Integrin α9β1 Directly Binds to Vascular Endothelial Growth Factor (VEGF)-A and Contributes to VEGF-A-induced Angiogenesis*

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Vascular endothelial growth factor A (VEGF-A) is a potent inducer of angiogenesis. We now show that VEGF-A-induced adhesion and migration of human endothelial cells are dependent on the integrin α9β1 and that VEGF-A is a direct ligand for this integrin. Adhesion and migration of these cells on the 165 and 121 isoforms of VEGF-A depend on cooperative input from α9β1 and the cognate receptor for VEGF-A, VEGF receptor 2 (VEGF-R2). Unlike α3β1 or αvβ3 integrins, α9β1 was also found to bind the 121 isoform of VEGF-A. This interaction appears to be biologically significant, because α9β1-blocking antibody dramatically and specifically inhibited angiogenesis induced by VEGF-A165 or -121. Together with our previous findings that α9β1 directly binds to VEGF-C and -D and contributes to lymphangiogenesis, these results identify the integrin α9β1 as a potential pharmacotherapeutic target for inhibition of pathogenic angiogenesis and lymphangiogenesis.

Angiogenesis requires the coordinated function of both cellular and extracellular regulatory and effector proteins to ensure optimal new vessel formation and function in both non-pathogenic and pathogenic settings (1). The identified molecular regulators of angiogenesis include the VEGF3 family of proteins and receptors, fibroblast growth factors, and integrins (2–4). VEGF-A is a potent pro-angiogenic stimulus and acts by potentiation of endothelial cell adhesion, migration, and proliferation (5, 6). Although VEGF-induced angiogenesis is principally mediated by interactions with its cognate receptor, VEGF-R2, its angiogenic effect may also be modulated through non-VEGF-R2 pathways, including neuropillin-2 (7, 8), heparin sulfate proteoglycans (8), and integrins (9–13).

Integrins are heterodimeric transmembrane proteins that can mediate cell adhesion, migration, and proliferation. Following activation by their respective ligands, integrins can also modulate these cell functions through coordinated cross-talk with growth factor receptors, including VEGF receptors (14, 15) often utilizing signaling proteins common to both receptor pathways (13, 16, 17). Indirect evidence suggests that inhibition of another β1 integrin, α3β1, can inhibit cell adhesion to VEGF-A, suggesting that VEGF-A could serve as an α3 ligand (18). We have previously shown that the integrin α9β1 directly binds to the growth factors, VEGF-C and -D (19), a finding that may help explain the abnormal lymphatic phenotype of mice expressing a null mutation of the α9 subunit. Because the VEGF homology domain of VEGF-C and -D shares 40% homology with VEGF-A, we hypothesized that VEGF-A might also be a ligand for α9β1 and could potentially modulate VEGF-A-induced angiogenesis.

VEGF-A can be synthesized in a variety of forms based on alternative splicing. A previous study has shown that the integrins α3β1 and αvβ3 specifically modulate cellular interactions with the 165-kDa form of VEGF-A but not the 121-kDa form (18). These results suggest that any association between these integrins and VEGF-A likely does not involve interaction with sequences encoded by exon 6. To determine whether interactions between α9β1 and VEGF-A involved similar sites in VEGF-A, we incorporated both splice variants into our studies.

Here we report that α9β1 does bind directly to VEGF-A and does cooperate with VEGF-R2 to modulate in vitro endothelial cell adhesion and migration on both VEGF-A165 and VEGF-A121. We also show that VEGF-A (but not bFGF)-induced angiogenesis in chick CAMs can be inhibited by antibody to α9β1, suggesting that this interaction could have in vivo relevance.

