Angiopoietin 1 Is Mitogenic for Cultured Endothelial Cells

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Abstract

The angiopoietin (Ang)/Tie2 system is implicated in blood vessel formation and maturation. However, the mitogenic effects of angiopoietins remain to be elucidated. Here, we show that Ang1 is mitogenic for cultured endothelial cells. Ang1 dose-dependently induced the proliferation and increased the labeling index of a murine brain capillary endothelial cell line, IBE cells. Ang1 also increased the labeling index of human umbilical vein endothelial cells (HUVEC). Ang1 up-regulated the expression of cyclin D1 in both of these cells. Ang1 activated mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) in IBE cells and HUVECs. Activated PI3K was associated with c-Fes protein tyrosine kinase in these cells, but not with Tie2. p70 S6 kinase (p70 S6K) was activated by Ang1-treatment, although this activation was blocked by a PI3K inhibitor, LY294002. Simultaneous treatment of cells with PD98059 (MAPK/ extracellular regulated kinase inhibitor) and rapamycin (mTOR inhibitor) completely blocked Ang1-induced mitogenic activity for IBE cells and HUVECs. Although Ang2 at high concentration weakly activated Tie2 and p70 S6K, it failed to activate Ras and MAPK, or to induce cell proliferation. Taken together, these findings indicate that Ang1 exerts mitogenic activity on endothelial cells, which requires activation of both MAPK and p70 S6K. (Cancer Res 2005; 65(15): 6820-7)

Introduction

Angiopoietin (Ang) family consists of four members, Ang1 to 4 (1, 2), whose specific receptor tyrosine kinase, Tie2, is exclusively expressed in vascular and lymphatic endothelial cells. Targeted disruption of genes encoding Tie2 as well as Ang1 in mice yielded similar defects of vascular complexity and integrity (3–5). In tetracycline-responsive transgenic mice, loss of Tie2 induced rapid endothelial cell apoptosis (6), suggesting that Tie2 signaling plays a role in the maturation and maintenance of normal vasculature in vivo. Ang2 was cloned by Ang1-based homology screening and was found to inhibit Ang1-mediated autophosphorylation of Tie2 in human umbilical vein endothelial cells (HUVEC; ref. 7). In addition, overexpression of Ang2 in transgenic mice yielded vascular defects similar to those observed in mice lacking Ang1 or Tie2, suggesting that Ang2 is a natural antagonist of Ang1 during embryonic development (7). In the postnatal period, Ang2, rather than Ang1, induces angiogenesis in coordination with vascular endothelial growth factor-A (VEGF-A; refs. 8, 9). Several studies have shown that Ang2 activates signaling pathways through Tie2 in endothelial cells, suggesting that Ang2 is an agonist of Tie2 in certain conditions (10, 11).

Downstream of Tie2, two major pathways, the phosphoinositide 3-kinase (PI3K)/c-Akt pathway and mitogen-activated protein kinase (MAPK) pathway, are activated and are involved in mediating several responses by endothelial cells (12, 13). Using a yeast two-hybrid system, pull-down assay with glutathione S-transferase (GST)-fusion proteins, and overexpression in 293T cells, COS1 cells, and EA.hy926 cells, it was found that the p85 subunit of PI3K, Grb2, Shp-2, and Dok-R, bound to the intracellular domain of activated Tie2 (14–16).

Angiogenesis is composed of a series of cellular responses. Pericytes leave the basement membranes of preexisting microvessels, and this is followed by activation of endothelial cells by proangiogenic factors. Activated endothelial cells produce pro- tease, which digest the basement membranes of blood vessels. Endothelial cells migrate into interstitial tissue, proliferate, and form lumen-containing, tube-like structures. Survival of endothelial cells is also required for maintenance of vascular integrity. Finally, the basement membranes of newly formed vessels are surrounded by pericytes (17, 18). The role of Ang1 in tumor angiogenesis is still controversial. It is widely accepted that Ang1 stabilizes the vascular wall to maintain vessels in static state, whereas Ang2 destabilizes the vasculature, making it angiogenic by releasing pericytes from endothelial cells (5, 7). The correlations between expression of Ang1 and 2 and tumor progression/angiogenesis have been extensively studied, and it has been found that the ratio of Ang2/Ang1 expression might determine tumor fate (19). A shift to a high Ang2/Ang1 ratio seems to be associated with tumor angiogenesis. However, some studies have clearly shown that Ang1 positively regulates tumor angiogenesis (20–22). Because angiogenesis involves new blood vessel formation from preexisting vessels, proliferation of endothelial cells must play a role in it. Recent extensive studies have provided evidence that Ang1 plays pivotal roles in controlling migration, survival, and tube formation of endothelial cells (2, 13). Ang2 also has been found to induce migration, survival, and tube formation by endothelial cells (10, 11, 23). Inhibition of the PI3K/c-Akt pathway impairs both the survival and migration of endothelial cells (10, 11, 24, 25). Blockade of MAPK activity also inhibits Ang1-promoted survival and migration of endothelial cells (26, 27). However, the effects of Ang1 and Ang2 on the proliferation of endothelial cells have yet to be established.

