Integrin Regulation by Vascular Endothelial Growth Factor in Human Brain Microvascular Endothelial Cells

ROLE OF $\alpha_6\beta_1$ INTEGRIN IN ANGIOGENESIS*

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Tae-Hee Lee,†,‡, Seyha Seng,‡, Huchun Li,‡, Stephen J. Kennel,‡, Hava Karsenty Avraham,§, and Shalom Avraham†,‡,§,‡

From the †Division of Experimental Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02115 and the §Life Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831

The precise role of vascular endothelial growth factor (VEGF) in regulating integrins in brain microvascular endothelial cells is unknown. Here, we analyzed VEGF effects on integrin expression and activation in human brain microvascular endothelial cells (HBMECs). Using human cDNA arrays and ribonuclease (RNase) protection assays, we observed that VEGF up-regulated the mRNA expression of $\alpha_\text{6}$ integrin in HBMECs. VEGF significantly increased $\alpha_\text{6}\beta_\text{1}$ integrin expression, but not $\alpha_\text{6}\beta_\text{3}$ integrin expression in these cells. Specific down-regulation of $\alpha_\text{6}$ integrin expression by small interfering RNA (siRNA) oligonucleotides inhibited both the capillary morphogenesis of HBMECs and their adhesion and migration. Additionally, VEGF treatment resulted in activation of $\alpha_\text{6}\beta_\text{1}$ integrins in HBMECs. Functional blocking of $\alpha_\text{6}$ integrin with its specific antibody inhibited the VEGF-induced adhesion and migration as well as in vivo angiogenesis, and markedly suppressed tumor angiogenesis and breast carcinoma growth in vivo. Thus, VEGF can modulate angiogenesis via increased expression and activation of $\alpha_\text{6}\beta_\text{1}$ integrins, which may promote VEGF-driven tumor angiogenesis in vivo.

Vascular endothelial growth factor (VEGF) contains potent mitotic activity specific to vascular endothelial cells (1) and plays an essential role in promoting vascularization during normal and pathological conditions (2, 3). Through its interaction with the VEGF receptor, KDR/Flk-1, VEGF mediates most angiogenic processes, such as proliferation, survival, adhesion, migration, capillary morphogenesis (tube formation), and the gene expression of endothelial cells (1, 2). One of the mechanisms by which VEGF promotes angiogenesis is via up-regulation of the expression and/or activation of integrins.

Integrins, the major receptors for extracellular matrix (ECM) components, mediate cell adhesion to the ECM (4, 5). The integrin family comprises at least 24 different heterodimers, with 18 $\alpha$- and 8 $\beta$-subunits and distinct and often overlapping specificity for ECM proteins (5). Integrins activated by engagement with their ligands can transduce various signals, such as calcium influx or activation of kinases, from the extracellular environment to the interior of the cells (6–8). This process is known as “outside-in” signaling. In addition, the active status of integrins can be achieved by growth factor-induced signaling pathways, a process known as “inside-out” signaling. Thus, blocking the functions of specific integrins can exert profound effects on the angiogenic response of endothelial cells, indicating that integrins are directly implicated in angiogenic processes (9–12).

Through “inside-out” signaling, VEGF activates various integrins. These integrins include the fibronectin receptor $\alpha_\text{5}\beta_\text{1}$, collagen receptor $\alpha_\text{2}\beta_\text{1}$, as well as the vitronectin receptors $\alpha_\text{v}\beta_\text{3}$ and $\alpha_\text{v}\beta_\text{5}$ in human umbilical vein endothelial cells (HUVECs) (13). Thus, the functional blocking of these integrins inhibits VEGF-induced angiogenesis. In addition to these integrin signalings, alterations in integrin expression were reported to contribute to angiogenesis. For example, VEGF increases the migration of human dermal microvascular endothelial cells (HDMECs) through the up-regulation of $\alpha_\text{v}\beta_\text{3}$ integrin expression (14). VEGF was also reported to increase the expression of $\alpha_\text{v}\beta_\text{3}$ and $\alpha_\text{v}\beta_\text{5}$ integrins in HDMECs, while blocking antibodies of these integrins markedly inhibited VEGF-induced angiogenesis and tumor growth in vivo (15, 16). Thus, VEGF modulates angiogenesis via regulation of the expression and/or activation of integrins.