**EXPERIMENTAL PROCEDURES**

*Materials—Human VEGF-A165, VEGF-A121, bFGF, and phycocerythrin-conjugated mouse monoclonal antibody to VEGF-R2 were purchased from R & D Systems (Minneapolis, MN). Production of Y9A2 (blocking antibody to α9β1), A9A1 (nonblocking antibody to α9β1) (19), CSβ6 (20), and purification of the integrins α9β1 (19) and αvβ6 (20) have

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; VEGF-R, vascular endothelial growth factor receptor; MEF, mouse embryonic fibroblasts; HMVEC, human microvascular endothelial cells; BSA, bovine serum albumin; bFGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; Ab, antibody; BSA, bovine serum albumin; ERK, extracellular signal-regulated kinase.
been described previously. Antibodies were purchased from the following companies as noted. Rabbit polyclonal antibody to VEGF-R2 (M-20) and goat polyclonal antibody to VEGF-R2pY951 (sc-16628) were from Santa Cruz Biotechnology, Santa Cruz, CA; VEGF-R2 kinase inhibitor (SU1498) was from Calbiochem; rabbit polyclonal anti-paxillin pY31, and anti-phospho-Y185/187, and VEGF-R2pY1054/59 were from BIOSOURCE; mouse monoclonal antibody to α3 and αvβ3 (LM609) integrins was from Chemicon; and anti-phosphotyrosine (clone 4G10) and mouse monoclonal anti-β-actin antibodies were from Sigma. Peroxidase-conjugated goat anti-rabbit, goat anti-mouse IgG, and donkey anti-goat IgG were from Jackson ImmunoResearch (West Grove, PA).

Cells and Cell Culture—α9 and mock-transfected mouse embryonic fibroblasts (MEF) and SW-480 cells were made and grown as described previously (21, 22). Primary adult human microvascular endothelial cells (HMVEC, Cambrex, East Rutherford, NJ) were grown in cell-specific growth factor-supplemented nutrient media (Cambrex, EBM-2).

Immunoprecipitation, SDS-PAGE, and Western Blot Analysis—For immunoprecipitation of VEGF-R2, HMVEC were grown in 6-well plates with full growth media until 70% confluent and subsequently in basal media with 0.1% BSA for 4 h. Cells were then added to 12-well dishes coated with VEGF-A (23, 24). Cells were exposed to VEGF-A or medium alone for 5–30 min, washed with phosphate-buffered saline/sodium orthovanadate (NaV 10 mM), and then lysed with buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X, 0.5% sodium deoxycholate, 10% glycerol, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaV, and protease inhibitors (Complete Mini EDTA-free, Roche Applied Science). Pre-cleared lysates were immunoprecipitated with 2 μg of antibody bound to protein A-Sepharose beads (Amersham Biosciences). The beads were washed with lysis buffer, resuspended in Laemmli sample buffer, boiled, resolved on 8% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA). The membrane was blocked in 5% milk/Tris-buffered saline with 0.1% Tween (TBST) for 1 h at room temperature and then probed with anti-VEGF-R2 antibody.

For immunoblotting of paxillin pY31, ERKpY185/187, 4G10, and β-actin, proteins were suspended in Laemmli sample buffer and resolved on 4–15% gradient SDS-PAGE, before transfer to polyvinylidene difluoride membrane. The membrane was blocked in 5% BSA, 0.1% Tween, probed with the appropriate specific primary antibodies, washed three times with TBST, and subsequently probed with horseradish peroxidase-conjugated secondary antibodies and developed using chemiluminescence (ECL, Amersham Biosciences).

**Flow Cytometry**—Cultured cells were trypsinized, washed with phosphate-buffered saline, and incubated with the appropriate primary antibodies and then appropriate phycoerythrin-conjugated secondary antibody. Phycoerythrin-conjugated VEGF-R2 was used to detect VEGF-R2. Fluorescence of labeled cells was determined with a flow cytometer (BD Biosciences).