We have previously shown that Ang2 at high concentrations (1 and 2 μg/mL) activated Tie2 in murine brain capillary endothelial cells (IBE cells; ref. 28), with subsequent activation of PI3K and c-Fyn (11). In that study, we found that Ang2 failed to induce proliferation and to activate MAPK. Because Ang1 is a potent activator of Tie2, we examined whether Ang1-mediated signaling could regulate proliferation of endothelial cells. We found

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that Ang1 induced proliferation of IBE cells and entry of HUVECs into S phase. Both MAPK and p70 S6 kinase (S6K) were activated in both of these types of cells. This mitogenic activity was almost completely abolished when cells were treated with PD98059 in combination with rapamycin, suggesting that Ang1-induced activation of MAPK and p70 S6K play roles in mitogenic activity.

Materials and Methods

Reagents. Antiphosphotyrosine (PY99) monoclonal antibody, anti-c-Fes polyclonal antibody, anti-phospho-p70 S6K (pS411) monoclonal antibody, and anti-Tie2 polyclonal antibody were purchased from Santa Cruz Biotechnologies, Santa Cruz, CA. Anti-FLAG monoclonal antibody (M2), anti-phospho-p70 S6K (pS411) monoclonal antibody, and anti-cyclin D1 monoclonal antibody, and bromodeoxyuridine (BrdUrd) were from Sigma-Aldrich, Inc., St. Louis, MO. Anti-phospho-MAPK polyclonal antibody was obtained from Cell Signaling Technology, Inc., Beverly, MA. Anti-p70 S6K polyclonal antibody was from Chemicon International, Temecula, CA, and anti–extracellular signal regulated kinase (Erk) 1/2 antibody from Upstate Cell Signaling Solutions, Lake Placid, NY. Anti-pan Ras monoclonal antibody was obtained from BD Bioscience, San Jose, CA. Histidine-tagged human recombinant Ang1 and Ang2, monoclonal anti-polyhistidine monoclonal antibody (for cross-linking of histidine-tagged proteins), and human recombinant VEGF-A were obtained from R&D Systems, Minneapolis, MN. Recombinant human fibroblast growth factor-2 (FGF-2) and recombinant human epidermal growth factor (EGF) were purchased from Roche Diagnostics GmbH, Mannheim, Germany. A PI3K inhibitor, LY294002, an mTOR inhibitor, rapamycin, and a MAPK/Erk kinase inhibitor, PD98059, were from Calbiochem-Novabiochem, La Jolla, CA. LY294002 and PD98059 were dissolved in DMSO as stock solutions and stored at −20°C until use. Stock solutions were further diluted with DMSO and dissolved in culture medium. Rapamycin was dissolved in methanol and kept at −20°C until use. Final concentrations of DMSO and methanol were 0.1% in all cases.

Cell culture. IBE cells, stable IBE cell lines expressing FLAG-tagged wild-type or kinase-inactive mutant c-Fes (denoted WT6-8 and KE5-15 cells, respectively; ref. 29), empty vector-transfected cells (mock cells), and deleted mutant p85 PI3K subunit, which does not interact with p110 subunit (denoted Δp85-8 cells; ref. 30), were cultured in Ham’s F-12 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS), 75 μg/mL endothelial cell growth supplement (Sigma), 5 μg/mL insulin, and 10 ng/mL EGF with or without 200 μg/mL of G418. HUVECs from three different individuals and their culture medium were obtained from Cambrex, Walkersville, MD, and cells were cultured in endothelial cell basal medium (EBM)-2 supplemented with 2% FBS, 10 ng/mL VEGF-A, 20 ng/mL FGF-2, 10 ng/mL EGF, 10 ng/mL insulin-like growth factor-1, 50 μg/mL ascorbic acid, 100 ng/mL heparin, and 10 pmol/L dexamethasone. HUVECs from different donors were separately used for each experiment.

