Direct Cell Adhesion to the Angiopoietins Mediated by Integrins*

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Genetic ablation of angiopoietin-1 (Ang-1) or of its cognate receptor, Tie2, disrupts angiogenesis in mouse embryos. The endothelial cells in growing blood vessels of Ang-1 knockout mice have a rounded appearance and are poorly associated with one another and their underlying basement membranes (Dumont, D. J., Gradwohl, G., Fong, G. H., Puri, M. C., Gertenstein, M., Auerbach, A., and Breitman, M. L. (1994) Genes Dev. 8, 1987–1999; Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995) Nature 376, 70–74; Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonneville, P. C., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1996) Cell 87, 1171–1180). It is therefore possible that Ang-1 regulates endothelial cell adhesion. In this study we asked whether Ang-1 might act as a direct substrate for cell adhesion. Human umbilical vein endothelial cells (HUVECs) plated for a brief period on different substrates were found to adhere and spread well on Ang-1. Similar results were seen on angiopoietin-2 (Ang-2)-coated surfaces, although cells did not spread well on Ang-2. Ang-1, but not Ang-2, supported HUVEC migration, and this was independent of growth factor activity. When the same experiments were done with fibroblasts that either lacked, or stably expressed, Tie2, results similar to those with HUVECs were seen, suggesting that adhesion to the angiopoietins was independent of Tie2 and not limited to endothelial cells. Interestingly, when integrin-blocking agents were included in these assays, adhesion to either angiopoietin was significantly reduced. Moreover, Chinese hamster ovary-B2 cells lacking the α5 integrin subunit did not adhere to Ang-1, but they did adhere to Ang-2. Stable expression of the human α5 integrin subunit in these cells rescued adhesion to Ang-1 and promoted an increase in adhesion to Ang-2. We also found that Ang-1 and Ang-2 bind rather selectively to vitronectin. These results suggest that, beyond their role in modulating Tie2 signaling, Ang-1 and Ang-2 can directly support cell adhesion mediated by integrins.

Angiogenesis, the de novo sprouting and remodeling of capillaries from preexisting blood vessels, is a critical process during both vertebrate development and adult life (4). This process can be divided into several distinct, often over-lapping, phases. In general, angiogenesis initiates with vasodilation and increases in endothelial permeability. Subsequently, endothelial cells begin to proliferate and migrate toward the angiogenic stimulus. During the final maturation stages, the endothelial cells acquire a more differentiated state marked by lumen formation and production and assembly of a complex basement membrane. Finally, periendothelial cells are recruited into the area thereby providing further support for the new vessel. A balance between stimulatory and inhibitory signals controls each step during angiogenesis. Such signals can arrive in the form of growth factors, cytokines, and extracellular matrix (ECM) proteins, to name a few. These extracellular cues are then transduced to the cytoplasm by various different classes of cell surface receptors, with the most common being members of either the receptor tyrosine kinase or integrin superfamilies (5).

The angiopoietins, along with their cell surface receptor tyrosine kinase, Tie2, comprise one of the most widely studied families of angiogenic factors. Unlike other well known angiogenic factors, the angiopoietins are not mitogenic for endothelial cells. Thus, their biological function is not well understood. The angiopoietin family members contain an N-terminal coiled-coil domain as well as a C-terminal fibronogen-like domain that shares a high degree of homology to the analogous domains in the ECM proteins tenascin-C and fibrinogen-γ and -β (6–9). Tie2 or Ang-1 knockout mice are embryonic lethal, with the most prominent defects involving the vasculature of the heart, brain, and yolk sac (1–3). In the heart, the endocardium appears to detach from the myocardium, and the atrial lining is almost collapsed (3). Ultrastructural analysis of the blood vessels in these animals reveals defects in endothelial cell interactions with their basement membranes as well as a marked decrease in the number of periendothelial support cells. Endothelial cells in the knockout embryos appear rounded, and there is poor organization of collagen-like fibers in the endothelial basement membranes (3). Conversely, overexpression of Ang-1 in transgenic mice leads to an increase in both vessel size and extent of branching (10) as well as to vessels that are resistant to inflammatory agent-induced leakage (11). Furthermore, adenoviral administration of Ang-1 is also an effective way to prevent plasma leakage in the adult vasculature (12).

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The role of Ang-2 in the developing vascular system is less well understood. *In vitro*, Ang-2 does not activate Tie2 in endothelial cells even though its affinity for the receptor is similar to that of Ang-1 (7). Moreover, the presence of excess Ang-2 can prevent Tie2 phosphorylation induced by Ang-1. Additionally, transgenic overexpression of Ang-2 in mice results in embryonic lethality, with the animals displaying a phenotype similar to that of Ang-1 knockout mice (7). These findings have led to the hypothesis that the role of Ang-2 *in vivo* is to serve as a natural antagonist for Ang-1.

Based on the aforementioned data with Ang-1 knockout and transgenic animals, it has been suggested that Ang-1 may be a critical mediator of endothelial cell-cell or cell-ECM interactions. Currently, however, very little data are available to support this hypothesis. In a study using a pro-B cell line stably expressing Tie2, it was found that the addition of Ang-1 to the culture medium enhances cell adhesion to fibronectin (13). In another report, fluorescence-activated cell-sorter Tie2-positive hematopoietic cells also responded to Ang-1 treatment with increased adherence to a fibronectin coated substrate (14). In both studies, excess Ang-2 or soluble Tie2 receptor was able to block the effect of Ang-1, suggesting that Ang-1 signals through Tie2 to modulate the activity of adhesion receptor(s) on the surface of hematopoietic cells.

