Chemotactic Properties of Angiopoietin-1 and -2, Ligands for the Endothelial-specific Receptor Tyrosine Kinase Tie2*

(Received for publication, January 7, 1998, and in revised form, May 6, 1998)

Bernhard Witzenbichler, Peter C. Maisonpierre‡, Pamela Jones‡, George D. Yancopoulos‡, and Jeffrey M. Isner§

From the Department of Cardiovascular Research, St. Elizabeth’s Medical Center of Boston, Tufts University School of Medicine, Boston, MA 02135 and Regeneron Pharmaceuticals, Inc., Tarrytown, New York 10591

Angiopoietin-1 and its putative natural antagonist, angiopoietin-2, were recently isolated, and the critical role of angiopoietin-1 in embryogenic angiogenesis was demonstrated by targeted gene disruption. Specific biological effects of angiopoietin-1, however, have yet to be defined. In this study we demonstrate that angiopoietin-1, but not angiopoietin-2, is chemotactic for endothelial cells. In contrast, angiopoietin-1 as well as angiopoietin-2 exhibit no proliferative effect on endothelial cells. Excess soluble Tie2, but not Tie1 receptor, abolish the chemotactic response of endothelial cells toward angiopoietin-1. Angiopoietin-2 dose-dependently blocks directed migration toward angiopoietin-1, consistent with the role of angiopoietin-2 as a naturally occurring inhibitor of angiopoietin-1. Fibroblasts stably transfected with Tie2 receptor exhibit chemotactic responses for both angiopoietin-1 and angiopoietin-2. Fibroblasts stably expressing a transfected chimeric receptor consisting of the extracellular domain of TrkC fused to the cytoplasmic domain of Tie2 also exhibit a chemotactic response to neurotrophin 3 (NT-3), a specific ligand for TrkC. Endothelial cells are shown to express angiopoietin-2 mRNA and protein, indicating the potential for autocrine activation of angiopoietin/Tie2. Finally, the demonstration that Tie2 as well as angiopoietin-1 are expressed in normal human arteries and veins suggests that the role of angiopoietin/Tie2 may extend beyond embryonic angiogenesis to maintaining integrity of the adult vasculature.

The receptor tyrosine kinase family of cell surface proteins is known to play key roles in transducing intercellular signals to the cytoplasm (1). A large diversity of receptor tyrosine kinases and receptor tyrosine kinase expression patterns, which are temporally modified during development and under pathologic conditions, determines cell fate and allows tissue-specific cell responses. Ligand binding to the large extracellular domain of receptor tyrosine kinases leads to receptor dimerization and autophosphorylation of tyrosine residues on the intracellular domain of the receptor (2). A variety of Src homology 2 domain-containing proteins, which are recruited to these phosphorylation sites, have been identified (3). These proteins are regarded as initiators of different signal cascades that finally lead to specific cellular responses including proliferation, migration, differentiation, and morphologic organization in the context of surrounding tissues (4).

On the basis of sequence similarity and structural characteristics, it is possible to classify receptor tyrosine kinases into subfamilies (1). Two receptor tyrosine kinase subfamilies are characterized by their largely endothelium-specific expression. One of them consists of the three known vascular endothelial growth factor (VEGF) receptors Flt-1/VEGF-R1, Flk-1/KDR/VEGF-R2, and Flt-4/VEGF-R3 (5, 6). The critical role of these receptors in promoting vasculogenesis and angiogenesis during normal embryogenesis was demonstrated using mutant mice with targeted disruption of the Flt-1 (7) or flk-1 gene (8). Moreover, expression of these receptors in the endothelial layer of adult blood vessels (9) is thought to mediate VEGF-induced post-natal angiogenesis in pathologic (10–12) or therapeutic settings (13, 14) but is also believed to be important in maintaining vessel integrity (15).