Cell proliferation assay. Cell proliferation assays were done as described previously (29). Briefly, IBE cells were inoculated into wells of human plasma fibronectin-coated 24-well culture plates at a density of 1.5 × 10^4 cells/cm² (3 × 10^3 cells/well) in Ham’s F-12 medium containing 0.25% BSA with or without indicated samples for 3 days. Cells were detached and cell numbers calculated. Columns, means; bars, ± SD. *, P < 0.05. Similar results were obtained in two independent experiments. B, IBE cells were cultured in Ham’s F-12 medium containing 0.25% BSA with or without indicated samples for 16 hours. Cells were pulse-labeled with BrdUrd for 4 hours, and nuclei which had incorporated BrdUrd were visualized by immunocytochemical staining. Labeling index was determined as the labeled nuclei/total nuclei ratio and expressed as a percentage. Columns, means; bars, ± SD. **, P < 0.01. Similar results were obtained in two independent experiments. C, HUVECs were cultured in EBM-2 supplemented with 0.5% FBS with or without indicated samples for 22 hours. Cells were pulse-labeled with BrdUrd for 4 hours, and labeling indices were determined. Columns, means; bars, ± SD. *, P < 0.05. Similar results were obtained in two independent experiments using cells obtained from two different donors. D, expression of cyclin D1 in IBE cells and HUVECs. Cells were seeded into 24-well plates and treated with the samples and periods indicated. Total cell extract was separated by SDS-PAGE, followed by transfer onto membranes. Cyclin D1 and β-actin were visualized by immunoblotting. Similar results were obtained in two independent experiments.
10% FBS and cultured. The following day, medium was changed to Ham's F-12 medium containing 0.25% fatty acid–free bovine serum albumin (BSA) with or without indicated samples, and culture was continued for 3 days. Cells were detached by trypsin and cell number was counted with a hemacytometer. The number of cells counted in untreated samples was considered 100%.

Labeling index. Labeling indices were computed to determine numbers of cells in S phase using the BrdUrd in situ detection kit (BD Biosciences PharMingen, San Diego, CA) with some modifications. Cells suspended in normal growth medium were seeded into wells of fibronectin-coated wells of 48-well culture plates at a density of 2 × 10^4 cells/cm^2. The following day, medium was changed to Ham's F-12 medium containing 0.25% BSA for IBE cells or EBM-2 supplemented with 0.5% FBS for HUVECs with or without indicated samples, and culture was continued. After 16 hours, IBE cells were pulse-labeled for 4 hours with 10 μmol/L of BrdUrd. For examination of HUVECs, pulse-labeling was started at 22 hours after sample addition. Cells were then fixed with the fixation buffer provided in the kit, treated with 70% ethanol for 20 minutes, and incubated with 4 mol/L HCl for 20 minutes (28). Cells were washed and incubated with biotinylated anti-BrdUrd antibody, followed by sequential incubation with streptavidin-horseradish peroxidase and diaminobenzidine. Finally, cells were counterstained with hematoxylin and photographs were taken under a light microscope. At least 1,000 cells were counted for each well, and labeling indices were determined as labeled nuclei / total nuclei ratios and expressed as percentages.

Immunoprecipitation and immunoblotting. IBE cells grown in fibronectin-coated culture dishes were incubated with Ham's F-12 medium containing 0.25% BSA for 2 hours and treated or not with Ang1 for 15 minutes. PI3K activity was immunoprecipitated with indicated antibodies and phosphorylation of phosphatidylinositol was examined in vitro. Reproducible results were obtained in two independent experiments.
products were detected and incorporation of $\gamma^{32}\text{P}\text{ATP}$ was measured with an Image Analyzer BAS 5000 (Fuji). Fold increase in Ang1- or Ang2-induced phosphorylation of phosphatidylinositol was calculated from the radioactivity.

**In vitro kinase assay for c-Fes.** Cells grown in 6 cm dishes were serum-starved for 2 hours and either stimulated or not with Ang1 for 15 minutes. Cells were then washed with TBS and lysed in Fes lysis buffer [50 mmol/L Tris-HCl (pH 7.5), supplemented with 0.15 mol/L NaCl, 10 mmol/L sodium fluoride, 1% Triton X-100, 1 mmol/L ethylenediamine-N,N,N',N'-tetraacetic acid, 0.1% SDS, 100 units/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.1 mmol/L orthovandate]. After centrifugation to remove insoluble materials, supernatant was incubated with indicated antibodies, followed by precipitation with GST Ras-binding domain (RBD) of c-Raf 1 fusion protein. Activated Ras bound to GST-RBD protein was detected by immunoblotting. The remaining 20% of lysates were used to monitor the presence of cross-linking antibody throughout the studies. Because recombinant Ang1 and Ang2 are 6× His-tagged proteins and their activities in the presence of cross-linking antibody had been assessed by the manufacturer, we examined their effects in the presence of cross-linking antibody throughout the studies.

