Induction of tube formation by angiopoietin-1 in endothelial cell/fibroblast co-culture is dependent on endogenous VEGF

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The angiopoietin-1 (Ang1)/Tie2 receptor system is known to be important for angiogenesis and vascular remodeling. However, its contribution to the survival and morphogenesis of endothelial cells is still not well elucidated. In this study, we analyzed the role of the Ang1/Tie2 pathway in cell survival and tube formation using a human umbilical vein endothelial (HUVE) cell and fibroblast co-culture system. In this system, which mimics angiogenesis *in vivo*, fibroblasts secrete a basal level of vascular endothelial growth factor (VEGF), and Ang1 stimulated tube formation. However, anti-VEGF or anti-VEGF receptor-2 neutralizing antibody blocked the Ang1-induced tube formation. Furthermore, other angiogenic factors such as hepatic growth factor (HGF) and basic fibroblast growth factor (bFGF) showed the same phenotype as Ang1, i.e., a stimulatory effect only in the presence of endogenous VEGF. The Ang1-promoted tube formation was mainly due to suppression of HUVE cell apoptosis in a PI3-kinase-dependent manner. These findings suggest that Ang1 stimulates tube formation *in vivo* via the PI3-kinase/Akt pathway, but this effect takes place only in a VEGF-dependent manner. (Cancer Sci 2003; 94: 782–790)

Angiogenesis plays an important role in embryogenesis and tumorigenesis.[1–5] It is a complicated multi-step process, which includes dynamic changes of cell-cell and cell-matrix interactions, endothelial cell proliferation and migration, the recruitment of peri-vascular supporting cells, and maturation.

Angiopoietin-1 (Ang1) and Ang2 have recently been identified as ligands of the endothelial cell-specific Tie2 receptor.[6, 7] *In vivo* analysis of targeted disruption and transgenic overexpression revealed that Ang1 is responsible for recruiting and sustaining peri-endothelial support cells and Ang2 disrupts blood vessel formation in the developing embryo by antagonizing the effects of Ang1 on Tie2.[7, 8] Interestingly, transgenic overexpression or gene transfer of Ang1 increases vascularization *in vivo*.[9, 10] Unlike most angiogenic factors, including basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF), Ang1 does not stimulate endothelial cell growth, but rather promotes sprouting, chemotactic responses and network stabilization.[11–15] Also, Ang1 inhibits apoptosis induced under conditions such as serum-depletion in endothelial cells.[16–18] However, the signaling pathways underlying these biological processes are still not well characterized.

The PI3-kinase/Akt pathway has been shown to be a common feature in the signal transduction of the anti-apoptotic effects of growth factors.[19] In the present study, we used a recently developed system for human umbilical vein endothelial (HUVE) cell and fibroblast co-culture, and compared the effects of Ang1 and other angiogenic factors on the survival and morphogenesis of HUVE cells. In this co-culture system of HUVE cells and fibroblasts, as well as a regular HUVE cell monolayer culture system, we demonstrate that Ang1 stimulates tube formation by blocking HUVE cell apoptosis via PI3-kinase-dependent survival signaling. Interestingly, however, these effects are highly dependent on the basal level of VEGF. These results suggest that Ang1 induces an anti-apoptotic effect on endothelial cells and promotes tube formation synergistically with endogenous VEGF *in vivo*.

Materials and Methods

Cell cultures. HUVE cells were purchased from Kurabo (Tokyo) and maintained in a HuMedia-EG2 medium (Kurabo) according to the manufacturer's instructions.

Recombinant proteins. Recombinant Ang1* was kindly provided by Dr. G. D. Yancopoulos (Regeneron Pharmaceuticals, Inc., Tarrytown, NY). Ang1* differs from native Ang1 in that it possesses a modified NH_2-terminal sequence and a mutation in Cys_{245}. These mutations were shown to be effective for production and purification of Ang1* protein. Ang2 was purchased from R&D Systems, Inc. (Minneapolis, MN).

To prepare soluble recombinant human Tie2-Fc, the ectodomain of Tie2 (the nucleotide residues 82 to 2340, the *Eco*RI to *Bst*EII fragment) was fused to the Fc portion (about 0.6 kb) of the human IgG heavy chain with a 33-mer oligonucleotide linker. Tie2-Fc protein was produced according to standard protocols in Sf9 cells infected with baculovirus vector bearing the Tie2-Fc construct, and then purified on a "HiTrap" rProtein A column (Amersham Pharmacia Biotech, Uppsala, Sweden).

Recombinant VEGF-A (165 amino acid form) was purified by heparin column chromatography from the conditioned medium of Sf9 insect cells expressing the *VEGF-A* gene.[20] Recombinant human hepatic growth factor (HGF) and bFGF were obtained from R&D Systems, Inc. and Oncogene Research Products (Cambridge, MA), respectively. VEGF-E_{NZ-7} which is a ligand specific to KDR (VEGFR-2) was prepared as described previously.[21]

Chemicals. Wortmannin, LY294002, PD98059 and Hoechst 33258 were purchased from Sigma Chemical Co. (St. Louis, MO).

Human VEGF assay. The concentration of human VEGF in the culture medium was determined by an ELISA kit (Quantikine Colorimetric Sandwich ELISA human VEGF Kit) obtained from R&D Systems, Inc.

Antibodies. Rabbit polyclonal anti-human Tie2 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antisera to KDR/Flk-1 were generated against a synthetic peptide of the kinase insert region of human KDR/Flk-1 (residues 947–966).[22] A monoclonal antibody specific to phosphotyrosine (PY-20) was obtained from ICN Biochemicals (Costa Mesa, CA). Anti-Akt antibody and anti-phospho-Akt (Ser473) antibody were purchased from New

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England Biolabs, Inc. (Beverly, MA). Secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from Amersham Pharmacia Biotech. Goat antihuman VEGF neutralizing antibody and anti-bFGF neutralizing antibody were from R&D Systems, Inc. Monoclonal antibodies neutralizing human KDR (KM1992) and human Flt-1 (KM1732) were prepared as described previously.[23] The efficiency of anti-Flt-1 antibody is about 5-fold higher than that of anti-KDR antibody.