In this study, we have examined the role of Ang-1 and Ang-2 in direct cell adhesion *in vitro*. We show that although both Ang-1 and Ang-2 serve as substrates for cell adhesion, they differentially regulate cell spreading, cell migration, and activation of intracellular signaling pathways. Additionally, we report that Tie2 is not required for cell adhesion or migration, as NIH 3T3 fibroblasts lacking this receptor adhere to Ang-1 and Ang-2 and migrate on Ang-1 as well as fibroblasts ectopically expressing Tie2. Therefore, we propose the existence of additional cell surface receptors for both Ang-1 and Ang-2, and we suggest that integrins may be involved directly in cell adhesion to these angiogenic proteins.

**EXPERIMENTAL PROCEDURES**

**Materials—**Ang-1 and Ang-2 were produced as described previously (7). The Ang-1 used in all experiments is a slightly modified version (Cys<sup>245</sup>→Ala) that is easier to purify and has been shown to have activities similar to those of the wild type protein. Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT). DMEM, α-MEM, medium 199, trypsin-EDTA, antibiotics-antimycotics, and G418 were obtained from Life Technologies, Inc. Mouse collagen type IV, mouse laminin, human vitronectin, human epidermal growth factor, bFGF, platelet-derived growth factor-AB, peptides GRGDSP and GRADSP, and the function-blocking anti-integrin α<sub>v</sub>β<sub>3</sub> antibody P1D6 were also from Life Technologies, Inc. Fibronectin was purified from human plasma by gelatin-agarose affinity chromatography (15). Nido- gen was purchased from Sigma, 96-well ChemoTx migration units, 8 μm pore size, were purchased from Neuro Probe, Inc. (Gaithersburg, MD). 4–20% gradient SDS-polyacrylamide gels were from Invitrogen (Omar, UT). Anti-phospho-p44/42 MAPK polyclonal antibody and anti-p44/42 MAPK polyclonal antibody were purchased from New England Biolabs (Beverly, MA). Anti-phospho-FAK (PY397) was obtained from Biosource International (Camarillo, CA), and anti-FAK monoclonal (clone 2A7) antibody was from Upstate Biotechnology (Lake Placid, NY). The β<sub>i</sub> integrin monoclonal antibodies Hα2/5 (function blocking) and HMβ1-1 (immunofluorescence) and the FITC-labeled anti-hamster IgG were obtained from PharMingen (San Diego, CA). Anti-Ang-1 polyclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The following function-blocking anti-integrin antibodies were purchased from Chemicon (Temecula, CA): LM609 (anti-α<sub>v</sub>β<sub>3</sub>), P1F6 (anti-α<sub>v</sub>β<sub>3</sub>), AV1 (anti-α<sub>v</sub>β<sub>6</sub>), and 656 (anti-α<sub>v</sub>β<sub>3</sub>). Horseradish peroxidase-conjugated secondary antibodies were obtained from ICN (Costa Mesa, CA). ECL Plus reagent and Hyperfilm were purchased from Amersham Pharmacia Biotech. Vectashield was purchased from Vector Laboratories (Burlingame, CA). All other chemicals were obtained from Sigma.

**Cells—**HUVECs were obtained from Clonetics (Walkersville, MD) and maintained medium 199 + 20% fetal bovine serum + antibiotics-antimycotics. HUVEC medium was supplemented with 100 μg/ml heparin and 400 μg/ml bovine hypothalamus extract prepared as described (16). For all experiments, HUVECs were at passage 7 or below and collected from a confluent dish. NIH 3T3 fibroblasts, obtained from the laboratory of Dr. Marsha Rosner (University of Chicago), MG Tie2 fibroblasts (7), IMR-90 human fibroblasts (ATCC, Manassas, VA), and CCD 45SK human skin fibroblasts, obtained from the laboratory of Dr. Janus Burkhart (University of Chicago), were maintained in DMEM + 10% fetal bovine serum + antibiotics-antimycotics. G418, at a concentration of 400 μg/ml, was added to the medium of the MG Tie2 cells. CHO-B2 (17), -B2/V7 (18), and -B2/α27 (19) cells, a generous gift of Erkki Ruoslahti (Burnham Institute, La Jolla, CA), were cultured in α-MEM + 10% fetal bovine serum + antibiotics-antimycotics. G418 (400 μg/ml) was added to the medium of the B2/V7 and B2/α27 cells.

**Cell Adhesion Assays—**Wells of a 96-well tissue culture plate were coated for 1 h at room temperature with protein, diluted into PBS to the final concentration indicated in the figure legends. Wells were then blocked for at least 30 min at room temperature with 0.5% heat-inactivated BSA in PBS (heat inactivated at 80 °C for 10 min) and washed three times with PBS before adding cells.

HUVECs were harvested by trypsin treatment, collected in medium 199 + 0.5% BSA + soybean trypsin inhibitor, washed three times with medium 199 + 0.5% BSA, and suspended in the same medium. 36,000 cells were added per well, and the plate was incubated at 37 °C for 30 min. Nonadhered cells were washed away with PBS and PBS rinses and quantitation of adhesion was performed by measuring endogenous alkaline phosphatase activity as described previously (20). A<sub>405</sub> values in all figures were calculated by subtracting the A<sub>405</sub> of wells containing buffer alone (no cells) from the absolute A<sub>405</sub> readings.

Adhesion assays with fibroblasts were performed similarly, except that DMEM replaced medium 199, and cells were added at 28,000 cells/well (MG Tie2 and NIH 3T3) or 14,000 cells/well (IMR-90 and CCD 45SK). The adhesion medium for CHO-B2, -B2/V7, and -B2/α27 cells was α-MEM + 0.5% BSA, and cells were added at a concentration of 36,000 cells/well.

For EDTA, GRGDSP, and GRADSP inhibition, the agent was added at the concentration indicated in the figure legends immediately before seeding the cells onto the 96-well dish. For blocking experiments with anti-integrin monoclonal antibodies, cells were incubated with antibody for 15 min at 37 °C before plating.

Molecular masses used for molarity calculations were as follows (in kDa): Ang-1, Ang-2, and vitronectin, 55; fibronectin, 250; collagen I, 137; collagen IV, 510; laminin, 800; nidogen, 100.