The Tie (tyrosine kinase with immunoglobulin (Ig) and ephringer growth factor (EGF) homology domains) receptor family comprises a second endothelial-specific subfamily of receptor tyrosine kinases termed Tie1 (16–19) and Tie2 (20). Similar to the VEGF receptors, both receptors have been shown to be critically involved in the formation of embryonic vasculature. Tie1 null mice (22, 23) as well as mice deficient in Tie2 (24) display a lethal phenotype caused by severe defects in embryonic vasculature. Moreover, an activating mutation in the Tie2 receptor was shown to cause inherited venous malformations in humans (25); these malformed vessels exhibited a disproportionately large number of endothelial cells (ECs) versus smooth muscle cells (SMCs), underlining the importance of an intact Tie2 receptor system for EC-SMC communication in vascular morphogenesis.

Although the ligands of the three VEGF receptors, their binding patterns, and their functions are relatively well characterized (5, 6, 26), the ligand for the Tie2 receptor, named angiopoietin-1 (Ang1), was only recently isolated (27), whereas the ligand(s) for the Tie1 receptors are still unknown. Targeted disruption of the Ang1 gene in mice resulted in an embryonic lethal phenotype by day 12.5 with generalized defects in vascular structure reminiscent of those previously observed in mice lacking Tie2 (28). Ang1 protein phosphorylated Tie2 receptor in a variety of ECs but did not induce EC growth or tube...

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* The work was supported in part by National Institutes of Health Grants HL 40518, HL 53354, HL 57516 and Academic Award in Vascular Medicine HL 02824 (from NHLBI). The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement is for information purposes only and has nothing to do with the advertised product, its quality, or acceptance or rejection of such product by the authors or editor. The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement is for information purposes only and has nothing to do with the advertised product, its quality, or acceptance or rejection of such product by the authors or editor.

§ To whom correspondence should be addressed: St. Elizabeth’s Medical Center of Boston, 736 Cambridge St., Boston, MA 02135. Tel.: 617-789-2392; 617-779-6362.

† VEGF, vascular endothelial growth factor; EC, endothelial cell; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; NT-3, neurotrophin 3; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; SMC, smooth muscle cells; Ang, angiopoietin; HM, human microvascular; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium; RT, reverse transcription; PCR, polymerase chain reaction; CM, conditioned media; rTie1-Fc, Tie1 receptor-Fc.
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MATERIALS AND METHODS

Cell Culture and Media—Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord vein by collagenase treatment as described previously (32). Human umbilical vein endothelial cells (Life Technologies, Inc.) supplemented with 20% fetal bovine serum (FBS) (Life Technologies), 100 μg/ml EC growth supplement, and 50 units/ml heparin (Sigma). HUVECs were used between passages 3 and 5. Human microvascular (HM) ECs of dermal origin were purchased from Clonetics, grown in EBM medium containing human epidermal growth factor (10 ng/ml), hydrocortisone (10 ng/ml), 5% FBS, and 0.4% bovine brain extract (Clonetics). Human microvascular endothelial cells were used between passages 4 and 6. Rat vascular smooth muscle cells were extracted from rats as described previously (33) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Human vascular smooth muscle cells were derived by explant outgrowth from unused portions of saphenous veins obtained at coronary bypass surgery as described previously (34) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% FBS. NIH 3T3 fibroblasts were purchased from the American Type Culture Collection and maintained in 10% FBS. Stable transfections of fibroblasts with an expression plasmid encoding full-length Tie2 receptor cDNA, a chimeric receptor consisting of the ectodomain of TrkC fused to the cytoplasmic domain of Tie2 or with empty vector (mock), were produced as before (29).

Recombinant Proteins—Ang1* and Ang2 recombinant protein were produced using baculovirus vectors and purified using protein A-Sepharose as described previously (27). (In the case of Ang1, the protein used for these in vitro studies was modified from the original and has been designated Ang1*) (29). The purity of proteins was greater than 95% as judged by reducing and nonreducing silver-stained SDS-polyacrylamide gel electrophoresis. Soluble Tie1 receptor-Fc (rTie1-Fc) and Tie2 receptor-Fc (rTie2-Fc) fusion proteins were constructed, purified, and produced as described recently (27). Heterodimeric recombinant human VEGF165, a protein purified from Escherichia coli was a generous gift of Drs. N. Ferrara, B. Keyt, and S. Bunting at Genentech Inc.