Immunoblotting and immunoprecipitation. HUVE cells were starved for 4 h in M199 (Nissui, Tokyo) containing 0.1% FBS. The cells were stimulated with Ang1 (450 ng/ml), VEGF-A (50 ng/ml) or HGF (50 ng/ml) for 10 min at 37°C. The preparation of cell lysates, immunoblotting and immunoprecipitation were carried out according to the method described by Takahashi *et al.*[24]

Detection of viability of HUVE cells. HUVE cells were seeded at 1×10^4 cells/well on collagen-coated 8-well culture slides in HuMedia-EG2 medium and were incubated for 24 h. After the incubation, the medium was changed to growth factor-containing M199-0.5% FBS with or without LY294002 or PD98059 (20 μ*M*) and the cells were incubated for 48 h. Subsequently, the floating apoptotic cells were collected and washed with PBS twice. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays were performed on both floating and adherent cells using the *in situ* Cell Death Detection kit (Roche Diagnostics Co., Mannheim, Germany) according to the manufacturer's instructions. The percentage of apoptotic cells is based on the sum of the apoptotic floating and adherent cells in a given cell population.

***In vitro* co-culture angiogenesis assay.** *In vitro* angiogenesis was assessed as formation of capillary-like structures of HUVE cells co-cultured with human diploid fibroblasts. The experimental procedure followed the instructions provided with the Angiogenesis kit (Kurabo). Briefly, cells were stimulated with the test substances at day 1 and the medium was replaced at days 4, 7 and 9. At day 11, the cells were fixed and HUVE cells were stained using an antihuman CD31 antibody (Kurabo) according to the protocol provided with the kit. To measure the formation of the capillary network, the total tube length per field was measured at ×40 magnification with the scale. Ten different fields were analyzed per well.

To detect apoptotic HUVE cells in tubules, the cells were triple-stained with antihuman CD31 antibody, Hoechst 33258 and TUNEL reagent. Cells that displayed condensed chromatin and blebbed nuclei were considered apoptotic. To determine apoptotic rates in HUVE cells, both total $CD31^+$ cells and $CD31^+$ apoptotic cells were counted in 20 different random fields. The percentage of apoptotic HUVE cells was expressed as the ratio of the number of $CD31^+$ apoptotic cells/the total number of $CD31^+$ cells.

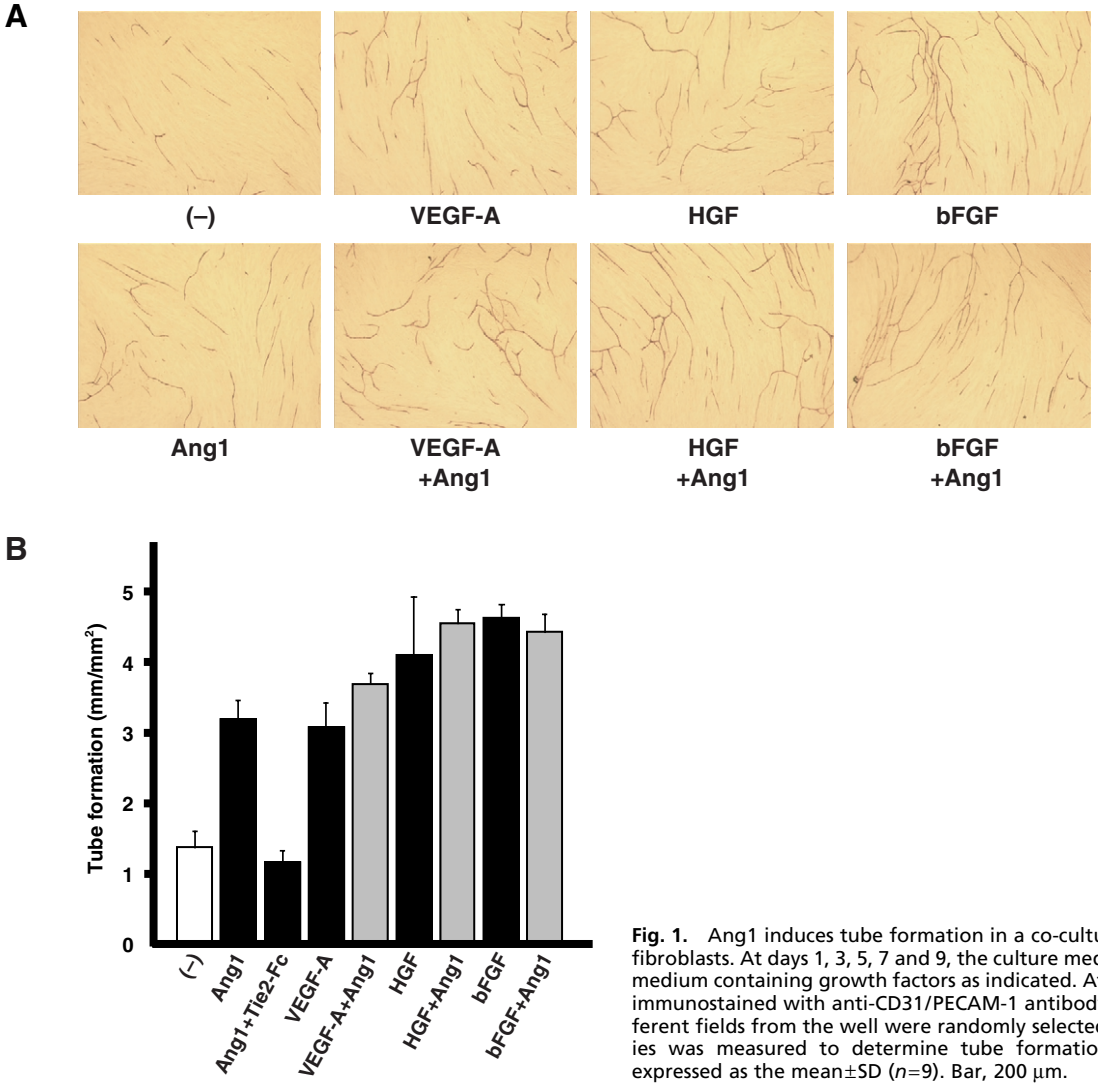

Fig. 1. Ang1 induces tube formation in a co-culture system of HUVE cells and fibroblasts. At days 1, 3, 5, 7 and 9, the culture medium was replaced with fresh medium containing growth factors as indicated. At day 11, cells were fixed and immunostained with anti-CD31/PECAM-1 antibody for HUVE cells (A). Ten different fields from the well were randomly selected, and the length of capillaries was measured to determine tube formation (mm/mm²) (B). Data are expressed as the mean±SD (n=9). Bar, 200 μm.