**Cell Migration Assays—**Migration in a modified Boyden chamber assay was performed essentially as described (21), with the following modifications. The underside of the ChemoTx filter was coated for at least 1 h at room temperature with the protein indicated in the legend to Fig. 2, rinsed with PBS, and dried under sterile air. The lower chamber medium was prepared, and 33 μl was added to each well. For HUVECs, this medium was medium 199 + 0.5% BSA plus or minus 20 ng/ml bFGF. For fibroblasts, DMEM + 0.5% BSA plus or minus 10 ng/ml bFGF + anti-α<sub>v</sub>β<sub>3</sub>-dependent growth factor-AB was used.

HUVECs were harvested by trypsin treatment, collected in medium 199 + 0.5% BSA + soybean trypsin inhibitor, washed three times with medium 199 + 0.5% BSA, and suspended in the same medium at a concentration of 1.7 × 10<sup>5</sup> cells/ml. 15 μl of this suspension was added to the top of the filter, and the entire apparatus was incubated at 37 °C. After 4 h, nonmigrated cells from the top of the filter were scraped away with a cotton applicator. The filter was fixed for 20 min at room temperature with 3.7% paraformaldehyde, 60 mm sucrose in TBS, pH 7.4, and stained with 4,6-diamidino-2-phenylindole (1 μg/ml in TBS). Individual nuclei from two fields/well were counted manually after taking photomicrographs with a Zeiss Axiosplan fluorescence microscope connected to a Photometrics PXL CCD camera on an Apple Macintosh computer using the Open Lab software suite.

Migration assays with fibroblasts were performed similarly, except that DMEM replaced medium 199, and cells were suspended at 1 × 10<sup>5</sup> cells/ml before being added to the top of the filter.

**Western Blotting—**Wells of a 6-well dish (see Fig. 4) or a 24-well dish (see Fig. 5B) were coated for 1 h at room temperature with protein diluted in PBS to the concentration indicated in the figure legends. The wells were blocked for 1 h at room temperature with 0.5% heat-inactivated BSA and washed three times with PBS before adding cells. HUVECs were harvested by trypsin treatment, collected in medium 199 + 0.5% BSA + soybean trypsin inhibitor, washed three times with medium 199 + 0.5% BSA, suspended in the same medium, and seeded at 300,000 cells/well (Fig. 4) or 60,000 cells/well (Fig. 5B). In Fig. 5B, EDTA (10 mM), GRGDSP (333 μg/ml), or GRADSP (333 μg/ml) was added.
added to cells immediately before plating. In both experiments cells were allowed to adhere for 30 min at 37 °C. Suspended cells were either stimulated with bFGF (20 ng/ml) or not stimulated 10 min before lysis. After the adhesion period, plates were placed on ice, unattached cells were collected and spun down, and cell monolayers were lysed with SDS/Polyacrylamide gel sample buffer (100 μl for Fig. 4 or 75 μl for Fig. 5B). Centrifuged pellets of unattached cells were combined with the appropriate monolayer lysate, and the samples were heated to 100 °C for 5 min. Samples were separated via SDS-polyacrylamide gel electrophoresis, transferred to Immobilon P membranes (Millipore, Bedford, MA), and blocked with 5% nonfat dry milk in TBS containing 1% Tween 20 for 1 h at room temperature. Incubation with primary antibody was overnight at 4 °C using the following antibody concentrations, diluted in blocking buffer: anti-phospho p42/44 MAPK (1:3,000), anti-phospho-FAK (1:5,000), or anti-FAK (1:1,000). Membranes were probed with secondary antibody (1,500 dilution in blocking buffer) for 1 h at room temperature. Blots were developed with the ECL Plus reagent and exposed to Hyperfilm. Blots were stripped according to the instructions in the ECL Plus reagent kit. For quantification, blots were scanned and imported into NIH Image 1.61 for analysis. The area covered by pixels was measured, and the ratio of phosphorylated to unphosphorylated protein was calculated.

For Fig. 5B, the ratio for “No block” was assigned a value of 1, and the values for all other conditions represent the ratio relative to this number in order to eliminate apparent differences caused by variation in MAPK levels across the blots.

Immunofluorescence—For all experiments, coverslips were coated for 1 h at room temperature with the protein indicated in the figure legend, blocked for at least 30 min at room temperature with 0.5% heat-inactivated BSA, and washed three times with PBS before adding cells.

In Fig. 2, HUVECs were harvested by trypsin treatment, collected in medium 199 + 0.5% BSA + soybean trypsin inhibitor, washed three times with medium 199 + 0.5% BSA, suspended in the same medium, and seeded at ~15% confluence. After 2 h at 37 °C, cells were fixed with 3.7% paraformaldehyde, 60 mM sucrose in TBS for 30 min at room temperature, permeabilized with 0.5% Triton X-100 in TBS for 5 min at 4 °C, and blocked for 30 min at room temperature with TBS + 4% goat serum. Cells were stained for 30 min at room temperature with Texas Red-conjugated phalloidin (1:300) and 4,6-diamidino-2-phenylindole (1:1,000) in TBS + 4% goat serum.

In Fig. 6C, MG Tie2 or NIH 3T3 fibroblasts were harvested by trypsin treatment, collected in DMEM + 0.5% BSA + soybean trypsin inhibitor, washed three times with DMEM + 0.5% BSA, suspended in the same medium, and seeded at ~15% confluence. After 2 h at 37 °C, cells were fixed and permeabilized as described above. Coverslips were stained for 1 h at room temperature with the anti-β3 integrin antibody HMβ1-1 (1:2500 in blocking buffer) followed by FITC-conjugated anti-hamster IgG (1:250) and Texas Red-conjugated phalloidin (1:500) for 1 h at room temperature.

Regardless of staining protocol, all coverslips were mounted with Vectashield and visualized with a Zeiss Axioplan fluorescence microscope. Images were captured using a Photometrics PXL CCD camera connected to an Apple Macintosh computer using the Open Lab software suite.