Cell Proliferation Assay—Cell proliferation was assayed using the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfoophenyl)-2H-tetrazolium) assay with the electron coupling reagent phenazine methosulfate (CellTiter 96 AQ, Promega). This assay was previously validated and demonstrated to positively correlate with cell number (35). Cells were seeded in a fibronectin-coated 96-well plate at 1×10^4 cells/well in 0.1 ml of serum-supplemented M199 and allowed to attach overnight. After 24-h incubation at 37 °C, 5% FBS was added to the wells for 48 h as indicated. MTS/phenazine methosulfate mixture (20 μl) was added to each well and allowed to incubate for 1 h at 37 °C before measuring absorbance at 490 nm in an enzyme-linked immunosorbent assay plate reader. Background absorbance from control wells was subtracted, and seven wells were performed in parallel for each condition.

Measurement of DNA Synthesis—EC DNA synthesis was evaluated by [3H]thymidine incorporation assay. A total of 2×10^4 cells/well in M199 containing 5% FBS were seeded in 24-well plates, coated with 1.5% gelatin, and allowed to attach overnight. Specific conditions (see below) were employed for 48 h before the addition of 2 μl/ml [3H]thymidine (Amersham Pharma Biotech). After 4 h, cells were washed with phosphate-buffered saline, precipitated with 10% trichloroacetic acid, lysed in 0.25 N NaOH, and then transferred into glass vials filled with scintillation liquid. Radioactivity was measured in a Beckman counter and expressed as counts/min (cpm). The addition of phorbol 12-myristate 13-acetate (Sigma) was used as a positive control to induce [3H]thymidine incorporation. Each sample was done in triplicate.

Cell Migration Assay—Migration assays were performed using a 48-well microchemotaxis chamber (Neuroprobe) (36). Polyvinylpyrrolidone-free polycarbonate filters with a pore size of 8 μm (Nuclepore) were coated with 50 μg/ml fibronectin and 0.1% gelatin in phosphate-buffered saline for at least 6 h at room temperature and dried under sterile air. Test substances were diluted to appropriate concentrations in M199 supplemented with 1% FBS, and 25 μl of the final dilution was placed in the lower chamber of a modified Boyden chamber. Confluent cell cultures were washed and trypsinized for the minimum time required to achieve cell detachment. After placing the filter between lower and upper chambers, 2.5×10^5 cells suspended in 50 μl of M199 containing 1% FBS were seeded in the upper compartment. The apparatus was then incubated for 5 h at 37 °C in a humidified chamber with 5% CO2 to allow cell migration. After the incubation period, the filter was removed from the filter hold, and the filter membrane with migrated cells was scraped with a rubber policeman. The filters were fixed with methanol and stained with Giemsa solution (Diff-Quick, Baxter). Migration was quantified by counting cells in three random high power fields (100×) in each well. All groups were studied in quadruplicate.

Analysis of Angiopoietin-1, Angiopoietin-2, and Tie2 Receptor mRNA in Cultured Cells and Human Vessel Specimens—Unseparated segments of human internal mammary artery, radial artery, and saphenous vein were obtained during bypass surgery, as described previously (9); these specimens were retrieved from the operating room at the time of excision and were promptly processed to avoid degradation of RNA. Care was taken to remove these segments in a nontraumatic manner, including limiting manipulation of the specimens to the ends of the vascular segments. Total RNA from these specimens or from cell cultures as indicated was immediately isolated by phenol/chloroform extraction (37), and RNA concentration was calculated from absorbance at 260 nm.