**Assay for the detection of activated Ras.** Activated Ras was detected by the precipitation with GST Ras-binding domain (RBD) of c-Raf 1 fusion protein followed by immunoblotting as described before (31). pGEX-RBD plasmid encoding amino acids 1 to 149 of c-Raf 1 (a kind gift from Dr. S.J. Taylor) was transformed into BL21 E. coli and after the induction with 1 mmol/L isopropyl-1-thio-β-D-galactopyranoside, GST-RBD fusion protein was extracted and purified by glutathione Sepharose beads (Amersham). IBE cells grown in 6 cm dishes were incubated with serum-free medium for overnight and were either stimulated or left unstimulated with 200 ng/mL Ang1 or 2 μg/mL Ang2 for 2 minutes. Cells were lysed in 20 mmol/L HEPES (pH 7.4), supplemented with 150 mmol/L NaCl, 1% Triton X-100, 100 mmol/L MgCl2, 2 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, 100 mmol/L MgCl2, 2 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, 100 mmol/L MgCl2, 2 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, 100 mmol/L MgCl2, 2 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, 100 mmol/L MgCl2, 2 mmol/L EGTA, 5 μmol/L leupeptin, and 0.05% 2-mercaptoethanol. After centrifugation, 80% of cleaved lysate was incubated with GST-RBD-bound glutathione Sepharose beads. Proteins were extracted from the beads and separated by SDS-PAGE. Activated Ras bound to GST-RBD protein was detected by immunoblotting. The remaining 20% of lysates were used to monitor the examined amount of Ras.

**Statistical analysis.** Values are presented as mean values ± SD. Differences between two groups were examined by Mann-Whitney U test. Differences were considered significant when the P value was <0.05.

**Results**

Ang1 has mitogenic activity for cultured endothelial cells. Ang1 activates MAPK and PI3K, which are involved in mitogenic responses to a number of external stimuli (32, 33). We first examined the effect of Ang1 on the proliferation of IBE cells. As shown in Fig. 1A, Ang1 significantly and dose-dependently induced the proliferation of IBE cells. Ang2 at 2 μg/mL failed to induce proliferation. It has been documented that super-clustering of Ang1 is required for full activation of Tie2 (34). To determine whether clustering of His-tagged Ang1 by cross-linking antibody to polyhistidine is required for mitogenic responses, we compared activities in the presence and absence of the antibody. It was found that Ang1 induced proliferation to similar extents even in the absence of antibody. This result suggested that recombinant Ang1 itself might cluster to efficiently activate Tie2. Because recombinant Ang1 and Ang2 are 6× His-tagged proteins and their activities in the presence of cross-linking antibody had been assessed by the manufacturer, we examined their effects in the presence of cross-linking antibody throughout the studies. Cell proliferation assay is affected by cell survival. We then determined the labeling index of IBE cells. As shown in Fig. 1B, treatment with Ang1 potently increased the number of these cells in S phase. We also examined the labeling index of HUVECs. As shown in Fig. 1C, Ang1 significantly increased labeling index.

Figure 3. Ang1-induced mitogenic activity is dependent on PI3K/p70 S6K in endothelial cells. A, stable IBE cell line expressing deleted p85 subunit (∆p85-8 cells) exerted a dominant-negative effect on Ang1-induced activation of PI3K. Cells grown in 6 cm dishes were serum-starved for 2 hours and either treated or not with Ang1 for 15 minutes. PI3K activity was examined by in vitro PI3K assay. B, Ang1-induced proliferation of IBE cells was impaired but not eliminated in the case of KE5-15 cells and ∆p85-8 cells. Cell proliferation assay was done as described in the legend to Fig. 1. Columns, means; bars, ± SD; *, P < 0.05. Similar results were obtained in two independent experiments.
which was higher than that of VEGF-A-treated cells. We also examined the expression of cyclin D1 protein, which is induced during G1 phase, in these cells. As shown in Fig. 1D, Ang1, as well as FGF-2, up-regulated the expression of cyclin D1 in both IBE cells and HUVECs.