In Vitro Binding Assays—Surface plasmon resonance assays were performed as described previously (6). The instrument for surface plasmon resonance reports the amount of protein associated with the substrate in resonance units, which physically represent a change in the cycle of minimum intensity light. One resonance unit corresponds to a density of protein of ~1 μg/mm². Briefly, protein was immobilized to the surface of a BIACore CM5 sensor chip (BIACore AB, Uppsala, Sweden) using standard amine chemistry (22). In Fig. 9A, each ECM protein was diluted to a concentration of 20 μg/ml in PBS and passed over the surface of the chip containing immobilized Ang-1 at a rate of 3 μl/min for 5 min. After 2 min of PBS washing, binding activity was calculated as the change in resonance units from before protein injection until after washing. Note that BSA was diluted in PBS to a concentration of 50 μg/ml before injection. In Fig. 9B, Ang-1 (20 μg/ml), Ang-2 (20 μg/ml), or BSA (50 μg/ml) was passed over the surface of a CM5 chip containing immobilized vitronectin, and binding activity was measured as above.

Alternatively, 96-well ELISA plates were coated for 1 h at room temperature with vitronectin either at 5 μg/ml (for Ang-1 dilutions), or 2-fold serial dilutions beginning at 5 μg/ml (for vitronectin dilutions). The entire plate was then blocked for 1 h at room temperature with 1% BSA in PBS. Ang-1 solutions were prepared in blocking buffer either at 5 μg/ml (for vitronectin dilutions) or 2-fold serial dilutions beginning at 5 μg/ml (for Ang-1 dilutions), added to the appropriate well, and incubated for 1 h at room temperature. After three PBS washes, anti-Ang-1 polyclonal antibody was added (1:100 in 1% BSA in PBS), and the plate was incubated for 1 h at room temperature. After three PBS washes, alkaline phosphatase-conjugated anti-rabbit IgG antibody was added (1:500 in 1% BSA in PBS), and the plate was incubated for another hour at room temperature. After extensive PBS washing, p-nitrophenyl phosphate was added (1 mg/ml in 0.1 M glycine, 1 mM MgCl₂, 1 mM ZnCl₂, pH 10.4), and A₄₀₅ values were recorded after 45 min.

RESULTS

Cell Adhesion to Ang-1 and Ang-2—To address the question of cell adhesion, HUVECs were plated onto wells of a tissue culture dish that had been precoated with either Ang-1 or Ang-2 or various other known ECM proteins. As shown in Fig. 1, HUVECs adhered well to both Ang-1 and Ang-2 in concentration-dependent manner within 30 min. On a molar basis, HUVECs adhered better to Ang-1 and Ang-2 than to the basement membrane proteins collagen IV, laminin, and nidogen but not as well as they did to collagen I, fibronectin, or vitronectin.

The role of Tie2 in cell adhesion to Ang-1 and Ang-2 was
Together, these findings suggest that Ang-1 and Ang-2 differ-
similar to that seen at the 2 h time point (data not shown).

time, as the relative extent of spreading at 1 or 4 h was
on morphology and actin dynamics was independent of adhe-

to Ang-2 display a discernible stellate morphology. The effect
on cortical actin. Similar to that seen on Ang-1, HUVECs attached

were precoated with Ang-1, Ang-2, collagen IV, fibronectin, or
vitronectin. As seen in Fig. 2, plating HUVECs onto Ang-1
for coated Ang-1 or Ang-2 to induce such morphological
changes, HUVECs were plated onto glass coverslips that had

examined next. This was done with NIH 3T3 cells, which do not
express Tie2, and MG Tie2 cells, an NIH 3T3 variant that has
been stably transfected with the Tie2 gene (7). Interestingly,
both cell lines adhered to Ang-1 and Ang-2, with the amount of
adhesion being comparable to that observed in cells plated onto
collagen I, collagen IV, or vitronectin (Fig. 1B). These results
demonstrated that both endothelial cells and fibroblasts can
attach directly to Ang-1 and Ang-2 and that Tie2 is not re-
quired for this adhesion.

Coated Ang-1, but Not Ang-2, Induces Cell Spreading—Cell
adhesion to ECM proteins has profound effects on overall cell
shape and cytoskeletal dynamics (23). To examine the potential
for coated Ang-1 or Ang-2 to induce such morphological changes,
HUVECs were plated onto glass coverslips that had been precoated with Ang-1, Ang-2, collagen IV, fibronectin, or
vitronectin. As seen in Fig. 2, plating HUVECs onto Ang-1
induced marked cell spreading and promoted the formation of
prominent actin stress fibers. The extent of spreading on Ang-1
was similar to that seen on collagen IV, fibronectin, or vitronec-
tin; however, cells adhering to Ang-1 displayed a much more
stellate morphology compared with the other known ECM pro-
teins. By contrast, HUVECs adhered to Ang-2 did not spread
well, and the cytoskeleton in these cells consisted primarily of
cortical actin. Similar to that seen on Ang-1, HUVECs attached
to Ang-2 display a discernible stellate morphology. The effect
on morphology and actin dynamics was independent of adhe-
sion time, as the relative extent of spreading at 1 or 4 h was
similar to that seen at the 2 h time point (data not shown).

Together, these findings suggest that Ang-1 and Ang-2 differ-
entially regulate actin dynamics and cell spreading.

Ang-1, but Not Ang-2, Supports Cell Migration—The ability
of cells to migrate on Ang-1 and Ang-2 was tested next. Ang-1
supported strong HUVEC migration in the absence of any
other ECM components or serum, and this migration was com-
parable to that seen on wells coated with fibronectin plus
gelatin (Fig. 3A). Addition of bFGF, a known endothelial che-
moattractant, increased the migratory response on Ang-1 or
fibronectin plus gelatin. In the absence of growth factor,
HUVECs did not migrate on Ang-2. HUVECs did respond to
bFGF with some migration on Ang-2; however, the extent of
this migration was markedly weaker than that seen with Ang-1
or fibronectin plus gelatin as the substrate.