Reverse transcription (RT) (38) was performed by heating a 10 μl reaction mixture containing 1 μg of total RNA and 20 μg/ml oligodeoxythymidine (Life Technologies, Inc.) at 70 °C for 10 min. After cooling, 20 μl of each reaction mixture was added to a mixture containing 6 μg Moloney’s murine leukemia virus RcNase H reverse transcriptase (Life Technologies, Inc.) were added in a final 20-μl reaction mixture containing 1 mmol/l each dNTP (Amersham), 10 mmol/l dithiorthioleter, 25 mmol/l Tris-HCl, pH 8.3, 75 mmol/l KCl, and 3mmol/l MgCl2, incubated for 1 h at 42 °C, heated 5 min at 95 °C, and diluted to 50 μl with double-distilled water. For the polymerase chain reaction

formation (27). In fact, no in vitro assay to date has demonstrated any biological function of Ang1 for ECs. Analysis of Ang1 mRNA expression in adult tissues revealed abundant expression in highly vascularized tissues such as skeletal muscle, prostate, ovary, uterus, and placenta (29), suggesting a role of Ang1 not only in embryonic angiogenesis but also in the adult vasculature. Given the paucity of in vitro data, however, the physiologic contribution of Ang1 to these processes remains enigmatic. The temporal appearance of embryonic Ang1 expression suggests that the Ang1/Tie2 system is not involved in the initial vasculogenic phase of vascular development (as shown for the VEGF system) but rather participates in angiogenic outgrowth (sprouts), vessel remodeling, and vascular maturation (22, 24, 8). The recent discovery of a natural antagonist for Tie2, named angiopoietin-2 (Ang2), that binds to Tie2 but does not transduce the receptor has added a further level of complexity to regulation by the Ang/Tie2 system (29).
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RESULTS

Effects of Angiopoietin-1 and -2 on Endothelial Cell Proliferation, DNA Synthesis, and Apoptosis—To evaluate cellular proliferation in response to Angi1* or Ang2, we stimulated HUVECs with increasing amounts of Ang1*, Ang2, or VEGF, a known EC-specific mitogen, for 48 h and performed MTS analysis. This assay was previously shown to accurately reflect cell number (35). VEGF treatment led to a significant dose-dependent increase in optical density, positively correlating with an increase in absolute cell number; a half-maximal response was between 1 and 10 ng/ml. In contrast, neither stimulation with Ang1* nor Ang2 over a wide dose range led to a significant increase in cell number (data not shown). In addition, the influence of Ang1* and -2 on DNA synthesis in HUVECs was evaluated by [3H]thymidine incorporation (data not shown). Phorbol 12-myristate 13-acetate (20 ng/ml) was used as a positive control and led to a 5.2 ± 0.2-fold increase in DNA synthesis compared with control. Whereas 10 ng/ml VEGF induced a 2.1 ± 0.03-fold significant increase in [3H]thymidine incorporation (p < 0.05), neither Ang1*, Ang2, nor a combination of both led to any change in DNA synthesis over base line. Moreover, combining VEGF with Ang1* (2.2 ± 0.1-fold) or Ang2 (2.1 ± 0.01) did not alter the effect of VEGF alone on DNA synthesis. Similar results were obtained using lower concentrations of Ang1* and Ang2 alone or for any combination tested (not shown).

The effect of 48-h stimulation with Ang1* and Ang2 on cell cycle progression and apoptosis induced by serum withdrawal (2% FBS) were investigated in HUVECs. Whereas VEGF stimulation increased the proportion of cells in S-phase (17.8 ± 3.0 versus 6.8 ± 2.2%; p < 0.05), results obtained for Ang1* and Ang2 stimulation did not exceed base-line values (Table I). Similarly, 10 ng/ml VEGF significantly reduced the amount of apoptotic cells in low serum condition (18.9 ± 3.5 versus 33.7 ± 6.7% for control, p < 0.05), whereas neither Ang1* nor Ang2 exerted any pro- or anti-apoptotic effects under the conditions tested.

Angiopoietin-1, but Not Angiopoietin-2, Is Chemotactic for Endothelial Cells—Using a modified 48-well chemotaxis Boyden chamber, we assessed the chemotactic response of HUVECs to increasing amounts of Ang1* and Ang2 and compared it to that of VEGF, which is known to be chemotactic for ECs (40). Ang1* led to a significant, dose-dependent increase in directed migration of HUVECs, with a half-maximal effect at a dose of 10 ng/ml (Fig. 1). Whereas at concentrations of 1 and 10 ng/ml the chemotactic effect of Ang1* seemed to be somewhat less than that of VEGF, the response at higher doses (100 and 1000 ng/ml) was indistinguishable. In contrast, Ang2 over a wide dose range did not exert any chemotactic properties.