**Adhesion Assay**—Assays were performed as described previously (19) with some minor modifications. After coating 96-well microtiter plates (ICN, Linbro/Titertek, Aurora, OH) with VEGF-A at 4 °C overnight, wells were blocked with 3% bovine serum albumin (BSA, Sigma) for 30 min at 37 °C. After

**FIGURE 1. α9β1 integrin mediates cell adhesion on VEGF-A165.** A, flow cytometry analysis of mock- (left) and α9-transfected (right) MEF used in cell adhesion assays. B, VEGF-A was used as substrate for cell adhesion assays with mock- (diagonal bars) or α9-transfected (black bars) MEF in the absence or presence of α9β1-blocking Ab. Cells were allowed to adhere to wells coated with a range of VEGF-A concentrations and then fixed and stained with crystal violet. Adhesion is expressed as absorbance at 595 nm. C, flow cytometry analysis of mock- (left) and α9-transfected (right) SW-480 cells used in cell adhesion assays. D, VEGF-A was used as substrate for cell adhesion assays with SW-480 mock cells in the absence of inhibitors (black bars) or the presence of α3 (white bars), α9 (diagonal bars), or both (brick bars) blocking Ab or VEGF-R2 inhibitor (diamond bars). E, similar adhesion assay to D but with α9-transfected SW-480 cells.
**a9β1 Integrin Is a Receptor for VEGF-A**

Trypsinization, cells were incubated with or without relevant antibodies (20 μg/ml) for 30 min on ice, and 5 × 10^4 cells/well were seeded. Adherent cells were fixed and stained with 1% formaldehyde, 0.5% crystal violet, 20% methanol for 30 min, and the number of adherent cells was evaluated by measuring absorbance at 595 nm in a microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA).

**Migration Assay**—Assays were performed as described previously (19) using 8-μm Transwell plates (Corning Costar, Cambridge, MA), either uncoated or coated, at 4 °C overnight with relevant ligand (VEGF-A) or 1% BSA as a binding control. After trypsinization, 5 × 10^4 cells were incubated with or without relevant inhibitors for 30 min on ice and seeded into the top chamber of the Transwell (22). 1% fetal calf serum or soluble VEGF-A was added to the bottom well, to serve as a chemottractant, and the plates were incubated at 37 °C for 3 h. Cells that migrated and adhered to the bottom surface of the Transwell membrane were fixed, stained (DiffQuik, Pierce), and counted in 10 high power (×25) fields for each condition.

**Binding Assay**—Purified α9β1 (19) and αvβ6 (20) integrins were used in solid phase binding assays with VEGF-A, as described previously (19). Recombinant VEGF-A (5 μg/ml) was coated on 96-well microtiter plates (Nunc ImmunoPlate, Naperville, IL), and purified α9β1 or αvβ6 at various concentrations was added for 2 h at room temperature in the presence or absence of α9 blocking antibody or 10 mM EDTA. The extent of α9β1 binding was detected using A9A1 antibody (20 μg/ml, 1 h at 37 °C). Following labeling with horseradish peroxidase (BD Biosciences), binding was quantified by measuring absorbance at 450 nm with a microplate reader (Molecular Devices).

**CAM Assay**—Chick eggs were maintained in a humidified 39 °C incubator (Lyon Electric, Chula Vista, CA). Pellets containing 0.5% methylcellulose plus recombinant human VEGF-A (50 ng) or bFGF (150 ng) were placed onto the CAM of 10-day-old chick pathogen-free embryos (SPAFAS; Charles River Breeding Laboratories, Wilmington, MA). The CAMs were exposed by cutting a small window in the egg shell to facilitate application of the pellet. Relevant antibodies or agonist/antagonist compounds were applied to the site 24 h after stimulation with VEGF protein. CAMs were imaged on day 13, both following fixation and excision or with real-time live imaging, using a digital camera (Canon Supershot6) attached to a Zeiss stereomicroscope. Angiogenesis was quantified by counting branch points arising from tertiary vessels from a minimum of 10 specimens from three separate experiments.

**Statistical Methods**—Data are presented as mean values ± S.D. from at least three separate experiments unless otherwise stated.