Ang1 activates a panel of signaling molecules. We examined the effects of Ang1 on signal transduction pathways in IBE cells and HUVECs. As shown in Fig. 2A, Ang1 induced autophosphorylation of Tie2 in IBE cells and HUVECs. The molecular weight of Tie2 differs between IBE cells and HUVECs, possibly as a result of alternative splicing (35). As shown in Fig. 2B, Ang1 activated MAPK in IBE cells, with a potency similar to FGF-2. Ang1 more potently induced MAPK activation than did EGF, of which mitogenic activity was weaker than FGF-2 (28). In HUVECs, Ang1 and FGF-2, and to a lesser extent, VEGF-A activated MAPK. Non–receptor protein tyrosine kinase c-Fes is activated by oligomerization, followed by autophosphorylation (36). We examined the effect of Ang1 on endogenous c-Fes. As shown in Fig. 2C, Ang1 induced autophosphorylation of c-Fes. We next examined the effect of Ang1 on PI3K activity in IBE cells and HUVECs. As shown in Fig. 2D, PI3K was activated by treatment with Ang1 in these cells. Ang1-promoted PI3K activity was coprecipitated with endogenous c-Fes, but not with Tie2 as has been shown before (11). Expression of kinase-inactive c-Fes (with substitution of lysine 590 by glutamic acid; ref. 36) in endothelial cells causes oligomerization with endogenous c-Fes to inhibit activation through trans-autophosphorylation (29). To determine whether Ang1-induced activation of PI3K is dependent on kinase-activity of c-Fes, Ang1-promoted activation of PI3K was examined in stable cell lines expressing either wild-type or kinase-inactive c-Fes. As shown in Fig. 2E, Ang1 induced activation of PI3K in empty vector-transfected (mock) cells and cells expressing wild-type c-Fes (WT6-8 cells), but not in cells expressing kinase-inactive c-Fes (KE5-15 cells). This result suggests that Ang1-induced activation of PI3K in endothelial cells may to a large extent depend on c-Fes activity.

p70 S6K is one of the downstream effectors of PI3K (37). p70 S6K is involved in G1 cell cycle progression (38). As shown in Fig. 2F, Ang1 increased the phosphorylation of these residues of p70 S6K in IBE cells and HUVECs, although this phosphorylation was abolished by treatment with LY294002. A more slowly migrating fraction of S6K was also observed, which probably represented S6K with various extents of phosphorylation (37). This result indicates that activation of p70 S6K is strongly dependent on PI3K activity.

Inhibition of both mitogen-activated protein kinase and p70 S6K abrogates Ang1-promoted mitogenic activity. We examined the signal transduction pathways responsible for Ang1-promoted mitogenic activity in endothelial cells. We previously established an IBE cell line stably expressing deleted mutant p85 subunit of PI3K, which does not associate with the p110 catalytic subunit (30). The Δp85-8 cell line exhibited a dominant-negative effect on Ang1-induced activation of PI3K (Fig. 3A). We next examined the proliferation of IBE cell lines in response to Ang1-treatment. As shown in Fig. 3B, Ang1 induced proliferation of mock cells and WT6-8 Fes cells. In Δp85-8 and KE5-15 Fes cells, Ang1-induced proliferation was decreased. In these cells, Ang1 efficiently activated MAPK (data not shown). We examined the effects of...
pharmacologic inhibitors on Ang1-induced proliferation of IBE cells. As shown in Fig. 4A, LY294002 and PD98059 partially but significantly inhibited Ang1-induced proliferation. FGF-2-induced proliferation was strongly inhibited by treatment with PD98059, but not by treatment with LY294002. We also examined effects of the combination of PD98059 and rapamycin. As shown in Fig. 4B, PD98059 and rapamycin each inhibited Ang1-induced proliferation. When PD98059 and rapamycin were simultaneously added to the cells, Ang1-induced proliferation was completely blocked. In FGF-2-treated cells, rapamycin inhibited proliferation weakly, although not to a significant extent. We also examined the effects of inhibitors on Ang1-induced increase in labeling index of HUVECs. As shown in Fig. 4C, PD98059 and rapamycin each weakly inhibited Ang1-induced increase in labeling index. When PD98059 and rapamycin were added simultaneously, Ang1-induced labeling index was significantly decreased. Taken together, these results suggest that MAPK and p70 S6K are involved in Ang1-induced mitogenic activity for endothelial cells.