To establish specificity of the observed effect on directed cell migration, we applied Ang1* at two different doses (0.1 and 1 μg/ml) with or without a 10-fold excess to reach maximal saturation concentration of either soluble Tie2 receptor (rTie2-Fc) or soluble Tie1 receptor (rTie1-Fc) (1 and 10 μg/ml) to the lower chambers of the Boyden apparatus. As shown in (Fig. 2), rTie2-Fc, competing off Ang1* from its cellular receptor due to its molar excess, completely blocked the chemotactic effect of

| Condition | Control | Ang1* | Ang2 | VEGF |
|-----------|---------|-------|------|------|
| S-Phase [%] | 6.8 ± 2.2 | 9.4 ± 3.1 | 7.1 ± 2.4 | 17.8 ± 3.0 |
| Apoptosis [%] | 33.7 ± 6.7 | 41.7 ± 7.0 | 36.3 ± 5.9 | 18.9 ± 3.5 |

*p < 0.05 versus control.
versus concentrations of VEGF, Ang1*, or Ang2 as indicated. Diluted test substances were placed in the lower chamber of a 48-well microchemotaxis Boyden apparatus. A total 2.5 × 10^5 cells/well were seeded into the upper chambers, and cells were allowed to migrate for 5 h through 8-μm pores of a fibronectin/gelatin-coated polycarbonate membrane placed between upper and lower chambers. After the migration period, nonmigrating cells were removed from the upper side of the membrane, the membrane was stained using a Giemsa solution, and migrated cells were quantified by counting nuclei in three random high power fields (HPF, 100×/well). Data points represent migrated cell numbers/ quadruplicate wells ±S.E. *p < 0.05 versus control (medium with vehicle solution, no factors added).

Ang1* on HUVECs. In contrast, rTie1-Fc, a closely related orphan receptor that does not bind Ang1, was not sufficient to inhibit the migratory response. Both results taken together indicate that the observed effects are due to Ang1 in the solution and cannot be explained by remaining impurities after the purification process of Ang1.

To distinguish between chemotactic and chemokinetic effects of Ang1 on ECs, we performed a modified checkerboard analysis in which the concentration of chemotactic above and below the filter was varied. Table II indicates that significant migration was observed only when a concentration gradient of Ang1* between upper and lower compartments was established, a finding typical of factors inducing chemotaxis. In contrast, adding equivalent concentrations of Ang1* to both sides of the filter did not enhance cell movement, thereby excluding a significant chemokinetic effect of Ang1.

To establish specificity, the chemotactic response of ECs and SMCs from different tissue origins toward Ang1* and Ang2 were determined. Again, Ang2 did not exert any effect on either cell type. Ang1*, however, stimulated directed migration in bovine aortic and HM ECs, whereas no effect on rat or human vascular SMCs was observed (Fig. 3). These results indicate that the chemotactic effect of Ang1 is cell-type specific but not limited to ECs of a certain origin.

Angiopoietin-2 Inhibits the Chemotactic Effect of Angiopoietin-1—Because previous findings have indicated that a naturally occurring antagonist for Tie2, we investigated if Ang2 is able to suppress the chemotactic response evoked by Ang1 in HUVECs. When increasing amounts of Ang2 were added to a constant amount of Ang1 (100 ng/ml) in the lower chamber of a Boyden chamber, the chemotactic effect of Ang1 on these cells was gradually inhibited (Fig. 4). To completely suppress the number of migrated cells to control value, an 8–10-fold excess of Ang2 over Ang1 was required. This result indicates that Ang2 is able to compete with Ang1 for Tie2 receptor binding sites, but binding of Ang2 to Tie2 does not lead to the same receptor signal transduction events necessary to stimulate chemotaxis.