Results

Ang1 stimulates tube formation in the co-culture system. Recently, a novel system to test for angiogenic substances has been developed, in which HUVE cells are co-cultured with human diploid fibroblasts as feeder cells. In this system, HUVE cells can form tubular structures for a long period (about 2 weeks) in response to angiogenic factors. Therefore, we tested Ang1 in this co-culture system to examine its activity for tube formation as compared with that of several angiogenic factors such as VEGF-A, HGF, bFGF and VEGF-E, which is a specific ligand for KDR.[21]

In this system, even without any supplemented factors, tube-like structures are formed to some extent as a background. The basal tube formation was significantly increased after stimulation with VEGF-A, HGF or bFGF at a concentration of 10 ng/ml. Also Ang1, at a concentration of 450 ng/ml, increased by 2.2-fold the formation of tubes, and this increase was inhibited by the addition of excess soluble Tie2-Fc protein, as expected (Fig. 1). The concentration of Ang1 used (450 ng/ml) was relatively high compared to those of other angiogenic factors such as VEGF-A and FGF (10–50 ng/ml). Since the biological and biochemical effects of Ang1 on Tie2 receptor autophosphorylation and Akt activation were weak at 50 ng/ml Ang1, but increased dose-dependently, we used Ang1 at 450 ng/ml.

Ang1-induced tube formation is highly dependent on endogenous VEGF but not on Ang1 or bFGF in the co-culture system. Ang1 has been reported to be unable to induce tube formation in endothelial cells within a Matrigel or collagen matrix.[6] Thus, we thought it possible that the Ang1-induced tube formation in the co-culture system is dependent on endogenous VEGF which may be secreted from feeder fibroblasts. To test this idea, we used anti-VEGF-A neutralizing antibody. As shown in Fig. 2, the background tube formation was completely blocked by anti-VEGF-A neutralizing antibody, strongly suggesting that most of this background is due to the endogenous VEGF. Interestingly, consistent with our hypothesis that the effect of Ang1 is dependent on VEGF, this neutralizing antibody strongly inhibited Ang1-induced tube formation.

Furthermore, anti-VEGF-A neutralizing antibody also almost abolished tube formation induced by HGF and bFGF, which have been thought to have a direct proliferative effect on HUVE cells. The blockage of tube formation by the neutralizing antibody was dose-dependent (Fig. 3A). Inhibition of tube formation by these factors was not due to an unexpected toxicity of the antibody, because VEGF-E could induce normal tubes to form even in the presence of anti-VEGF-A neutralizing antibody (Fig. 2). We confirmed these results by using soluble Flt-1, which efficiently blocks VEGF-A. Soluble Flt-1 also strongly inhibited the stimulatory effect on tube formation by

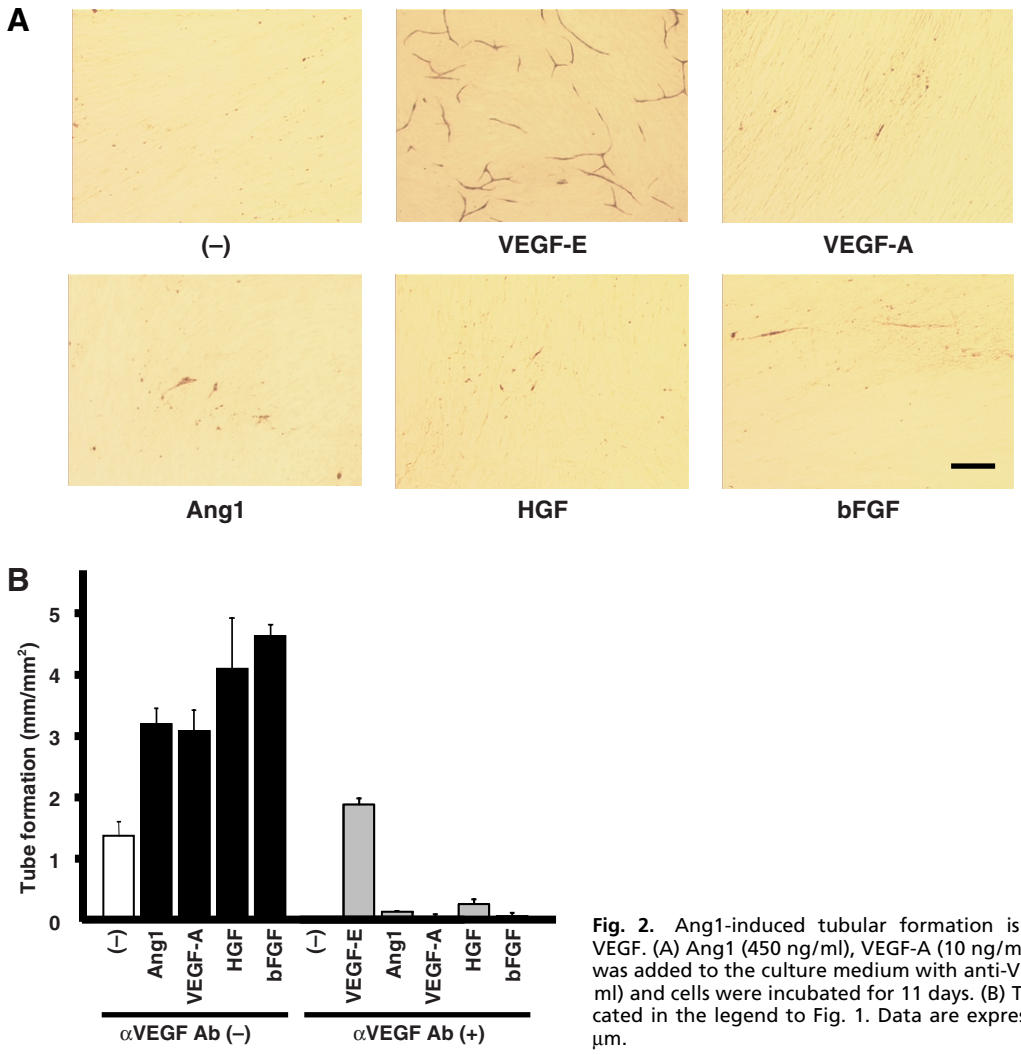

Fig. 2. Ang1-induced tubular formation is highly dependent on endogenous VEGF. (A) Ang1 (450 ng/ml), VEGF-A (10 ng/ml), HGF (10 ng/ml) or bFGF (10 ng/ml) was added to the culture medium with anti-VEGF-A neutralizing antibody (500 ng/ml) and cells were incubated for 11 days. (B) Tube formation was measured as indicated in the legend to Fig. 1. Data are expressed as the mean±SD (n=9). Bar, 200 μm.