**Comparison of signal transduction pathways activated by Ang1 and Ang2.** We compared the effects of Ang1 and Ang2 on signal transduction pathways. As shown in Fig. 5A, Ang1 and Ang2 each induced autophosphorylation of Tie2. However, induction of phosphorylation by Ang2 was weaker than that by Ang1. We next examined the activation of Ras by Ang1 or Ang2. As shown in Fig. 5B, Ang1, but not Ang2, induced the activation of Ras. Subsequently, MAPK was activated by Ang1 treatment (Fig. 5C). Ang2 increased PI3K activity (Fig. 5D). However, this activity was higher in Ang1-treated cells than in Ang2-treated cells. Accordingly, Ang2-induced activation of p70 S6K was much weaker than that by Ang1 (Fig. 5E).

**Discussion**

In the present study, we examined the effects of Ang1 on two different types of endothelial cells. Although IBE is an immortalized cell line originating from mouse brain capillaries, it retains many characteristics of normal endothelial cells, such as the expression of von Willebrand factor, ability to bind to BS-I and UEA-I lectins, tube formation, uptake of acetylated low-density lipoprotein, and maintenance of gap junctional intercellular communications through connexin 43 (28). Ang1 dose-dependently stimulated the proliferation and increased the labeling index of IBE cells (Fig. 1A and B). We were unable to determine culture conditions suitable for examination of the proliferation of HUVECs. However, Ang1 dose-dependently promoted the entrance of HUVECs into S phase (Fig. 1C). Previous studies found that Ang1 failed to induce mitogenesis (as measured by DNA synthesis, proliferation, and labeling index) in HUVECs (23, 25, 39). In these studies, genetically modified Ang1 (denoted Ang1*) had little effect on the efficiency of production and purification. However, one study found that Ang1* weakly, but significantly, induced proliferation of HUVECs (40). In the present study, we used 6× His-tagged Ang1. It is unclear why discrepancies in these findings exist. One possible explanation is the difference among culture conditions. In many studies, HUVECs were cultured in medium supplemented with a high concentration of FBS (15-20%) without recombinant growth factors (23, 25, 39). The HUVECs we used were cultured in medium containing only 2% FBS, but supplemented with FGF-2, VEGF-A, insulin-like growth factor-I, and EGF. During culture, HUVECs used in previous studies might have grown highly dependent on FBS rather than pure proangiogenic factors. In vivo, overexpression of Ang1 caused increase in vascularity (41). In a rat aorta ring model, Ang1 induced sprouting of endothelial tubules (42). In these studies, proliferation of endothelial cells probably contributed to the findings obtained. However, the effects of the presence of several kinds of cells and of mitogenic factors that may be secreted by these cells cannot be completely excluded. Thus, our findings indicate that Ang1 is able to induce mitogenesis of endothelial cells.

In the present study, we compared the driving of signal transduction pathways by treatment with Ang1 and that with Ang2. Ang1 and Ang2 induced tyrosine phosphorylation of Tie2 and activation of PI3K (Fig. 5A and D). Ang2 did not activate Ras and MAPK (Fig. 5B and C), although the reason for this is unclear. Ang2-induced tyrosine phosphorylation of Tie2 was markedly lower than that by Ang1. Because receptor tyrosine kinases are

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1 S. Kanda, unpublished observations.
activated by dimerization or oligomerization (43, 44), it is likely that activation of Tie2 also requires this process. Testing with a number of recombinant proteins composed of various domains of Ang1 and 2 revealed that tetramer or more clustered forms of Ang1 strongly activated Tie2 in endothelial cells (34). Ang2 also formed tetramer or more clustered forms, similar to Ang1. However, this multimeric Ang1 also activated Tie2 in fibroblasts, but not in endothelial cells. These results suggest that oligomerization of Tie2 may be induced to distinct extent by Ang1 or Ang2. It is also possible that yet undefined cell surface proteins determine the quality of clusters containing Tie2.

In addition, the cytoplasmic region of Tie2 has a specific autoinhibitory function, unlike other receptor tyrosine kinases (45). In the COOH-terminal tail of Tie2, there are three autophosphorylation sites, Y1101, Y1107, and Y1112 (14). In unphosphorylated state, Y1101 and Y1112 are hydrogen-bonded to surrounding residues to stabilize the COOH-terminal tail in the inhibitory conformation of tyrosine kinase of Tie2. Deletion of the COOH-terminal tail (amino acid residues 1,108-1,123) increased the inhibitory conformation of tyrosine kinase of Tie2. Deletion of the COOH-terminal tail of Tie2, the three autophosphorylation sites are located nearby each other, binding of multiple signaling molecules determine the quality of clusters containing Tie2.

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