VEGF induces significant changes in the permeability of the endothelium and is expressed in some breast cancer cells (17, 18). Breast cancer cells such as MDA-MB-231 cells secrete high levels of VEGF (18). We previously reported that VEGF significantly increased penetration of the highly metastatic MDA-MB-231 breast cancer cells across the HBMEC monolayer (18). We also observed that VEGF modulated the transendothelial migration (TM) of breast tumor cells by regulating the integrity.
of the HBMEC monolayer (17, 18). Thus, VEGF might contribute to breast cancer metastasis by enhancing the TM of tumor cells through its effects on endothelial integrity.

In this study, we examined the effects of VEGF on integrin expression and activation in HBMECs. Until recently, the role of VEGF in the regulation of integrins was not well defined because of their high redundancy. To directly investigate this topic, we first used human cdNA arrays containing 12 α- and 8 β-subunits as well as RNase protection assays (RPAs) covering 16 α- and 8 β-subunits, which include most of the known integrins. By using these tools, we observed that VEGF potently induced the expression of α6 integrin mRNA in these cells. The α6 integrin, which constitutes a subunit of the α6β1 and α6β4 integrins, is known to be a receptor for laminins (19, 20) and to mediate several biological activities, such as embryogenesis (21), invasion of human carcinoma cells (19, 22, 23), and the survival of oligodendrocytes (24). However, the precise role of α6 integrin in angiogenesis has not yet been addressed.

Here, we report that the α6 integrin mediated VEGF-induced angiogenesis through α6β1 integrin. We demonstrated that blocking of α6 integrin with its specific antibody inhibited VEGF-induced adhesion and migration as well as in vivo angiogenesis, and markedly suppressed tumor angiogenesis and breast carcinoma growth in vivo. These results strongly suggest that breast cancer cells secreting VEGF are able to induce the expression and activation of α6 integrin in brain microvascular endothelial cells, which may facilitate breast cancer cell migration and metastasis to the brain.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant VEGF165 was obtained from Genentech Inc. (San Francisco, CA). Growth factor-reduced Matrigel was purchased from BD Biosciences Inc. (Palo Alto, CA). Fibronectin, Collagen IV, and Laminin-1 were purchased from Roche Applied Sciences. Polyclonal antibodies for human von Willebrand factor were purchased from Dako Inc. (Carpinteria, CA). Monoclonal antibodies for the α6 [MAB1378 (GoH3) for functional blocking and MAB1356 for Western blot analysis], β1 (MAB2253) and β4 (MAB2059) integrins were obtained from Chemicon International Inc. (Temecula, CA). Polyclonal antibodies for the α5 integrin (AB1949-20) were also purchased from Chemicon International Inc. Rat monoclonal antibody (clone 135-13C) against mouse α6 integrin was produced as described previously (25, 26).

**Cell Culture**—HBMECs were purchased from Cell Systems Inc. (Kirkland, WA) and maintained according to the method described previously (17, 18). HBMECs were used from passage three up to passage seven and were checked routinely for the expression of von Willebrand factor. HUVECs were purchased from Cambrex Bio Science Inc. (Walkersville, MD) and cultured with EGM-2 medium.

**Cytokine cdNA Array Analysis**—HBMECs were grown subconfluently onto attachment factor-coated 100-mm dishes. The cells were starved in serum-free CSC medium containing 0.5% fetal bovine serum (assay medium) for 4 h and then stimulated with VEGF (30 ng/ml) for 5 h. Total RNA preparation and hybridization procedures were performed according to the manufacturer’s protocol. Equal amounts (2 μg) of total RNA from unstimulated or VEGF-stimulated HBMECs were used for preparation of the cdNA probes. The cytokine cdNA membranes (R&D Systems) were hybridized with the cdNA probes at 68 °C for 18 h. After washing, the membranes were exposed on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and analyzed using ArrayVision™ software (Imaging Research Inc., Ontario, Canada).

**RNase Protection Assay**—The assay was performed on total RNA using the RPA kit (BD Pharmingen, San Diego, CA). Briefly, HBMECs were starved in assay medium for 4 h and then stimulated with VEGF (30 ng/ml) for the indicated times. The total RNA was isolated, and 5 μg of the isolated RNA was hybridized with 32P-labeled antisense RNA probes prepared using the Human Integrin Template Sets (BD Pharmingen). After digestion by RNase, the samples were separated on a 5% acrylamide gel containing 48% urea. The gel was dried and exposed on a Phosphorimager. The intensity of the protected bands was measured using Scion Image Beta 4.02 software (Scion Corp., Frederick, MD).