**RESULTS**

**Integrin α9β1 Mediates Cell Adhesion and Migration on VEGF-A**—To determine whether cells use integrin α9β1 to adhere to VEGF-A, adhesion assays were performed using two different cell lines, α9- and mock-transfected MEF and SW-480 cells (Fig. 1). Flow cytometry with the anti-α9β1 antibody, Y9A2, showed robust expression of α9β1 in the α9-transfected cell types and no expression in mock transfectants (Fig. 1, A and C). α9-Transfected MEF demonstrated concentration-dependent adhesion to VEGF-A (Fig. 1B) that was abolished by the α9β1 blocking antibody Y9A2. In contrast, mock-transfected MEF did not adhere to VEGF-A above background levels of attachment to BSA-coated wells. Because cells have also been shown to adhere to VEGF-A in an α3β1 integrin-dependent manner, we performed assays in a second α9-transfected cell line (SW-480) known to express α3β1 (Fig. 1C). α9β1-dependent adhesion on VEGF-A was also demonstrated in these cells. In mock-transfected cells, blocking α3β1 inhibited cell adhesion, whereas blockade of...
α9β1 or VEGF-R2, neither of which are expressed in these cells, had no effect (Fig. 1D). In α9, compared with mock-transfected cells, adhesion to VEGF-A in the absence of blocking antibodies was substantially enhanced, as demonstrated by higher absorbance values. In these cells, blockade of α9β1 caused substantial inhibition of adhesion, whereas blocking antibody to α3β1 had no detectable effect (Fig. 1E), perhaps because α9β1 was expressed at substantially higher levels than α3β1 (Fig. 1C). Because these transfected cells do not express VEGF-R2, the cognate receptor for VEGF (25, 26), these findings suggest that α9β1 might interact directly with VEGF-A.

To confirm these findings in a physiologically relevant cell type, separate experiments were performed using HMVEC that express both VEGF-R2 and α9β1 (Fig. 2A). Concentration-dependent adhesion of HMVEC to VEGF-A165 was inhib-
ited by antibody to α9β1 (Fig. 2B). Because α3β1 and αvβ3 also interact with VEGF-A (18), we performed adhesion assays using HMVEC expressing these two integrins and α9β1 (Fig. 2, A and C) in the presence of blocking antibodies to each of the three integrins. Fig. 2D shows that inhibition of any single integrin inhibited adhesion of these cells to VEGF-A, with nearly complete inhibition caused by antibodies to α9β1 or αvβ3 and partial inhibition with antibody to α3β1. This was not because of artifactual effects of antibody binding to integrins on these cells, because a nonblocking antibody to α9β1 had no effect.

The Integrin α9β1 Directly Binds VEGF-A—To determine whether the findings from adhesion assays were a result of direct binding of VEGF-A165 to α9β1, we performed solid phase protein-protein binding assays. α9β1 bound to VEGF-A in a concentration-dependent fashion, and binding was inhibited by blocking antibody to α9β1 or by chelating divalent cations (10 mM EDTA), as expected for authentic integrin-ligand interactions (Fig. 3A). In contrast, the irrelevant integrin, αvβ6, showed no binding to VEGF-A.

Because we have shown that the homologous proteins VEGF-C and -D also bind to α9β1 integrin, we wished to better localize potential α9-binding sites for VEGF, using splice variants of VEGF-A. In contrast to VEGF-A165, which is missing exon 6, the 121 isoform is missing both exons 6 and 7 (Fig. 3B). Mock-transfected SW-480 cells, which express α3β1 but not α9β1 or VEGF-R2, adhered to VEGF-A121, and this binding was unaffected by antibody to α3β1, confirming previous reports (18) that α3β1 specifically mediates adhesion to the 165-kDa splice variant (Fig. 3C, top panel). In contrast, α9-transfected SW-480 cells, which also demonstrated increased adhesion to VEGF-A121, was specifically inhibited by antibody to α9β1 (Fig. 3C, bottom panel). Fig. 3D shows that in physiologically relevant HMVEC, cell adhesion to either VEGF-A165 or -121 isoform was partially α9-dependent.