Table II: Checkerboard analysis of HUVECs in response to angiopoietin-1 using modified Boyden chamber

| Ang1* in lower chamber | 0 | 0.1 | 1 |
|------------------------|---|-----|---|
| 0                      | 60 ± 5 | 58 ± 4 | 64 ± 5 |
| 0.1                    | 120 ± 10⁺ | 62 ± 5 | 60 ± 3 |
| 1                      | 145 ± 20⁺ | 90 ± 4 | 59 ± 4 |

⁺ p < 0.05 each (Fig. 5A, left bars). In contrast, NIH 3T3 fibroblasts stably expressing transfected Tie2 receptors demonstrated a marked increase in cell migration to 466 ± 6% for 100 ng/ml Ang1* and to 410 ± 13% for the same amount of Ang2 compared with control buffer solution (p < 0.05 each (Fig. 5A, middle bars)). A 10-fold excess of rTie2-Fc applied to the lower compartment of the Boyden apparatus completely abolished the chemotactic effect of both Ang1* and Ang2 on 3T3-Tie2 fibroblasts (147 ± 16% for Ang1*, 120 ± 21% for Ang2, p = not significant versus control), whereas chemotaxis toward PDGF-BB was not influenced by excess rTie2-Fc (Fig. 5A, right bars). Thus, fibroblasts ectopically expressing Tie2 receptor demonstrated a specific chemotactic response to both Ang1* and Ang2; in contrast, ECs exhibited a specific chemotactic response only to Ang1.

Moreover, we investigated other potential ways in which activation of the Tie2 catalytic domain could produce chemotactic responses. MG87 fibroblasts (a 3T3 variant cell line)
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Migration assays were performed as described in Fig. 1 utilizing human microvascular cells (HMECs), bovine aortic endothelial cells (BAECs), and rat and human vascular smooth muscle cells (VSMCs). Ang1* or Ang2 at 0.2 μg/ml was added to the lower chambers of the Boyden apparatus. Bars represent the mean ± S.E. from quadruplicate wells. HPF, high power field (100×). *p < 0.05 versus control. n.s., not significant.

Angiopoietin-1, -2, and Tie2 Receptor mRNA Expression in Human Vascular Specimens and Cultured Cells Analyzed by RT-PCR—Ang1, Ang2, and Tie2 mRNA expression was investigated using RT-PCR in explanted human specimens of internal mammary artery, radial artery, and saphenous vein. As shown in (Fig. 6), both Ang1 as well as Ang2 mRNA were detectable in all three tissues. Furthermore, Tie2 mRNA expression was documented in all specimens investigated. Non-transfected NIH 3T3 fibroblasts did not express Ang1/2 or Tie2 and served as negative controls (Fig. 6, lane 4), whereas amplification of glyceraldehyde-3-phosphate dehydrogenase was used as control for comparable amount and quality of mRNA among samples. Positive controls included PCR amplification of cloning vectors containing full-length cDNAs for Ang1, Ang2, and Tie2, yielding PCR products in agreement with the predicted sizes (not shown). Successful expression of Tie2 receptor mRNA in the stably transfected 3T3 fibroblasts was also confirmed by RT-PCR (Fig. 6, lane 5). As expected, Tie2 mRNA was easily detectable in different ECs (HUVECs, human microvascular endothelial cells, BAECs; in Fig. 6, lane 6 only shown for HUVECs). Ang1 as well as Ang2 mRNA were observed not only in human vascular SMCs but in ECs as well (lanes 6 and 7).

Angiopoietin-1, -2, and Tie2 Receptor mRNA Expression in Cultured Endothelial Cells Analyzed by Northern Blot—When Northern blot analysis was performed on cultured ECs, expression of Ang2 as well as Tie1 and Tie2 was confirmed at the mRNA level in HUVECs, cultured human aortic ECs, and cultured human microvascular ECs (Fig. 7). In contrast, human cutaneous fat pad ECs expressed an extraordinary amount of Ang1 transcripts but were negative for Ang2.