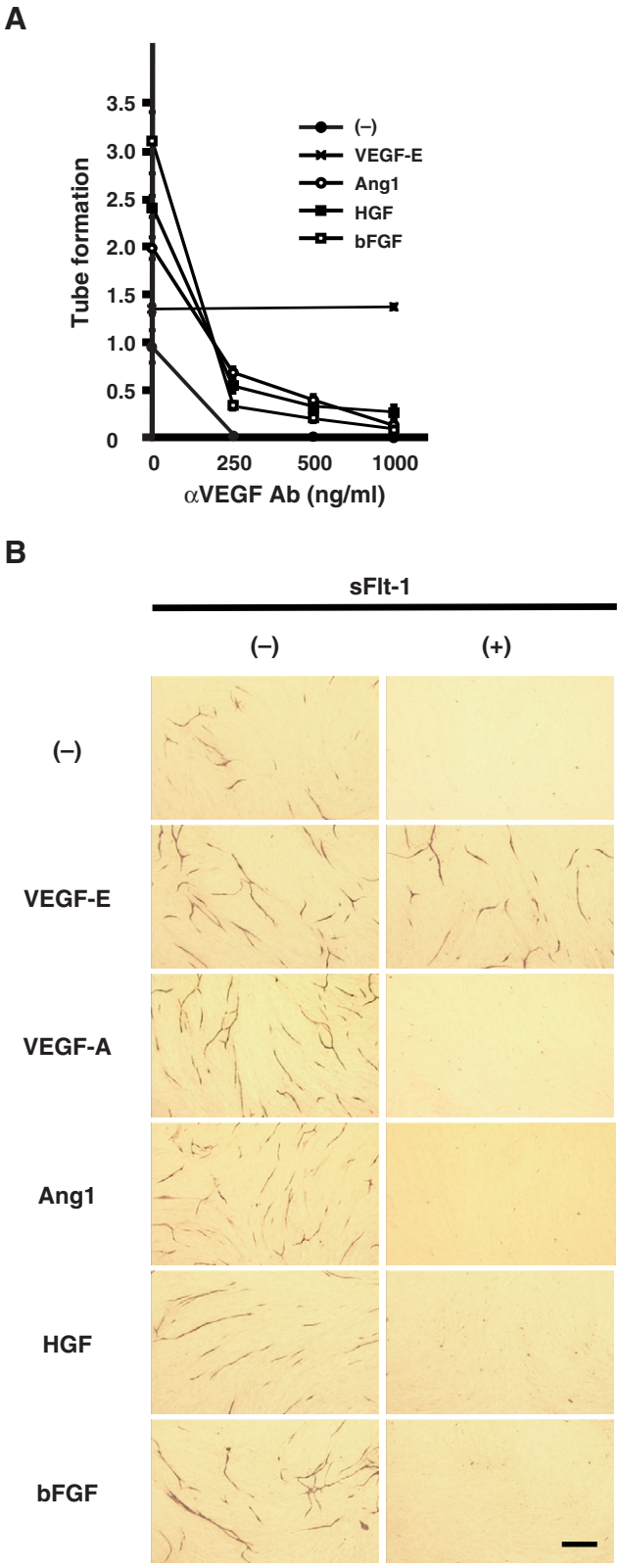

Fig. 3. Effects of anti-VEGF neutralizing antibody and soluble Flt-1 on tubulogenesis induced by Ang1. Cells were incubated for 11 days in medium containing Ang1 (450 ng/ml), VEGF-A (10 ng/ml), VEGF-E (10 ng/ml), HGF (10 ng/ml) or bFGF (10 ng/ml) with various amounts of anti-VEGF-A neutralizing antibody (A) or soluble Flt-1 (500 ng/ml) (B). Tube formation was measured as indicated in the legend to Fig. 1. Tube formation under basal conditions is arbitrarily defined as 1. Data are expressed as the mean±SD (n=9). Bar, 200 μm.

Ang1 and other factors (Fig. 3B). These results clearly indicate that the tube formation induced not only by Ang1, but also by HGF or bFGF is highly dependent on the endogenous VEGF in this co-culture angiogenesis system.

The concentration of endogenous VEGF in the medium of the co-culture system after 11-day culture was 2.5 ng/ml (±0.2 ng/ml), and this level did not change significantly during the culture (from day 1 to day 11) (see "Materials and Methods"). The level was also not altered after addition of Ang1. In a separate culture of human fibroblasts, we found that most of the endogenous VEGF was secreted from fibroblasts.

To determine whether some other endogenous angiogenic factor, such as bFGF or Ang1, is necessary for the tube formation induced by Ang1, anti-bFGF neutralizing antibody or soluble Tie2-Fc protein was used. As shown in Fig. 4A, however, the basal tube formation was not affected by treatment with excess Tie2-Fc protein, even at a concentration of 5 μg/ml. When we used anti-bFGF neutralizing antibody at a concentration of 500 ng/ml, at which bFGF (10 ng/ml)-induced activation of MAP kinase is completely inhibited, a significant decrease of tube formation was not observed (Fig. 4B). These results suggest that neither the endogenous Ang1 nor bFGF contributes to Ang1-induced tube formation.