To investigate the relative role of α9β1, α3β1, and αvβ3 integrins in cell migration, we performed HMVEC migration assays on either immobilized VEGF-A165 (Fig. 3E, top panel) or VEGF-A121 (Fig. 3E, bottom panel) in the absence or presence of inhibitory antibodies to either of the three integrins. Consistent with the specialized role of α9β1 in facilitating accelerated cell migration, inhibition of α9 resulted in the greatest reduction in cell migration on VEGF-A165. HMVEC migration on VEGF-A121 was also α9 integrin-dependent and, as expected, not dependent on integrins α3β1 or αvβ3. Because VEGF-A121 and VEGF-A165 are physiologically active in a soluble form, we tested whether soluble VEGF-A-induced cell migration was also α9β1-dependent. Fig. 3F (top and bottom panels) shows that both soluble VEGF-A165- and -121-induced endothelial cell migration was inhibited by α9β1 blocking antibody. As for responses to immobilized
VEGFA isoforms, inhibition of α3β1 or αvβ3 integrins decreased migration induced by VEGF-A165 (Fig. 3F, top panel) but not VEGF-A121 (bottom panel).

These findings taken together with our previous report showing that the homologous proteins VEGF-C and -D also bind to α9β1 (19) suggest that the α9β1 binding domain resides within the VEGF homology domain (exons 1–5). However, a binding site in exon 8, which is present in both VEGF165 and VEGF121, cannot be excluded.

α9β1 and VEGF-R2 Act Coordinate to Mediate Endothelial Cell Adhesion and Migration—To assess the relative contribution of VEGF-R2 and α9β1 to HMVEC adhesion
ERK and Paxillin—To determine whether VEGF-R2 and α9β1 coordinate signal in response to VEGF-A, we examined the effects of VEGF-A165 on phosphorylation of the downstream signaling intermediates ERK and paxillin, in the presence or absence of inhibitors of either receptor (Fig. 5). In the absence of VEGF-A, tyrosine phosphorylation of each protein was minimal. When these cells were plated on immobilized VEGF-A, both ERK1/2 and paxillin (Fig. 5A, top panel) were phosphorylated maximally after 15 min. Phosphorylation of each protein was inhibited by α9β1 blocking antibody at all time points. Following HMVEC binding to immobilized VEGF-A, ERK and paxillin phosphorylation was also inhibited by the VEGF-R2 kinase inhibitor (Fig. 5B). When cells were exposed to soluble VEGF-A (Fig. 5C), paxillin was maximally phosphorylated after 5 min, an effect that was inhibited by either blocking α9β1 or VEGF-R2. However, ERK phosphorylation in response to soluble VEGF-A was not affected by inhibition of α9β1 (data not shown).

We also wished to determine whether co-ligation of VEGF-R2 and α9β1 by VEGF-A leads to physical association of these two receptors. By co-immunoprecipitation, we could detect minimal association of VEGF-R2 and α9β1 in the absence of VEGF-A, and this association was substantially increased when plated on immobilized VEGF-A (Fig. 5D). Using irrelevant antibody, VEGF-R2 was unable to be immunoprecipitated in the absence or presence of VEGF-A. Also, we were not able to co-immunoprecipitate VEGF-R2 and α9β1 from cells exposed to soluble VEGF-A (data not shown). Furthermore, using either VEGF-R2-phosphospecific antibodies (pY951, 1054/1059) or immunoprecipitation, we found that in the presence of VEGF-A or the α9β1-specific ligand Tnfn3RAA (where Tnfn3RAA is α9-specific ligand, recombinant third fibronectin repeat of tenascin C in which arginine-glycine-aspartic acid is mutated to RAA), VEGF-R2 phosphorylation was not α9β1-dependent (data not shown).