**Northern Blot Analysis**—HBMECs were starved in assay medium for 4 h and then stimulated with VEGF (30 ng/ml). The isolated total RNA (10 μg) was separated on an agarose gel and transferred to a hybrid N membrane (Amersham Biosciences). The membrane was hybridized with full-length cdNA to human α6 integrin (a generous gift of Dr. Leslie M. Shaw, Beth Israel Deaconess Medical Center, Boston, MA) and, after washing, the blot was exposed to x-ray film. The intensity of the bands was measured using Scion Image Beta 4.02 software.

**mRNA Stability Analysis**—HBMECs were grown subconfluently onto attachment factor-coated 100-mm dishes. The cells were starved in assay medium for 4 h and then treated with VEGF (30 ng/ml) or PBS. After 24 h, actinomycin D (5 μg/ml) was added, and the cells were harvested for RNA isolation at 0, 1, 3, 5, 7, and 9 h after the addition of actinomycin D. Northern blot analysis was performed according to the method described above. The densities of α6 integrin mRNA were measured using Scion Image Beta 4.02 software.

**Biotinylation and Immunoprecipitation**—HBMECs were starved in assay medium for 4 h, treated with VEGF (30 ng/ml) for 24 h, and dispersed by brief treatment with 0.05% trypsin solution. Cell surface labeling with biotin was performed according to the method described previously (15). For the immunoprecipitations, 50 μg of total protein lysate was incubated with 2 μg of monoclonal antibody against α6, β1, and β4 integrins, respectively, and the immune complex was precipitated with protein G-Sepharose. The precipitated samples were separated by SDS-PAGE under non-reducing conditions and then transferred onto Immobilon-P membranes (Millipore, Boston, MA). The membranes were incubated with avidin-horseradish peroxidase conjugate (Bio-Rad) for 1 h, and the biotinylated proteins were detected by enhanced chemiluminescence.

**Western Blot Analysis**—HBMECs were starved in assay medium for 4 h and then stimulated with VEGF (30 ng/ml). HBMECs were lysed in commercial lysis buffer (New England Biolabs, Beverly, MA) and Western blot analysis was performed.
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as described previously (18). The intensity of the bands was measured using Scion Image Beta 4.02 software.

Adhesion Assay—HBMECs were starved in serum-free CSC medium containing 1% BSA (adhesion buffer) for 4 h and dispersed for resuspension in adhesion buffer. 24-Well plates were coated with matrix proteins in adhesion buffer (50 mM NaHCO$_3$, 150 mM NaCl, pH 8.0) for 1 h at 37 °C, and nonspecific binding sites on the plates were blocked with 3% BSA in adhesion buffer for 1 h at 37 °C as described previously (13, 27). Prior to the assays, $1 \times 10^5$ cells in 100 µl were preincubated with test antibodies (20 µg/ml) for 30 min and, in some experiments, were treated with VEGF (30 ng/ml), phorbol 12-myristate 13-acetate (PMA, 200 nM), or MnCl$_2$ (1 mM) for 30 min, and then added to matrix protein-coated 24-well plates at a final volume of 0.5 ml. Alternatively, siRNA-treated HBMECs were starved in assay medium for 4 h and treated with VEGF (30 ng/ml) for 24 h, then added to Laminin-1-coated 24-well plates after suspension in adhesion buffer. After 30 min of incubation, the cells were fixed with 3.7% formaldehyde and washed extensively with PBS. Attached cells were stained by incubation, the cells were fixed with 3.7% formaldehyde and observed under an inverted microscope and counted from 15 random fields of ×400 magnification.

Migration Assay—HBMECs were dispersed in 0.05% trypsin solution and resuspended in assay medium. Prior to the assays, $1 \times 10^5$ cells in 100 µl were incubated with test antibodies (20 µg/ml) for 30 min and added to Laminin-1-coated 24-well Transculture inserts with a pore size of 8 µm (Costar Corp.). Alternatively, siRNA-treated HBMECs were directly added to the Transculture inserts after suspension in assay medium. VEGF (30 ng/ml) was added to the basolateral chambers at a final volume of 0.6 ml. After 3 h of incubation, the apical chambers were fixed and stained by using a Hema-3 Stain Set (Fisher Inc.), then observed under an inverted microscope and counted from 15 random fields of ×400 magnification.