DISCUSSION

Recent gene targeting experiments disrupting either Tie2 (22, 24) or Ang1 (28) have clearly established a significant role for the Tie2/Ang1 system in embryonic angiogenesis. Whereas

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3 S. Davis, unpublished data.
ECs are absent from VEGF-R2 null mice (8), ECs in Tie2 null mice are present in normal numbers and are assembled into tubes; the vessels of Tie2 mice, however, appear immature, lacking the extent of branching and organization typical for normal vessels. Similar vascular defects, although less severe, are seen in Ang1 null mice (28): ECs are aberrantly rounded, do not spread and/or flatten normally, are loosely associated with the underlying matrix, and do not properly associate with periendothelial cells. These findings were interpreted as evidence that the physiologic roles of the Tie2/Ang system in angiogenesis come into play subsequent to regulation by VEGF, although the exact nature of Tie2/Ang remained to be clarified. Our finding of a strong and specific chemotactic effect of Ang1 on ECs is to our knowledge the first description of a direct biological function of the Tie2/Ang1-system, implies a...
close spatial and polarized interaction between Ang1-secreting cells and ECs expressing the Tie2 receptor, and thus establishes putative basis for defective angiogenesis in mice lacking Tie2 or Ang1.

The critical contribution of EC migration, as opposed to proliferation, in angiogenesis was previously demonstrated in a classic experiment performed in the rat cornea by Sholley et al. (43). Vascular sprouting could be induced and continued for more than 2 days, despite irradiation treatment sufficient to suppress DNA synthesis. Initiation of angiogenesis in this model was thus interpreted to reflect the dominant impact of EC migration.

Tie2 receptor and Ang1 mRNA were easily detectable in normal human arterial and venous specimens. Tie2 expression was recently shown by immunohistochemical staining to be present in quiescent adult tissues, including vascular endothelium, (15); the extent of Tie2 phosphorylation was interpreted as inferential evidence for the presence of Tie2 ligand(s) in quiescent adult tissues, consistent with previous findings that Ang1 is widely expressed in adult tissues (29). Our direct demonstration of Ang1 in the adult vessel wall is in agreement with this notion.

Tie2/Ang1 expression pattern, together with the novel finding of an EC-specific chemoattractive effect of Ang1, suggests two possible functions of the Tie2/Ang1 system. First, in normal adult quiescent vessels, periendothelial cells (pericytes, SMCs) constitutively secrete Ang1, enhancing contact between neighboring ECs and between ECs/periendothelial cells, thus maintaining endothelial integrity and orientation of ECs on the basal lamina. Disruption of the endothelial monolayer could lead to up-regulation of Ang1 expression in surrounding cells and/or changes in Tie2 receptor expression in ECs, which have lost contact with adjacent ECs. Up-regulation of VEGF expression, resulting in a strong mitogenic effect on ECs and subsequent reendothelialization of denuded areas, was recently demonstrated in response to balloon injury (15). The chemotactic effect of Ang1 may be involved as an adjunct to VEGF in recruiting ECs to initiate and accelerate reendothelialization. In the Ang1 and Tie2 knockout mice, the absent impact of Ang1 on ECs may contribute to the poor association between ECs and periendothelial cells as well as the lack of EC spreading, resulting in immature appearing vessels.

Second, under conditions of postnatal angiogenesis, such as tissue ischemia or tumor growth, the chemoattractive effect of Ang1 on ECs may be important for initiation of new capillary sprouting, as well as movement of ECs toward each other required for fusion into capillary structures. Such three-dimensional EC fusion processes may be responsible for the cork-screw-like appearance typical of collaterals in ischemic areas or neovascularized tumors. In this regard, the effects of Ang1 may be achieved in a tightly localized fashion, possibly requiring a concentration gradient of Ang1 to induce an effect. In vitro systems such as the tube formation assay may fail to disclose an effect of Ang1 because exogenous factors are introduced “on top” of the cells, disregarding concentration gradients of molecules that may be more typical of angiogenesis in vivo. The trigger for increased responsiveness of the Tie2/Ang system remains enigmatic; unlike VEGF, which is known to be positively regulated by hypoxia (44), Ang1 seems not to be up-regulated by hypoxia (45). Enhanced expression of Tie2, however, has been observed in the vascular endothelium of metastatic melanomas (46) and during ovulation and wound healing (47), suggesting the possibility that Tie2/Ang1 is regulated at the level of receptor expression.