Tie2 was not required for cell migration on Ang-1, as shown
by the comparable migration of both NIH 3T3 and MG Tie2
cells on Ang-1 (Fig. 3B). As expected, both cell lines responded
positively to the chemoattractant platelet-derived growth factor-
AB. Consistent with the data obtained with HUVECs, mi-
gation on Ang-1 was similar to that seen on fibronectin plus
gelatin, and neither fibroblast cell line migrated well on Ang-2
in the absence of a chemoattractant. Thus, although cells can
adhere to both Ang-1 and Ang-2, only Ang-1 supports robust
cell migration independent of growth factor activity.
Adhesion to Ang-1 and Ang-2 Stimulates FAK and MAPK Activation—Cell adhesion to ECM proteins stimulates the activation of numerous intracellular signal transduction pathways (23). Therefore, the effect of HUVEC adhesion to Ang-1 and Ang-2 on the activation of the FAK and MAPK pathways was tested. Adhesion to Ang-1, Ang-2, or various ECM proteins had differential effects on activation of FAK and MAPK. The extent of FAK phosphorylation was highest in HUVECs plated onto fibronectin, vitronectin, and Ang-1, whereas MAPK activation was most pronounced when the cells were adhered to Ang-1, Ang-2, or fibronectin (Fig. 4). As expected, HUVECs held in suspension or plated onto poly-L-lysine did not activate either FAK or MAPK. Thus, cell adhesion to both Ang-1 and Ang-2 stimulates FAK and MAPK activation to levels comparable to those seen in cells adhering to other ECM proteins.

Integrin-blocking Agents Inhibit Adhesion and Signaling on Ang-1 and Ang-2—As shown in Figs. 1B and 3B, adhesion to Ang-1 and Ang-2 and migration on Ang-1 did not require the presence of the only known receptor for the angiopoietins, Tie2. To test whether or not integrins may serve as additional receptors for Ang-1 and Ang-2, cell adhesion experiments were performed in the presence of the calcium chelator EDTA, a universal inhibitor of integrins, or RGD peptides, which can inhibit integrins that bind this sequence. As shown in Fig. 5A, EDTA completely inhibited HUVEC adhesion to Ang-1, vitronectin, and type I collagen. On the other hand, EDTA only partially blocked HUVEC adhesion to Ang-2. RGD peptides, although completely blocking adhesion to vitronectin, were about 50% effective in blocking adhesion to Ang-1 and only 25% effective in blocking adhesion to Ang-2. As expected, RGD peptides had no inhibitory effect on HUVEC adhesion to type I collagen, and the negative control RAD peptide had no effect on cell adhesion to any of the test substrates. The degree of MAPK activation in the presence of these inhibitory agents was also reduced (Fig. 5B). The reduction in signaling on vitronectin closely paralleled the extent of cell adhesion; however, the reduction in MAPK activation on Ang-1 and Ang-2 was much greater than the amount that might be predicted from the corresponding adhesion data. In the presence of EDTA, MAPK activation was virtually abolished when HUVECs were plated on either Ang-1 or Ang-2, even though EDTA only blocked about 50% of cell adhesion to Ang-2. RGD peptides, although less effective than EDTA in blocking cell adhesion to Ang-1 or
Ang-2, also almost completely blocked MAPK activation. Taken together, these data suggest that integrins are involved in HUVEC adhesion to Ang-1 and Ang-2 and that MAPK activation on both substrates may lie downstream of integrin-mediated cell adhesion.

**β1 Integrins Participate in Cell Adhesion to Ang-1**—The role of integrins in cell adhesion to Ang-1 and Ang-2 was also studied in MG Tie2 and NIH 3T3 fibroblasts (Fig. 6, A and B). EDTA completely inhibited NIH 3T3 cell adhesion to Ang-1 and Ang-2, but interestingly, it only blocked about 50% of MG Tie2 cell adhesion to either angiopoietin. This finding suggests a possible role for Tie2 in mediating adhesion to the angiopoietins and is similar to that seen when HUVECs adhere to Ang-2 in the presence of EDTA (Fig. 5A). Unlike the findings with HUVECs, RGD peptides had no significant inhibitory effect on cell attachment to Ang-1 or Ang-2. However, when a function-blocking anti-β1 integrin antibody was included in adhesion assays, NIH 3T3 cell adhesion to Ang-1 and Ang-2 was reduced significantly. This antibody has less of an inhibitory effect on cell adhesion with MG Tie2 cells. As expected, the β1-blocking antibody completely inhibited MG Tie2 and NIH 3T3 cell adhesion to collagen IV, an ECM ligand for the integrins α5β1 and α6β1 (24).

Next, the localization of β1 integrins in MG Tie2 and NIH 3T3 fibroblasts adhering to Ang-1 and Ang-2 was studied. Both fibroblasts spread well on Ang-1 and produced actin stress fibers emanating from β1 integrin-enriched focal contacts (Fig. 6C). A similar pattern of actin and β1 staining was seen when these cells were spread on fibronectin, an ECM substrate for β1 integrins. On the other hand, neither MG Tie2 nor NIH 3T3 fibroblasts spread well on Ang-2; their actin cytoskeleton consists primarily of cortical actin, and focal β1 staining was not observed. Overall, the degree of spreading and stress fiber formation was slightly less on Ang-1 compared with fibronectin; however, both spreading and stress fiber formation were much greater on Ang-1 than on Ang-2. Thus, adhesion to Ang-1, but not Ang-2, induces β1 integrin localization to focal contacts, and this does not require Tie2.