Small Interfering RNA (siRNA)—The siRNA oligonucleotides for human $\alpha_\text{v}$ integrin (CAACUGAAGUCACCUCUUUG-AUU; GGAAUAGGCUCAGGUAAUU; CAAGACAG-CUCAUAAUGAUUU; GAAAGGAUGUUGUUGUAUU) (cat. M-007214-00) and nonspecific control (cat. D-001206-13) siRNA were purchased from Dharmacon Inc. (Lafayette, CO). Transfection procedures were performed with oligofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Cells were grown subconfluently onto 100-mm dishes or 6-well plates and transfected with 200 nM of siRNA. After 72 h of transfection, cells were washed and analyzed further as specifically indicated.

Capillary Morphogenesis Assay—150 µl of Matrigel (9 mg/ml) was added to a 24-well plate and allowed to gel for 5 min at 37 °C. The HBMECs were detached by brief treatment with 0.05% trypsin solution and resuspended in CSC medium containing 10% fetal bovine serum. Prior to the assays, $5 \times 10^4$ cells in 100 µl were incubated with test antibodies (20 µg/ml) for 30 min and added to the plate at a final volume of 0.5 ml. Alternatively, siRNA-treated HBMECs were directly added to the plate after suspension in CSC medium containing 10% fetal bovine serum. After 6 or 12 h of incubation, the cells were fixed with 3.7% formaldehyde and observed under an inverted microscope. The lengths of the tubular-like structures were measured using Scion Image Beta 4.02 software.

In Vivo Angiogenesis Assay—Assays were performed according to the method described previously (15, 16) with the following modifications. MDA-MB-231 breast cancer cells were injected with adenovirus encoding VEGF (a generous gift of Dr. Harold F. Dvorak, Beth Israel Deaconess Medical Center, Boston, MA) or control adenovirus at a multiplicity of infection (MOI) of 1,000 for 24 h. The cells (1 × 10$^6$) were resuspended in 0.25 ml of growth factor-reduced Matrigel (at a final concentration of 9 mg/ml) and injected subcutaneously to the midline of athymic nude mice (female, 11 weeks-old). Soon after injection, animals were co-injected peritonally with 250 µg of the $\alpha_\text{v}$ integrin antibody or with control antibody, each of which was administered repeatedly at an interval of 2 days. In these experiments, we used rat monoclonal antibody (clone 135-13C) for the mouse $\alpha_\text{v}$ integrin. Specifically, the 135-13C antibody is similar to the commercially available rat monoclonal antibody (GoH3) for mouse $\alpha_\text{v}$ integrin. Furthermore, this antibody inhibited the capillary morphogenesis of HBMECs on Matrigel as well as VEGF-induced adhesion and migration. After 7 days, the animals were euthanized and dissected. Implants together with associated skin were fixed overnight in 10% paraformaldehyde and embedded in paraffin. The tissues were cut and deparaffinized, then treated with 0.1% trypsin for 30 min to improve antigen accessibility. Subsequently, the sections were treated with H$_2$O$_2$ to inhibit endogenous peroxidase. After blocking the nonspecific binding to the secondary antibodies by treatment with normal goat serum, polyclonal antibodies against human von Willebrand factor (1:100, Dako Inc., Carpinteria, CA) were incubated for 2 h at room temperature. The tissues were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories) for 1 h. After washing, staining was performed using the Vectastain ABC kit (Vector Laboratories).

Tumor Growth Assay—MDA-MB-231 breast cancer cells (3 × 10$^6$) were injected subcutaneously into both flanks of 6-week-old female nude mice. The next day after implantation, animals (5 mice per group) were injected peritoneally with 250 µg of the $\alpha_\text{v}$ integrin antibody (clone 135-13C) or with control antibody, each of which was administered repeatedly at an interval of 3 days. After 10 days of implantation, tumor mass was measured with calipers at an interval of 2 days and the tumor volume (mm$^3$) was estimated by the following formula: volume = $(4/3)(\pi)(1/2 \times \text{smaller diameter})^2(1/2 \times \text{larger diameter})$. The differences between the experimental groups were analyzed by the Student’s $t$ test. After 25 days, tumors were dissected from the euthanized animals, embedded in OCT compound, and immediately frozen. 6-µm sections were obtained with a cryostat microtome and fixed with cold acetone. To estimate tumor vascularization, sections of nine tumors per group were stained with 10 µg/ml mouse anti-CD31 monoclonal antibody (Abcam). Blood vessels were observed under a light microscope and counted from three random fields of ×200 magnification. The lengths of the blood
vessels were measured using Scion Image Beta 4.02 software. The differences between the experimental groups were analyzed by the Mann-Whitney U test.

