmol/L) or LY294002 (30 nmol/L) produced 34% suppression in the basal secretion of TIMP-2 (Figure 6C). Under these conditions, addition of Ang1* and VEGF produced a further 45% to 50% suppression in TIMP-2 secretion. These results suggest that activation of PI 3'-kinase may be involved in Ang1*- and VEGF-induced plasmin and MMP-2 secretions, but it may not be involved in suppression of TIMP-2 secretion.

α2-Antiplasmin, a Combination of TIMP-1 and TIMP-2, or PI 3'-Kinase Inhibitors Suppress Ang1*-Induced Sprouting Activity

Because Ang1* induced the secretion of plasmin, MMP-2, and TIMP-2, we examined the effect of α2-antiplasmin (100 mU, added daily) on sprouting activity of cells grown on microcarrier beads in fibrin gels. Addition of α2-antiplasmin produced ∼53% suppression of Ang1*-induced sprouting activity (Figures 8A and 8B). Given that the secretion ratio of MMPs to TIMPs was increased by Ang1*, we examined the effect of TIMPs on Ang1*-induced sprouting. Although the addition of either TIMP-1 (100 ng/mL) or TIMP-2 (100 ng/mL) did not produce a significant suppression of sprouting, a combination of TIMP-1 and TIMP-2 produced ∼36% suppression of Ang1*-induced sprouting activity (Figures 8A and 8B). Given that PI 3'-kinase inhibitors suppress migratory activity and secretion of plasmin and MMP-2, we examined the effect of PI 3'-kinase inhibitors on sprouting. Addition of wortmannin (10 nmol/L) or LY294002 (30 nmol/L) produced 68% or 61% suppression of Ang1*-induced sprouting activity, respectively (Figures 8A and 8B).

Discussion

Endothelial cell sprouting is an initial step in angiogenesis and neovascularization. This process requires cell migration and invasion into the extracellular matrix beneath the basement membrane. During initial angiogenesis and vasculogenesis, a variety of growth factors and cytokines are upregulated and exert their functions through autocrine or
paracrine actions. Of these, VEGF and Ang1 may be the key molecules, because their receptors are selectively located in endothelial cells.\textsuperscript{25,26} Recent reports indicate that transgenic overexpression or gene transfer of Ang1 increases vascularization.\textsuperscript{4,5} Thus, Ang1 is a reasonable candidate for therapeutic neovascularization for ischemic hearts or limbs. However, the exact mechanisms governing the increased vascularization with Ang1 overexpression are not yet known. Here, our findings explain how Ang1 induces sprouting in endothelial cells for increasing vascularization.

A member of the non–receptor protein-tyrosine kinases, p125\textsuperscript{FAK}, plays a key role in regulating dynamic changes in actin cytoskeleton organization during migration.\textsuperscript{15} Our results indicate that Ang1 induces tyrosine phosphorylation of p125\textsuperscript{FAK} and paxillin. These phosphorylation events take place rapidly, in a time- and concentration-dependent manner in endothelial cells. Thus, the migratory effect of Ang1 in endothelial cells may be mediated through actin cytoskeleton reorganization by tyrosine-phosphorylated p125\textsuperscript{FAK} and paxillin. We next investigated how Ang1 phosphorylated p125\textsuperscript{FAK}.

Recent studies indicated that Tie2 activates PI\textsubscript{3} kinase through an association with the p85 regulatory unit.\textsuperscript{27,28} Our results indicate that the PI\textsubscript{3} kinase inhibitors completely inhibit Ang1-stimulated tyrosine phosphorylation of p125\textsuperscript{FAK} in endothelial cells and migration. This result suggests that PI\textsubscript{3} kinase lies upstream in the signal transduction pathway linking Tie2 to the tyrosine phosphorylation of p125\textsuperscript{FAK} and migration. Therefore, we conclude that PI\textsubscript{3} kinase activation is an essential intracellular element in Ang1-induced cell migration through tyrosine phosphorylation of p125\textsuperscript{FAK}.

To produce sprouting in response to Ang1 or VEGF stimulation in an in vitro fibrin gel, endothelial cells must secrete fibrinolytic enzymes. To date, the ability of endothelial cells to mediate fibrinolytic activity has been largely attributed to the powerful fibrinolysin, plasmin.\textsuperscript{29} As we expected, Ang1 and VEGF induced plasmin secretion. However, a recent study identified MMP-dependent fibrinolytic pathways in the endothelial cells during neovascularization in fibrin gels.\textsuperscript{30} However, our PPAECs secrete mainly MMP-2, which exhibits a lack of fibrinolytic activity.\textsuperscript{30} Indeed, our fibrin zymography did not produce fibrinolytic bands where MMP-2 was active, whereas it produced strong fibrinolytic bands where plasmin was active. Our gelatin-zymographic assay reveals that Ang1, like VEGF, is a stimulant for secretion of proMMP-2. Although plenty of proMMP-2 is secreted and accumulated in the medium by Ang1 or VEGF stimulation, the conversion from proMMP-2 to active MMP-2 is not proportional. Cell membrane–associated processing with membrane type 1-MMP and TIMP-2 may be required for conversion to active MMP-2.\textsuperscript{31} Some larger (>74 kDa) gelatinolytic bands of unknown nature are observed in the culture medium of PPAECs. They could be proMMP-2 bound with an unknown protein or unknown gelatinase. The balanced ratio between the levels of MMPs and TIMPs is a critical factor in regulating the breakdown of matrix proteins by MMPs.\textsuperscript{21,22} Our results indicate that TIMP-1 secretion in PPAECs. Ang1, like VEGF,\textsuperscript{32} decreases TIMP-2, but not TIMP-1, secretion. Therefore, the increased ratio between MMPs and TIMPs by Ang1 is favorable for the degradation of matrix proteins. Notably, our results indicate that
activation of PI 3′-kinase could be involved in Ang1*- and VEGF-induced plasmin and MMP-2 secretion but not in TIMP-2 secretion. The mechanisms by which PI 3′-kinase is involved in Ang1*- and VEGF-induced plasmin and MMP-2 secretions will be examined in future studies.

Given that our sprouting activities were measured in fibrin gels, Ang1-induced plasmin secretion, rather than Ang1-induced MMP-2 secretion, could be a major determinant for sprouting. Consistent with this idea, addition of α2-antiplasmin produced a more pronounced suppressive effect than combination of TIMP-1 and TIMP-2 on Ang1-induced sprouting activity. However, the suppressive effect of the combination of TIMP-1 and TIMP-2 was unexpected. Previous studies indicate that TIMPs have several other functions, including inhibition of basic fibroblast growth factor–induced endothelial cell proliferation and endothelial tube formation. This work was supported by the Creative Research Initiatives of the Korean Ministry of Science and Technology. We thank Peter C. Maisonpierre and George D. Yancopoulos (Regeneron Pharmaceuticals, Inc) for providing critical angiopoietins and Tie reagents.

In summary, the present results explain how Ang1 induces sprouting in endothelial cells. Ang1 induces endothelial cell migration mediated through Tie2 receptor binding and PI 3′-kinase activation. The Ang1-induced migratory effect might be mediated through tyrosine phosphorylation of p125FAK in a manner that requires PI 3′-kinase activity. Increased plasmin and MMP-2 secretion from endothelial cells by Ang1 is also an important determinant for inducing sprouting. These secretions are inhibited by PI 3′-kinase inhibitors. Taken together, Ang1-induced sprouting process in vivo may be accomplished by enhanced cytoskeletal changes and secretion of proteinases mainly mediated through intracellular PI 3′-kinase activation.

Acknowledgments

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