**Integrins Serve as Receptors for Ang-1 and Ang-2 in Human Fibroblasts**—The data in Fig. 6 suggest that β1 integrins are involved in fibroblast adhesion to Ang-1 and Ang-2. Opportunities to perform more extensive studies employing additional integrin-blocking antibodies with NIH 3T3 and MG Tie2 fibroblasts are limited because of the lack of available antibodies suitable for use with mouse cells. To circumvent this problem, cell adhesion experiments were performed with two different human fibroblasts. Both IMR-90, a human lung fibroblast (Fig. 7), and CCD 45SK, a human skin fibroblast (not shown), were found to adhere well to Ang-1 and Ang-2. As seen in Fig. 7, inclusion of EDTA, RGD, or function-blocking monoclonal antbody directed against β1, α5, or α6β1 integrins nearly completely abolished IMR-90 adhesion to Ang-1. RAD, anti-α1β6 or anti-α6, had no significant inhibitory effect on adhesion to Ang-1. Similar results were seen on Ang-2, although RGD peptides were less effective, and anti-α6 integrin antibodies were more effective at preventing cell adhesion compared with Ang-1. Nearly identical results were obtained using this panel of integrin-blocking agents with CCD 45SK fibroblasts in cell adhesion experiments on Ang-1 and Ang-2 (data not shown). Thus, β1 and α5β1 integrins are likely to mediate fibroblast adhesion to Ang-1 and Ang-2.

**Integrins Mediate CHO-B2 Cell Adhesion to Ang-1 and Ang-2**—Adhesion experiments were also performed with CHO-B2 cells that lack the α5 integrin subunit (17), CHO-B2/α6 cells, which stably express the human α6 subunit (18), and CHO-B2/α27 cells, which stably express the human α5 subunit (19). As seen in Fig. 8, the parental B2 cells did not adhere to fibronectin or Ang-1, but they did attach to vitronectin and Ang-2. Stable expression of the human α5 integrin subunit, which heterodimerizes with endogenous β1 (18), rescued cell adhesion to fibronectin but not to Ang-1. On the other hand, expression of the human α5 subunit rescued cell adhesion to Ang-1. Inclusion of P1D6, a function-blocking anti-α5 integrin monoclonal antibody, in adhesion assays with B2α27 cells completely blocked adhesion to Ang-1, suggesting that the human α5 subunit is responsible for mediating this adhesion and not some other cell surface protein that may be differentially expressed in these cells compared with parental B2 cells. Considering that B2 cells adhere to Ang-2 and that this adhesion is inhibited by EDTA (data not shown), that B2α27 cells adhere better than the parental cells, and that P1D6 only partially inhibits B2/α27 adhesion to Ang-2, it is likely that both α5β1 and some other integrin are involved in CHO-B2 cell adhesion to Ang-2.

**Ang-1 and Ang-2 Bind to Vitronectin in Vitro**—Many ECM proteins have specific binding sites for other ECM proteins. To test whether Ang-1 can bind any ECM proteins, two different *in vitro* binding assays were performed. The first assay utilized surface plasmon resonance and started with the immobilization of Ang-1 to the surface of a Biacore CM5 sensor chip using standard amine chemistry (22). Subsequently, various purified ECM proteins were passed over the surface of this chip to identify binding activities. As shown in Fig. 9, Ang-1 specifically bound to vitronectin but had little to no affinity for any of the other ECM proteins tested. When the experiment was performed in the reverse order, Ang-2, as well as Ang-1, bound to vitronectin immobilized on the surface of a sensor chip (Fig. 9B). Note that it was not possible to test the potential for collagen IV binding to Ang-1 or Ang-2 in this system because this protein is retained within the carboxymethylcellulose gel used to covalently immobilize the test protein at the chip surface and gives a high background measurement (data not shown).

The second set of experiments employed an enzyme-linked immunosorbent assay, where Ang-1 was allowed to bind to vitronectin-coated plastic. The results, shown in Fig. 9C, confirm that Ang-1 binds to vitronectin. This association was concentration-dependent and could be observed either when a fixed concentration of Ang-1 was allowed to bind to 2-fold serial dilutions of vitronectin or when 2-fold serial dilutions of Ang-1 were added to vitronectin that had been coated at a constant concentration. Control experiments demonstrated that neither the anti-Ang-1 antibody nor the secondary antibody bound to vitronectin. Also, secondary antibody alone did not react with Ang-1 (data not shown). Taken together, these findings suggest that both Ang-1 and Ang-2 can bind vitronectin *in vitro*.

**DISCUSSION**

Our results show that cells adhere directly to Ang-1 and Ang-2 and that this adhesion is mediated, at least in part, by integrins. We also find that Ang-1, but not Ang-2, can support robust cell migration independent of Tie2 function. Adhesion to Ang-1 or Ang-2 results in differential effects on FAK and MAPK activation. Adhesion to Ang-1, but not Ang-2, can support cell migration independent of Tie2 function. Adhesion to Ang-1, but not Ang-2, can support cell migration independent of Tie2 function. Adhesion to Ang-1, but not Ang-2, can support cell migration independent of Tie2 function. Adhesion to Ang-1, but not Ang-2, can support cell migration independent of Tie2 function.
A and D, and F similarly are shown. In Representative results from three independent experiments performed at 37 °C, cell adhesion was measured as described in the legend to Fig. 6. For the signal seen in C the presence of EDTA (10 mM), RAD or RGD (500 µg/ml), 6S6 (anti-β1, 50 µg/ml), LM609 (anti-α, 50 µg/ml), AV1 (anti-α, 1:4 dilution of tissue culture supernatant), P1F6 (anti-α, 50 µg/ml), or P1D6 (anti-α, 1:25 dilution of mouse ascites). Cells were preincubated with antibody for 15 min at 37 °C and allowed to adhere for 30 min at 37 °C. Cell adhesion was quantified as described in the legend to Fig. 1. Data are the mean ± S.D. for each condition performed in duplicate. Similar results were obtained in an identical experiment with CCD 45SK fibroblasts.

was shown to bind latent transforming growth factor β1 in vitro (26). Additionally, β1-transfected cells bound to dishes coated with large latent transforming growth factor β1 complexes, and this adhesion induced the phosphorylation of both FAK and paxillin. Thus, although our findings with Ang-1 and Ang-2 are novel, they do not represent the first report of a cytokine supporting cell adhesion in an integrin-dependent fashion.