We also examined the effect of Ang2 on the tube formation in this co-culture system. Ang2 (450 ng/ml) increased the tube formation by1.8-fold (2.60±0.21 mm/mm^2), whereas Ang1

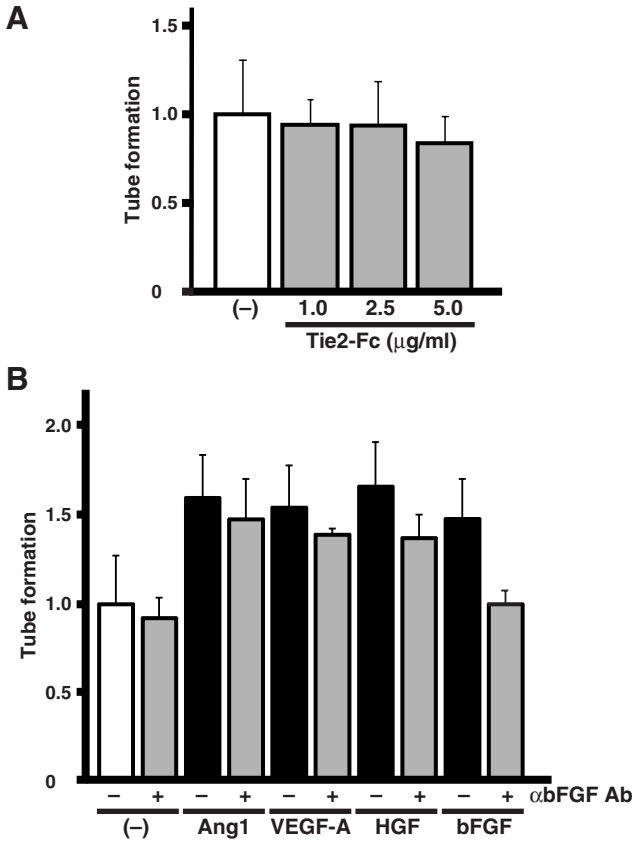

Fig. 4. Effects of inhibition of endogenous Ang1 or bFGF on tube formation in a co-culture system. (A) Cells were incubated for 11 days with various amounts of Tie2-Fc. (B) Ang1 (450 ng/ml), VEGF-A (10 ng/ml), HGF (10 ng/ml) or bFGF (10 ng/ml) was added to the medium with or without anti-bFGF neutralizing antibody (500 ng/ml). Cells were then incubated for 11 days. Tube formation was measured as indicated in the legend to Fig. 1. Tube formation under basal conditions is arbitrarily defined as 1. Data are expressed as the mean±SD (n=3).

(450 ng/ml) did so by 2.4-fold (3.46±0.59 mm/mm^2) as compared to the control (1.43±0.10 mm/mm^2). Thus, the stimulatory effect of Ang2 on this system was a little weaker than that of Ang1. In addition, soluble Tie2-Fc, which binds to and blocks both Ang1 and Ang2, did not suppress the background tube formation (Figs. 1 and 4). These results suggest that both endogenous Ang1 and Ang2 had only minor effects, if any, on the tube formation induced by endogenous VEGF.

VEGF-KDR signaling is necessary for Ang1-induced tube formation in the co-culture system. To determine the role of VEGF receptors in the process of tube formation in the co-culture system, we used anti-KDR neutralizing antibody and anti-Flt-1 neutralizing antibody. As shown in Fig. 5, the basal or Ang1-induced tube formation was severely impaired in the presence of anti-KDR neutralizing antibody. In contrast, blockage of Flt-1 by anti-Flt-1 neutralizing antibody did not cause a significant decrease in tube formation even at a concentration of 2 μg/ml, at which phosphorylation of Flt-1 in response to VEGF was completely inhibited.[23, 25] These results suggest that VEGF/KDR signaling is mainly used in the tube formation and is required for Ang1-induced upregulation of the formation.

Ang1 promotes the survival of HUVE cells in tube formation through the PI3-kinase pathway. To examine whether Ang1 promotes a survival response in HUVE cells in the co-culture angiogenesis system, we evaluated the rate of apoptosis in HUVE cells in the presence of Ang1, VEGF-A or HGF at days 4, 7, 9 and 11. At day 4, the number of HUVE cells on tubules in the presence of each factor was essentially the same as that in the

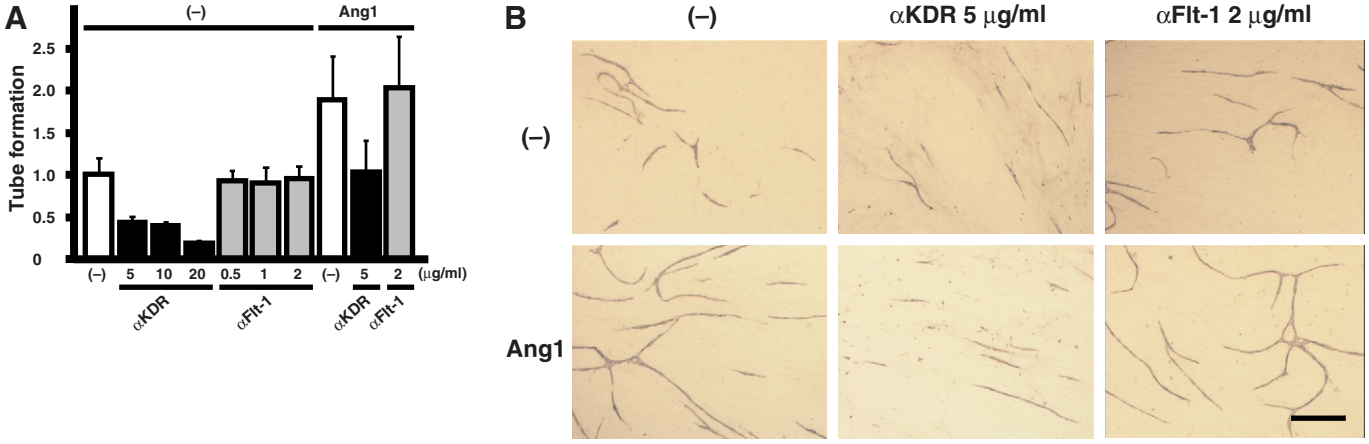

Fig. 5. Anti-KDR antibody but not anti-Flt-1 antibody significantly inhibits Ang1-induced tube formation. (A) Cells were cultured for 11 days in medium with or without Ang1 (450 ng/ml) in the presence of various amounts of anti-KDR or anti-Flt-1 neutralizing antibody. Tube formation was measured as indicated in the legend to Fig. 1. Tube formation under basal conditions is arbitrarily defined as 1. Data are expressed as the mean±SD (n=3). (B) Representative examples are shown. Bar, 200 μm.