VEGF-A-induced Angiogenesis Is α9β1-Dependent—To determine the in vivo relevance of our in vitro findings, we performed chick CAM assays to measure VEGF-A-induced angiogenesis, in the presence or absence of α9β1 blocking or non-blocking antibody. VEGF-A induced significant angiogenesis that was inhibited by the α9β1 blocking antibody, Y9A2, but not by the nonblocking antibody, A9A1 (Fig. 6, A and B), or by

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and migration on VEGF-A, cells were treated with α9β1 blocking antibody and/or the VEGF-R2 tyrosine kinase inhibitor SU1498 (27). Inhibition of either α9β1 or VEGF-R2 significantly decreased endothelial cell adhesion to VEGF-A165 (Fig. 4A), and the combination of both inhibitors caused maximal inhibition. Neither inhibitor affected adhesion to the irrelevant ligand, collagen (Fig. 4B). Inhibition of α9β1 or VEGF-R2 also decreased endothelial cell migration on VEGF-A (Fig. 4C), and again inhibition was maximal by the combination of both inhibitors. Inhibition of either α9β1 or VEGF-R2 also decreased endothelial cell migration in response to soluble VEGF-A165 or -121 (Fig. 4D), although in this case the effects of both inhibitors together were not different from the effects of each individual inhibitor. Taken together, these findings suggest that both α9β1 and VEGF-R2 are required for maximal cell adhesion and migration on immobilized VEGF-A and in response to soluble VEGF-A.

In Response to Immobilized VEGF-A, α9β1 and VEGF-R2 Become Physically Associated and Both Contribute to VEGF-A-induced Phosphorylation of the Signaling Intermediates

ERK and Paxillin—To determine whether VEGF-R2 and α9β1 coordinate signal in response to VEGF-A, we examined the effects of VEGF-A165 on phosphorylation of the downstream signaling intermediates ERK and paxillin, in the presence or absence of inhibitors of either receptor (Fig. 5). In the absence of VEGF-A, tyrosine phosphorylation of each protein was minimal. When these cells were plated on immobilized VEGF-A, both ERK1/2 and paxillin (Fig. 5A, top panel) were phosphorylated maximally after 15 min. Phosphorylation of each protein was inhibited by α9β1 blocking antibody at all time points. Following HMVEC binding to immobilized VEGF-A, ERK and paxillin phosphorylation was also inhibited by the VEGF-R2 kinase inhibitor (Fig. 5B). When cells were exposed to soluble VEGF-A (Fig. 5C), paxillin was maximally phosphorylated after 5 min, an effect that was inhibited by either blocking α9β1 or VEGF-R2. However, ERK phosphorylation in response to soluble VEGF-A was not affected by inhibition of α9β1 (data not shown).

We also wished to determine whether co-ligation of VEGF-R2 and α9β1 by VEGF-A leads to physical association of these two receptors. By co-immunoprecipitation,
\(\alpha 9\beta 1\) Integrin Is a Receptor for VEGF-A

In this study we have identified the angiogenic growth factor VEGF-A as a ligand for the integrin \(\alpha 9\beta 1\). Experiments with \(\alpha 9\)-transfected cells and primary human endothelial cells demonstrated that they adhere to and migrate on VEGF-A using \(\alpha 9\beta 1\). In response to immobilized VEGF-A, VEGF-R2 and \(\alpha 9\beta 1\) assemble into a macromolecular complex to cooperatively signal in an additive manner through phosphorylation of the downstream signaling intermediates ERK and paxillin. In vivo, endothelial cells respond to both immobilized VEGF-A, bound to the extracellular matrix, and to soluble VEGF-A, which may either be secreted from cells or released from the matrix through the action of matrix-degrading proteases. It appears that endothelial cell responses to soluble VEGF-A also depend on signals from \(\alpha 9\beta 1\). In contrast to immobilized VEGF-A, we could not demonstrate critical roles for ERK phosphorylation or the formation of a physical complex between VEGF-A and \(\alpha 9\beta 1\) following soluble VEGF-A stimulation.

Using an in vivo angiogenesis assay, we provide evidence that the interactions between both immobilized and/or soluble VEGF-A and \(\alpha 9\beta 1\) are likely to be biologically significant.