The concentrations of soluble Ang-1 necessary to modulate downstream activities such as increased cell survival (27) or enhanced cell migration (21) were 0.2 µg/ml or 0.1–1 µg/ml, respectively. These concentrations are considerably lower than those required to support good cell adhesion in this report (6–10 µg/ml). There are at least two important points to consider when comparing these concentration differences. First, protein adsorption to a hydrophobic surface results in irreversible denaturation of native protein conformation. The extent of denaturation varies from protein to protein and from surface to surface (28). However, changes in protein structure upon adsorption can have a significant impact on protein function. For example, specific activity of the proteolytic enzyme α-chymotrypsin disappeared almost completely upon adsorption to polystyrene (29). The extent of adsorptive denaturation and subsequent loss of activity can be decreased by using more concentrated coating solutions as demonstrated for α-chymotrypsin and the lipolytic enzyme, cutinase (29). It is possible that in our studies, the relatively high coating concentrations used are necessary to prevent denaturation at the substrate surface. The second point to consider regards the affinities of growth factor receptors versus integrins for their respective ligands. The dissociation constant of Ang-1 or Ang-2 binding to Tie2 was calculated to be ~3 × 10⁻⁹ M (7). By comparison, integrins typically have lower affinities for their ligands, with dissociation constants typically ranging from 10⁻⁹ to 10⁻¹⁰ M (30, 31).

Considering the potential for adsorptive protein denaturation and that integrins have lower affinities for ligand than do growth factor receptors, it is not surprising that relatively high concentrations of the angiopoietins are required to support good cell adhesion.

Upon initial contact with the ECM, cells generally extend finger-like filopodia and actin-rich lamellipodia. The spreading process then culminates with the formation of focal contacts.
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Fig. 8. Adhesion of CHO-B2, -B2/v7, and -B2/α27 to Ang-1 and Ang-2. Panel A, adhesion of CHO-B2 (no α5 integrin), -B2/v7 (stable α5 transfectant), and -B2/α27 (stable α5 transfectant) to fibronectin (FN), vitronectin (VN), Ang-1, and Ang-2 was measured. All proteins were coated at 10 μg/ml. Panel B, adhesion of CHO-B2/α27 cells to the indicated proteins, coated as in panel A, in the presence of P1D6 (anti-α5 integrin, 1:25 dilution of mouse ascites) was measured. Cells were preincubated with antibody for 15 min at 37 °C before plating. In both panel A and panel B cells were allowed to adhere for 30 min at 37 °C, and the extent of adhesion was quantified as described in the legend to Fig. 1.

These focal contacts contain proteins such as talin and vinculin that serve as linkers between the extracellular environment and the cytoskeleton through their direct interactions with integrins and actin, respectively, and signal transduction proteins such as FAK and members of the Rho family of GTPases. Together, this complex of proteins transduces signals from the ECM and mediates changes in cell morphology, migration, proliferation, and differentiation (23). As seen in Figs. 2 and 6C, cell adhesion to Ang-1 induces morphological changes similar to those seen when cells are attached to known ECM proteins. On the other hand, cells attached to Ang-2 do not spread well, nor do they form actin-rich focal contacts. The biochemical nature of this effect on spreading is currently unknown, but based on findings in other cell systems it is possible that the difference is the result of differential regulation of signaling via the GTPases Rho, Rac, and Cdc42. For example, expression of dominant negative mutants of either Rac or Cdc42 was found to inhibit NIH 3T3 cell spreading on fibronectin (32). In another report, expression of dominant negative Rac or Cdc42 significantly inhibited Rat1 cell spreading on fibronectin but had no effect on overall cell adhesion (33). Thus, it is possible that cell adhesion to Ang-1 and Ang-2 has distinct effects on the activity of these GTPases.

An equally plausible, non-mutually exclusive explanation for the differences observed in cell spreading, focal contact formation, and migration on Ang-1 versus Ang-2 may be the preferential use of different integrins for adhesion to the two proteins. Although the data in Figs. 7 and 8 suggest that both β1 and αβ integrins mediate cell adhesion to Ang-1 and Ang-2, we hypothesize that cells may rely more heavily on β1 integrins to bind Ang-1, but utilize primarily αβ2 to bind Ang-2. Several lines of evidence support this hypothesis. First, CHO-B2 cells adhere well to Ang-2 but not to Ang-1. Stable expression of the α2 integrin subunit rescues cell adhesion to Ang-1, and complete inhibition of CHO-B2/α27 cell adhesion to Ang-1 is observed in the presence of anti-α5 integrin blocking antibodies. Considering that B2 cells attach to Ang-2 and that only partial inhibition of CHO-B2/α27 cell adhesion to Ang-2 is observed with anti-α5, it is likely that some other integrin is primarily responsible for CHO-B2 adhesion to Ang-2. Because CHO-B2 cells express no cell surface β1 integrins and produce no detectable β1 mRNA, but do make β3 and adhere to vitronectin (18), it is possible that these cells rely heavily on αβ2 to adhere to vitronectin and Ang-2. Indeed, inhibition of the function-blocking anti-αβ2 integrin antibody P1D6 completely inhibited CHO-B2 adhesion to vitronectin and partially blocked adhesion to Ang-2 (data not shown). Initial studies of αβ2-mediated cell adhesion to vitronectin established that αβ2 does not localize to focal contacts or associate with the actin cytoskeleton (34). Subsequent studies found that activation of protein kinase C indirectly via epidermal growth factor stimulation or directly with phosphol esters promoted αβ2-mediated cell spreading, focal contact formation, and migration on vitronectin (35, 36). These findings are similar to our data with cells migrating or spreading on Ang-2. As seen in Fig. 3, cells do not migrate on Ang-2 in the absence of growth factor, but they do migrate to some extent in the presence of growth factor. In addition, cells do not spread on Ang-2 (Figs. 2 and 6C), but our preliminary results show that the addition of lysophosphatidic acid, an activator of the Rho GTPase which has been shown to induce focal adhesion and actin stress fiber formation (37, 38), induces substantial spreading on Ang-2 (data not shown). Taken together, these results suggest that the integrins responsible for adhesion to Ang-2 require activation of distinct intracellular signaling pathways to mediate migration and spreading. On the other hand, the primary Ang-1-binding integrins do not require activation of other intracellular signaling pathways to induce migration, spreading, or focal contact formation. Our data, together with published data indicating that exogenous growth factor is not required for β1-mediated spreading or migration on collagen (35), are consistent with the hypothesis that β1 integrins serve as the primary Ang-1 adhesion receptors.