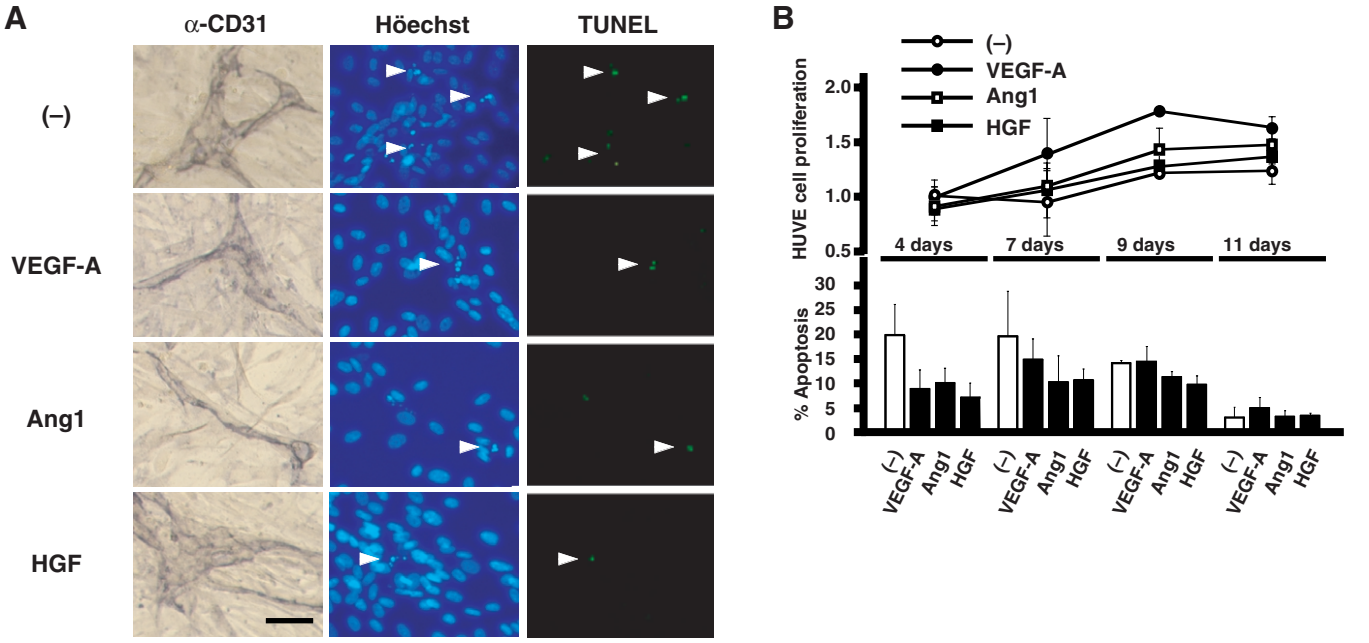

Fig. 6. Ang1 inhibits apoptosis of HUVE cells in a co-culture system. Cells were incubated with Ang1 (450 ng/ml), VEGF (10 ng/ml) or HGF (10 ng/ml) and fixed at day 4, 7, 9 or 11. Cells were triple-stained with anti-CD31 antibody, Hoechst 33258 and TUNEL reagent. (A) Representative examples of the cells stained at day 4 are shown. (B) Apoptotic HUVE cells were quantified as described in "Materials and Methods." The proliferation of HUVE cells at day 4 under basal conditions is arbitrarily defined as 1. Data are expressed as the mean±SD (n=3). Bar, 5 μm.

absence of any ligands, and the proportion of apoptotic cells among total cells was approximately 20% under basal conditions. As compared with the control, Ang1 decreased the apoptotic HUVE cells to 10.1%, and the anti-apoptotic potency of VEGF or HGF was similar to that of Ang1 at day 4. The rate of apoptosis of HUVE cells in the presence of each factor was almost the same in the later period (days 7–11) of culture (Fig. 6).

To examine the contribution of the PI3-kinase pathway to the Ang1-induced survival response in this co-culture system, we used LY294002. As shown in Fig. 7A, 1 μ*M* LY294002 significantly inhibited the Ang1-induced anti-apoptotic effect on HUVE cells at day 4. Cell culture for 11 days in the presence of LY294002 slightly suppressed tube formation, but damage to the co-cultured fibroblasts was not observed. The Ang1-promoted tube formation was almost completely blocked by the addition of LY294002 (Fig. 7B). These findings suggest that Ang1 induces an anti-apoptotic response via the PI3-kinase pathway in HUVE cells during the early period of culture, resulting in an increase in tube formation.

Ang1 inhibits apoptosis induced by serum depletion through activation of the PI3-kinase/Akt pathway in HUVE cells. To examine further the *in vitro* signaling of Ang1 and its effect on VEGF signaling, we used a regular HUVE cell monolayer culture system and compared the Ang1-induced anti-apoptotic effect with that of other angiogenic factors, VEGF, HGF and bFGF. As shown in Fig. 8A, 65 to 70% of the total cells underwent apoptosis at 48 h after serum depletion. Ang1, as well as VEGF, HGF and bFGF, inhibited apoptosis in HUVE cells, but a higher concentration of Ang1 (500 ng/ml) was required to induce a clear survival response compared to VEGF, HGF or bFGF.

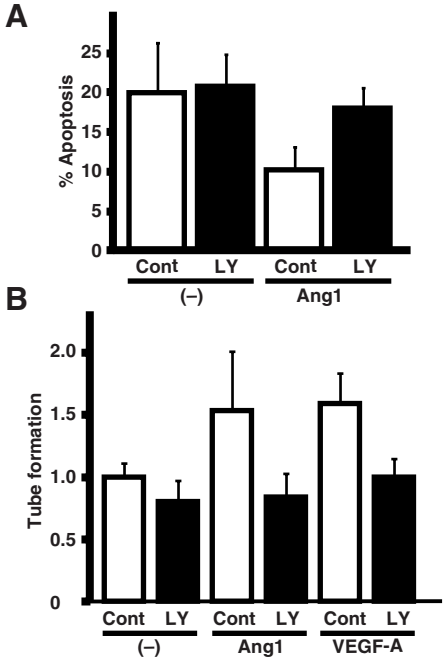

Fig. 7. Ang1 promotes the survival of HUVE cells in tube formation through the PI3-kinase pathway. (A) Cells were cultured for 4 days with or without Ang1 (450 ng/ml) in the presence of only DMSO (Cont) or LY294002 (LY, 1 μ*M*). Apoptotic HUVE cells were quantified as described in "Materials and Methods." (B) Cells were incubated for 11 days with or without Ang1 (450 ng/ml) or VEGF (10 ng/ml) in the presence of only DMSO (Cont) or LY294002 (LY, 1 μ*M*). Addition of wortmannin in this long-term co-culture system showed nonspecific cytotoxic effects, so we used only LY294002 for this experiment. The medium was changed every 2 days. Tube formation was measured as indicated in the legend to Fig. 1. Tube formation of mock-treated HUVE cells is arbitrarily defined as 1. Data are expressed as the mean±SD (*n*=3).