A second ligand for the Tie2 receptor, named Ang2, having nearly 60% homology in amino acid sequence with Ang1, was recently identified (29). Two experimental findings were interpreted as evidence to suggest that Ang2 might represent a naturally occurring antagonist for Tie2. First, Ang2 was shown to bind to Tie2 expressed on ECs without transducing the receptor and was capable as well of blocking Ang1-induced receptor phosphorylation (29). Second, transgenic overexpression of Ang2 led to disruption of blood vessel formation in the mouse embryo (29). Our in vitro finding that Ang1 chemotactic activity for ECs can be blocked by adding increasing amounts of Ang2 is consistent with these results and suggests a negative regulatory function of Ang2 versus Ang1 by competitive binding to Tie2 without subsequent autophosphorylation of the cytoplasmic tyrosine kinase domain. Moreover, our demonstration of Ang2 expression in the normal quiescent vessel wall has not been previously reported and suggests a delicate balance of vessel maintenance by positive and negative regulators.

The lack of available antibodies for immunostaining precluded identification of cell type-specific Ang2 expression in intact vascular specimens. Studies of cultured cells, however, disclosed that Ang2 expression was not limited to SMCs but could be detected in ECs as well. Specifically, Ang2 mRNA was identified by RT-PCR in HUVECs, by Northern analysis in HUVECs, human aortic endothelial cells, and dermal microvascular endothelial cells, and by Western analysis in HUVECs. In contrast, although Ang1 was identified by RT-PCR in HUVECs, it was not detected by Northern analysis of HUVECs, human aortic endothelial cells, or human microvascular endothelial cells nor Western analysis of conditioned media of HUVECs. These findings appear to corroborate the results of in situ hybridization demonstrating endothelial expression of Ang2 transcripts in vivo (29). An apparent exception are cutaneous fat pad microvascular endothelial cells, which by Northern analysis were shown to express an extraordinary amount of Ang1 transcripts but were negative for Ang2. It is unclear what this finding means since detection of Ang1 expression in vivo has generally been shown to localize to nonendothelial cells in vivo (27). For those ECs that are able to express Ang2, it is intriguing to propose an autocrine mode of Ang2 expression whereby ECs might block themselves from the influence of externally derived Tie2 ligand(s).

Previous reports (29) have indicated that although Ang2 antagonizes Ang1-induced receptor phosphorylation in ECs, it is surprisingly equivalent to Ang1 for promoting Tie2 phosphorylation when the receptor is ectopically expressed in fibroblasts. Our demonstration of a similar chemotactic response of Tie2-expressing fibroblasts for Ang1 and Ang2 extends these previous findings to a functional level. Thus fibroblasts appear to express a sufficient “repertoire” of intracellular adaptor proteins to effectively interact with ectopically expressed Tie2 receptor and trigger a signal cascade, resulting in cell migration. This is confirmed in particular by our finding that fibroblasts transfected with a chimeric receptor encoding the cytoplasmic domain of Tie2 fused to TrkC extracellular binding domain, a neuron-specific receptor for NT-3, can be stimulated to migrate by adding NT-3. The differential effect of Ang2 in endothelial cells and nonendothelial cells may be due to a lack of critical signaling element(s) in the former. An alternative explanation is likely required, however, given that chemotaxis is the end result of Tie2 receptor activation in both cell types. One potential explanation for this paradox is that binding of Tie2 by Ang2 leads to preferred heterodimerization of the receptor with another membrane component such as Tie1 or an as yet unidentified EC-specific receptor, “trapping” Tie2 monomers and thereby preventing reciprocal phosphorylation of cytoplasmic receptor kinase domains. Further experiments, in-
including concomitant transfection of fibroblasts with Tie2 and Tie1, will be required to substantiate this concept.

Acknowledgments—The authors are grateful to Drs. Samuel Davis, Terence Ryan, Thomas Daly, and Nick Papadopoulos of the Protein Sciences Division of Regeneron for recombinant AngI*, Tie1-Fc, and Tie2-Fc and to Tammy Huang for technical assistance.

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