Endothelial cell migration is an important aspect of the angiogenic process, and therefore much research is focused on the intracellular mechanisms that control vascular cell motility. Substantial amounts of data implicate both ECM-integrin and growth factor-receptor interactions as critical mediators of this process (4). Recently, two groups have shown that soluble Ang-1 potentiates cell migration on fibronectin (21, 39). In another report, it was demonstrated that Ang-1 induces capillary sprouting of endothelial cells cultured in a three-dimensional collagen gel (40). In each of these systems, the migratory phenotype induced by Ang-1 was blocked by excess soluble Tie2, suggesting that Ang-1 was acting through this receptor to modulate the function of the cellular migration machinery. In this report, we show that Ang-1 can support directed cell migration in vitro independent of Tie2 function (Fig. 3). Our experiments were performed in the absence of any additional ECM components, added serum, or growth factors, suggesting that Ang-1 alone supports cell migration. Considering that both NIH 3T3 and MG Tie2 fibroblasts migrate on Ang-1, it is
likely that this process is also mediated primarily by member(s) of the integrin superfamily.

Integrins are known to activate intracellular signaling pathways, including the FAK and ras/MAPK pathways (23). As seen in Fig. 4, HUVEC adhesion to Ang-1 and Ang-2 induces phosphorylation of both FAK and MAPK. EDTA and RGD peptides, agents that have distinct inhibitory effects on HUVEC adhesion to Ang-1 and Ang-2, also block activation of MAPK (Fig. 5B). These findings suggest that signaling to FAK and MAPK is mediated, at least in part, by integrins. We cannot, however, rule out the possibility of cross-talk between integrins and Tie2 in signaling to these molecules. In fact, stimulation of endothelial cells with Ang-1 has been shown to induce activation of both MAPK and FAK (39, 41). The precise role of Tie2 in these studies is not clear because neither group used excess soluble receptor to block Ang-1-mediated activation of MAPK or FAK. Therefore, the possibility exists that Ang-1 action is also mediated via direct interaction with additional cell surface receptors in the endothelial cell. Elucidation of the signaling induced by cell adhesion to Ang-1 and Ang-2 will require careful analysis of the pathways in multiple cell types that adhere well to these substrates.

A common property of ECM proteins is that many contain distinct binding sites for not only cell surface integrins, but other ECM proteins as well. For example, it is well established that nidogen can bind both laminin and collagen IV in vitro, and it is thought that this activity is necessary for the stabilization of complex basement membranes structures in vitro (42). Our data indicate that both Ang-1 and Ang-2 can bind to vitronectin in vitro. The functional significance of the Ang-1 and Ang-2 interaction with vitronectin is currently unknown, although the recent suggestion that both Ang-1 and vitronectin can be found within platelet α-granules indicates a potential role in hemostasis (43, 44).

The finding that Ang-1 serves as an adhesive protein helps to explain several key findings in the angiogenesis field. First, Ang-1 knockout mice are embryonic lethal and possess defects in the angiogenic process (3). Endothelial cells in these embryos are rounded and appear detached from their basement membranes. Our demonstration that Ang-1 can directly support cell adhesion and spreading may provide a mechanistic explanation for the endothelial cell morphology observed in these embryos. Second, overexpression of Ang-2 in endothelial cells of transgenic mice resulted in an embryonic lethal phenotype that was more severe than that seen in Ang-1 or Tie2 knockout embryos (7). It is possible that some of the phenotype in these animals resulted from the vast abundance of Ang-2 directly altering endothelial cell adhesivity and spreading, independent of its effects on Tie2 signaling. Third, two different mouse models that overexpress Ang-1 produce blood vessels that are leakage-resistant (11, 12). However, no explanation for this reduction in leakiness exists. Blood-borne molecules can leak through the vessel wall via several pathways (45). One of these pathways is directly through endothelial clefts at sites of weak cell-cell or cell-ECM contacts (46). Based on our findings, it is possible that Ang-1 is directly ligating endothelial cell surface adhesion molecules in the Ang-1 overexpressor animals, thereby enhancing the integrity of the vessel wall and preventing leakage. Finally, the results presented here are consistent with reports that Ang-1 enhances hematopoietic cell adhesion (13, 14) and cell migration (21, 39); but because we have found that Tie2 is not required for either adhesion or migration, the possibility remains that Ang-1 action is also mediated via direct interaction with additional cell surface receptors in the published experiments, as well. It will be important to determine the relative contributions of signaling through Tie2 versus signaling through adhesion receptors in these types of
experiments. Of course, this would first require identification of the sequence(s) responsible for both cell adhesion and Tie2 binding. Notwithstanding, the findings presented herein open the door for investigation into such possibilities and offer new insights into the role of Ang-1 and Ang-2 in vascular biology.

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Direct Cell Adhesion to the Angiopoietins Mediated by Integrins
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