Activation of PI3-kinase initiates the Akt-dependent survival pathway.[26] To address the question of whether the PI3-kinase/Akt pathway is involved in the anti-apoptotic signaling of Ang1, we used an inhibitor of PI3-kinase, LY294002. The anti-apoptotic effect of Ang1 was completely blocked by the treatment with LY294002. In contrast, PD98059 did not affect Ang1-induced HUVE cell survival (Fig. 8B). Second, we examined whether Ang1 activates Akt with anti-phospho-Akt antibody. Ang1 activated Akt similarly to HGF, which is known to induce activation of the PI3-kinase/Akt pathway (Fig. 8C).

Dose-dependence analysis of Tie2 activation by Ang1 indicated that at least 150 ng/ml of Ang1 was required to detect clear Tie2 autophosphorylation and Akt phosphorylation (Fig. 8D). Interestingly, VEGF was found to induce a very weak activation of Akt at a concentration of 50 ng/ml, at which VEGF induces a strong survival response in HUVE cells. When we used an inhibitor of PI3-kinase, wortmannin, at a concentration of 50 μ*M*, Ang1-induced activation of Akt was abrogated (Fig. 8C). Another PI3-kinase inhibitor LY294002 gave essentially the same results (data not shown). Therefore we suggest that Ang1 inhibits the apoptosis induced by serum depletion through activation of the PI3-kinase/Akt pathway in HUVE cells.

As we showed before, Ang1 upregulates tube formation of HUVE cells in combination with endogenous VEGF. To see whether Ang1 upregulates VEGF-induced MAP kinase activation in HUVE cells, we examined the levels of VEGF-induced phospho-MAP kinase in the presence or absence of Ang1. However, we did not detect a clear increase in phospho-MAP kinase after addition of Ang1 (Fig. 8E). Further, VEGF did not increase the Ang1-induced phospho-Akt (data not shown).

Discussion

Vasculogenesis, angiogenesis and microvascular remodeling are complex processes involving endothelial cell proliferation, migration and differentiation.[2,27] The complex behavior that endothelial cells exhibit during neovascularization has been studied by using *in vitro* models. Three-dimensional (3-D) extracellular matrix culture systems have been developed to simulate natural interactions between cells and the extracellular environment. However, capillary-like structures are quite unstable in the current *in vitro* assays such as the Matrigel assay and the collagen overlay assay. In the present study, we used a co-culture system of HUVE cells and fibroblasts as a model of angiogenesis *in vivo*. The culture conditions in this system are more similar to the environment *in vivo* and capillary-like structures are more stable than those in current *in vitro* assays.[28]

We therefore chose to analyze the Ang1-induced anti-apoptotic effect and tube formation in the co-culture system. In contrast to a previous report which suggested that Ang1 does not induce tube formation in Matrigel or in collagen gel,[6] Ang1 could induce tube formation similarly to VEGF, HGF and bFGF in this co-culture system. Since endothelial cells receive and integrate information from multiple angiogenic signals during angiogenesis, we hypothesized that Ang1-induced tube formation is dependent on the endogenous VEGF secreted from fibroblasts. As expected, endogenous VEGF was secreted at low levels (2.5 ng/ml), and more than 95% of this VEGF was secreted from fibroblasts by a human VEGF-detection system (see "Materials and Methods"). The amount of VEGF in the culture medium was not increased by Ang1, and anti-VEGF neutralizing antibody as well as soluble Flt-1 strongly suppressed tube formation induced by Ang1. These results are con-