\(\alpha \nu\beta 5\), \(\alpha 5\beta 1\), and \(\alpha 4\beta 1\) (9–11, 28–30) are at least three other integrins that have been implicated in angiogenesis. One could thus argue that the findings reported here are not novel or unexpected. However, each of the integrins thus far shown to contribute to angiogenesis appears to do so by distinct mechanisms (10, 11, 28, 29). One such novel mechanism is through direct binding of growth factor to the integrin. Hutchings et al. (18) showed in protein-protein binding assays that VEGF-A165 bound directly to \(\alpha 9\beta 3\). However, there has been only indirect evidence that growth factors may directly bind to \(\beta 1\) integrins. In the same study by Hutchings et al. (18), human umbilical artery endothelial cells were reported to adhere to immobilized VEGF-A in an \(\alpha 3\beta 1\)-dependent fashion. The authors suggested this adhesion was independent of VEGF-R2 based on the results

**FIGURE 6.** VEGF-A- and not bFGF-induced angiogenesis is \(\alpha 9\beta 1\)-dependent. A and B, chick CAMs were exposed to 50 ng of VEGF-A165 alone (A, left panel, and B, black bar) or in the presence of either \(\alpha 9\) inhibiting (A, center panel, and B, diamond bar) or noninhibiting Ab (A, right panel, and B, diagonal bar) or VEGF-R2 inhibitor alone (B, brick bar) or VEGF-R2 inhibitor and \(\alpha 9\) inhibiting Ab (B, hatched bar) on incubation day 10 and imaged 3 days later for quantification of angiogenesis. C, in separate CAMs under the same conditions angiogenesis was induced by VEGF-A121 in the absence (white bar) or presence of blocking Ab for the integrins \(\alpha 9\beta 1\) (diagonal bar), \(\alpha 3\beta 1\) (diamond bar), or \(\alpha 9\beta 3\) (brick bar). D, in separate CAM experiments bFGF (150 ng) was used to induce angiogenesis, in the absence (black bars) or presence of \(\alpha 9\) blocking (white bar) or nonblocking (diagonal bar) Ab or \(\alpha 9\beta 3\) blocking Ab (diamond bar). Values are reported as mean ± S.E.

isotype-matched control antibody (data not shown). Also, as expected, VEGF-induced angiogenesis was inhibited by blocking VEGF-R2 (SU1498). Further inhibition was achieved by blocking both VEGF-R2 and \(\alpha 9\beta 1\). In separate experiments, similar results were found when the CAMs were stimulated with VEGF-A121 (Fig. 6C). Consistent with previous reports and our in vitro work, inhibition of \(\alpha 3\beta 1\) and \(\alpha 9\beta 3\) did not result in inhibition of VEGF-A121-induced angiogenesis. In contrast, induction of angiogenesis by bFGF (Fig. 6D) was not inhibited by the \(\alpha 9\) blocking antibody. These results suggest that the interaction between \(\alpha 9\beta 1\) and VEGF-A is relevant to in vivo angiogenesis, and the role of \(\alpha 9\beta 1\) in this process may be specific for angiogenesis induced by VEGF-A.
of presumably down-regulating the receptor following cell pretreatment with VEGF-A. However, the authors did not provide biochemical evidence of direct binding in cell-free experiments. In this study, we show that purified α9β1 directly binds to recombinant VEGF-A and that cell adhesion to VEGF-A is not α3-dependent in the presence of α9β1, suggesting the interaction with α9β1 is more robust than with α3β1.

Although both α3β1 and αβ3 integrins were found to bind the 165 isoform of VEGF-A, they did not bind VEGF-A121, suggesting the binding site for these integrins is encoded within exons 6–8. In contrast, α9β1 does bind to VEGF-A121 and, as we have shown previously, binds the homologous VEGF-C and -D growth factors (19), suggesting that a unique α9β1-binding site is encoded within the VEGF homology domain (exons 1–5). Not only is the interaction of the α9β1 integrin with VEGF-A121 unique for β1 integrins but also biologically significant as demonstrated by inhibition of cell migration and in vivo angiogenesis following blockade of α9β1 activity.