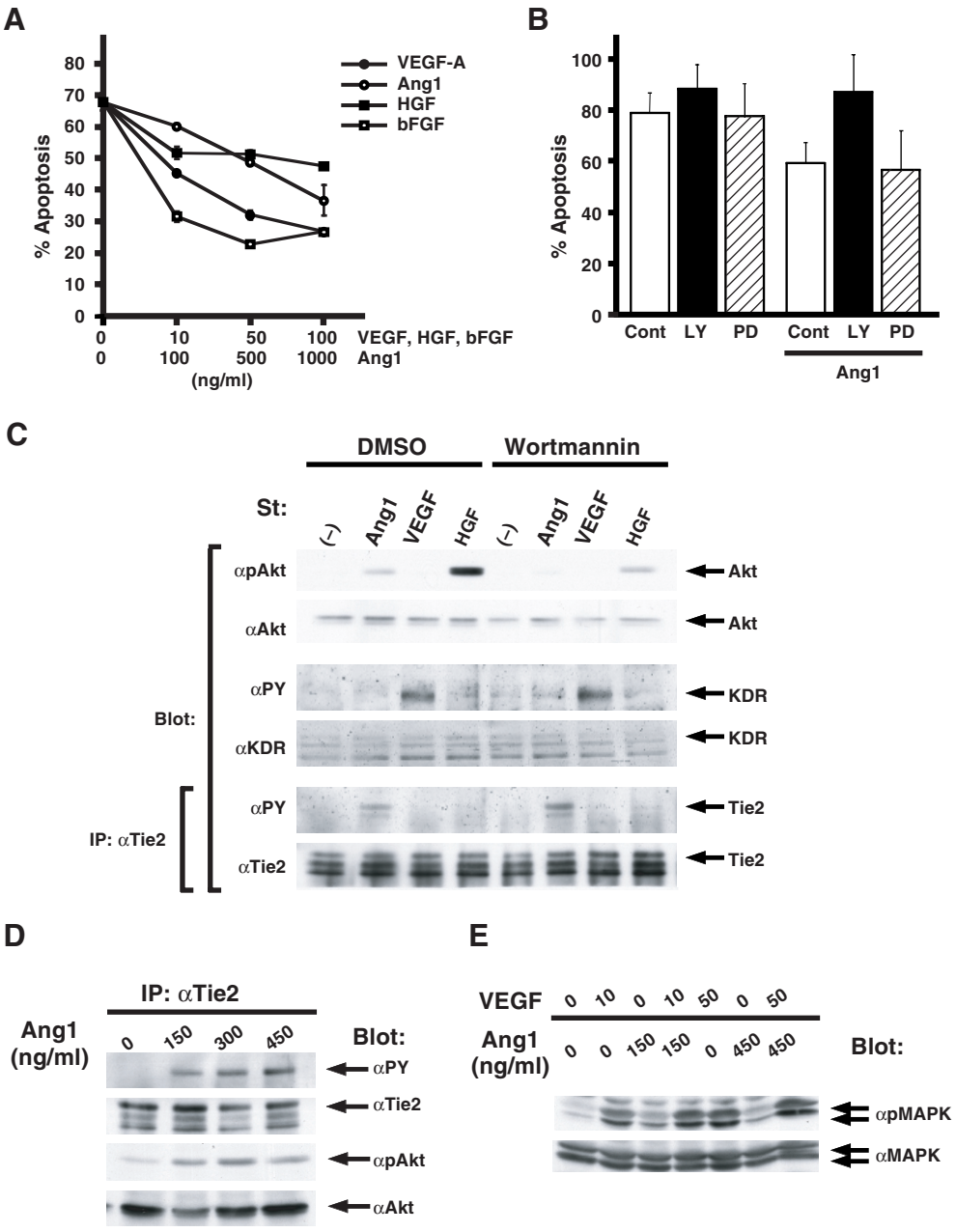

Fig. 8. Activation of the PI3-kinase/Akt pathway is required for Ang1-induced survival in monolayer-cultured HUVE cells. (A) Ang1 is a survival factor for serum-depleted HUVE cells. Cells were incubated for 48 h in 0.5% FBS-M199 with various amounts of Ang1, VEGF, HGF or bFGF. (B) Cells were incubated for 48 h with or without Ang1 (500 ng/ml) in the presence of only DMSO (Cont), LY294002 (LY, 20 μ*M*) or PD98059 (PD, 20 μ*M*). Apoptotic cells were quantified as described in "Materials and Methods." Results represent the mean of triplicate experiments with the standard deviation. (C) Ang1 induces PI3-kinase-dependent activation of Akt. HUVE cells were stimulated with Ang1, VEGF or HGF for 10 min after treatment with only DMSO or wortmannin in DMSO (50 μ*M*) for 1 h. Cell lysates were used directly for SDS-PAGE or immunoprecipitated with anti-Tie2 antibody. Then, they were analyzed by western blotting using the antibodies indicated on the left of the figure. (D) A high dose of Ang1 is required for autophosphorylation of Tie2 and phosphorylation of Akt. HUVE cells were stimulated with Ang1 for 10 min, analyzed by SDS-PAGE and western-blotted with antibodies indicated on the left of the figure. (E) Ang1 does not significantly increase the level of MAP kinase activation induced by VEGF. HUVE cells were stimulated with VEGF or Ang1 or both, and examined by blotting with anti-phospho-MAP-kinase or MAP-kinase antibody.

sistent with recent reports suggesting that Ang1 *in vivo* acts synergistically with VEGF to promote endothelial cell sprouting and angiogenesis.[13, 29)]

To understand the stimulatory effect of Ang1 on tube formation, we examined whether Ang1 upregulates the major signaling pathway of MAP kinase activation induced by VEGF. In addition, we also tested whether VEGF upregulates the Ang1-dependent survival signal. However, we could not detect any such cooperative effects (Fig. 8E). Thus, we suggest that VEGF and Ang1 signaling for cell proliferation and cell survival, respectively, are mostly independent, and the suppression of apoptosis by Ang1 results in the upregulation of tube formation.

To observe a stimulatory effect of Ang1 in tube formation assay, we needed to use a high concentration of Ang1 (450 ng/ml). There are several possible explanations for this requirement; (1) Since Ang1 requires a polymeric (6-mer) structure for

activity, only a portion of the protein may be biologically active. (2) The protein used here as Ang1 is Ang1*, which contains a mutation to facilitate its production, so its activity might be reduced. (3) Since the kinase activity of the Ang1 receptor Tie2 is relatively weak, the physiological signaling from Tie2 may be weaker than that of other representative tyrosine kinase receptors. To examine these possibilities, further studies, particularly measurement of the physiological concentration of Ang1 *in vivo*, are required.

To our surprise, HGF- and bFGF-induced tube formation was also markedly suppressed by anti-VEGF antibody. The blockade of endogenous bFGF or Ang1 did not cause a significant decrease in the basal level of tube formation in the co-culture system. Therefore, based on these results, we suggest that *in vivo*, Ang1, bFGF and HGF may induce their angiogenic effects in a highly VEGF-dependent manner, even though they stimulate cell proliferation and survival without VEGF in cultured HUVE cells *in vitro*.

In the remodeling of the capillary network structure, programmed cell death in the endothelium plays an important role, in addition to cell proliferation.[30, 31] A recent study showed that TGF-α promotes endothelial cell survival via the activation of the PI3-kinase and MEK pathways during *in vitro* angiogenesis in collagen gels. In the present study, we demonstrated that Ang1 induces an anti-apoptotic effect through activation of PI3-kinase on HUVE cells in both a regular culture system (Fig. 8) and in a co-culture system (Fig. 1). These results suggest that Ang1 promotes HUVE cell survival and tube formation synergistically with endogenous VEGF *in vivo*. Some reports have shown that not only survival signals, but also other signals such as expression of cytokines and matrix proteins, stimulation of integrins and release of matrix metalloproteinases (MMPs) are important for capillary network formation and remodeling. In fact, the activation of PI3-kinase is also involved in the expression of proangiogenic tissue factors in endothelial cells.[32] Ang1 has been shown to induce the secretion of plasmin and MMP2 from endothelial cells.[12] These findings suggest that not only the survival signal, but also other signals might be involved in Ang1-promoted tube formation.

In a recent report, VEGF enhanced the expression of the apoptosis inhibitor survivin, and suppression of survivin enhanced caspase-3 activity and regressed the capillary network in HUVE cells.[33] Papapetropoulous *et al*. showed that an Ang1-induced anti-apoptotic effect was associated with enhanced expression of survivin through the PI3-kinase/Akt pathway.[34] This, together with our results, suggests that an anti-apoptotic response via activation of PI3-kinase is necessary for Ang1-induced tube formation. Furthermore, our results indicate that Ang1 alone (in the presence of VEGF neutralizing antibody or soluble VEGFR-1) cannot induce stable tubular structure of HUVE cells, and basal VEGF signaling is always required for the detection of Ang1-induced stimulation on tube formation.

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