There have been numerous previous reports of “cross-talk” between tyrosine kinase growth factor receptors and integrins (9, 13, 16, 17). In some cases, this cross-talk has been associated with either growth factor or integrin ligand-induced co-association of both receptors (31). In the case of cross-talk between integrin α5β1 and epidermal growth factor receptor, ligation of the integrin induces phosphorylation of the receptor that appears to be independent of binding of the epidermal growth factor receptor itself (32, 33). In contrast, activation of α9β1 by its specific ligand, Tfn3RAA, does not appear to lead to phosphorylation of VEGF-R2, and blocking α9β1 has no effect on VEGF-A-induced phosphorylation of VEGF-R2. It thus appears that the signaling pathways activated by these two receptors intersect downstream of activation of VEGF-R2.

The role of α9β1 in signaling responses to immobilized and soluble VEGF-A appears to be dissimilar. In response to immobilized VEGF-A, α9β1 seems to play an important role in downstream phosphorylation of both paxillin and ERK and leads to the formation of a macromolecular complex containing both the integrin and VEGFR-2. In response to soluble VEGF-A, α9β1 also contributes to phosphorylation of paxillin, but it does not appear to enhance ERK phosphorylation or induce the formation of a dual receptor complex. These differences might reflect the influence of the extracellular matrix and its associated receptors such as heparin sulfate proteoglycans (34) that facilitate immobilization of a high density of VEGF-A, which in turn may stimulate an increase in both α9β1 and VEGFR-2 clustering. Nonetheless, the findings suggest that although important for full signaling through VEGF-R2 the integrin is not necessary and that paxillin phosphorylation may be the most relevant end point leading to enhanced cell migration in response to VEGF-A.

Our findings that blocking α9β1 specifically inhibited CAM angiogenesis induced by the 165 and 121 isoforms of VEGF-A and not bFGF suggest that the selective interaction of VEGF-A with this integrin might be the mechanism by which α9β1 enhances in vivo angiogenesis. This is supported by our findings that blocking both α9β1 integrin and VEGF-R2 resulted in greater inhibition of angiogenesis than blocking either receptor alone. Of course, in vivo angiogenesis is a complex process, and we cannot be certain that inhibition of α9β1 only inhibits angiogenesis by interfering with binding to VEGF-A. For example, the closely related integrin, αβ1, was recently shown to contribute to angiogenesis following binding to one of its ligands, VCAM-1 (10). We have previously shown that α9β1 is also a receptor for VCAM-1 (35), so it is conceivable that a similar mechanism contributes to the in vivo role of α9β1 in angiogenesis. However, in contrast to results for α9β1, blockade of αβ1 inhibits angiogenesis induced by bFGF, so this effect is clearly not specific for VEGF-A. Furthermore, mice lacking VCAM-1 or the integrin α4 subunit (36, 37) develop similar defects in vascular development, whereas development of the nonlymphatic vasculature appears to be normal in α9 knock-out mice (38). These results are most consistent with the hypothesis that the mechanisms by which α9β1 and αβ1 contribute to angiogenesis are distinct.

The absence of major vascular defects in α9 knock-out mice might suggest that the α9β1 integrin is not essential for normal vascular development. However, this should not be taken as evidence that α9β1 would not contribute to pathologic angiogenesis (39) as has been clearly demonstrated in the case of the αv integrins. Antagonists of αβ3 or αβ5 inhibit pathologic angiogenesis, but mice lacking β3 and/or β5 integrins have normal vascular development and even demonstrate enhanced tumor and ischemic angiogenesis (12). Additional experiments will be required using inhibitors of α9β1 that are effective in mammalian disease models of angiogenesis such as tumor growth and metastasis to more definitively address this question. The results presented here, in our previous reports (19, 38) and by others (40), demonstrate the unique role of α9β1 in not only lymphatic development but also VEGF-induced angiogenesis and lymphangiogenesis and suggest that inhibition of this integrin could affect pathologic vasculogenesis supporting cancer cell growth and metastasis.

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