RESULTS

**VEGF Induces Integrin mRNA Expression in HBMECs**—By using human cDNA arrays containing integrins, we found that VEGF significantly up-regulated the mRNA expression of $\alpha_2$ and $\alpha_6$ integrins in HBMECs. Based on RNase protection assays, we obtained similar results demonstrating that VEGF induced $\alpha_2$ and $\alpha_6$ integrin mRNA expression in a time-dependent manner (Fig. 1, A and B). These results are consistent with a previous report showing that VEGF induced the expression of $\alpha_2$ integrin in HDMECs (15). We also observed that the expression of $\beta_3$ integrin reached a maximal level of transcription at 3 h (2.5-fold increase compared with the control) and decreased to control levels at 12 h, while VEGF decreased $\beta_3$ integrin mRNA expression in a time-dependent manner (Fig. 1, B and C). Interestingly, both experiments revealed that VEGF significantly increased mRNA expression of the $\alpha_6$ integrin in HBMECs. To confirm these results, we performed Northern blot analysis with full-length cDNA encoding the human $\alpha_6$ integrin. As expected, the mRNA expression of the $\alpha_6$ integrin began to increase 1 h after the treatment with VEGF and reached a maximal level of transcription at 12 h (7-fold increase compared with control) (Fig. 2A).

To examine whether VEGF can affect $\alpha_6$ integrin expression by enhancing the stability of its mRNA, HBMECs were starved for 4 h and treated with VEGF (30 ng/ml). After 24 h, cells were treated with actinomycin D, harvested, and subjected to Northern blot analysis for $\alpha_6$ integrin expression. As shown in Fig. 2B, VEGF had no effect on the mRNA stability of $\alpha_6$ integrin in HBMECs. Thus, these data indicate that VEGF increases $\alpha_6$ integrin expression at the transcriptional level. The induction
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Figure 2. Expression of α6 integrin in VEGF-stimulated HBMECs. A. Northern blot analysis. HBMECs were starved in assay medium for 4 h and then stimulated with VEGF (30 ng/ml) for the indicated times. The isolated total RNAs (10 μg) were separated on an agarose gel. Northern blot analysis was performed according to the procedures described under “Experimental Procedures.” The actin mRNA is shown to verify equal loading. B, mRNA stability analysis. HBMECs were grown subconfluently onto attachment factor-coated 100-mm dishes. Cells were starved in assay medium for 4 h and then treated with VEGF (30 ng/ml) or PBS. After 24 h, actinomycin D (5 μg/ml) was added, and the cells were harvested for RNA isolation at 0, 1, 3, 5, 7, and 9 h after the addition of actinomycin D. Northern blot analysis was performed according to the procedures described under “Experimental Procedures.” The relative densities of α6 integrin mRNA were measured using Scion Image Beta 4.02 software. C, Western blot analysis. HBMECs were starved in assay medium for 4 h, stimulated with VEGF (30 ng/ml) and then lysed in lysis buffer. Proteins were separated by SDS-PAGE under reducing conditions, and Western blot analysis was performed with α6 antibody. The membrane was stripped and reprobed with α6 integrin antibodies. To verify equal loading, the membrane was stripped once more and probed with human Csk antibodies. WB, Western blot. D, biotinylation and immunoprecipitation. HBMECs were biotinylated, lysed, and immunoprecipitated with α6 antibody. The immune complex was precipitated with protein G-Sepharose. Precipitated samples were separated by SDS-PAGE under non-reducing conditions. To verify equal loading and biotinylation efficiency, cell lysates (5 μg) were subjected to SDS-PAGE, and the total biotinylated proteins visualized with enhanced chemiluminescence (first panel). αα, a variant of α6 integrin having a small molecular weight; IP, immunoprecipitation; Ab, antibody. E, expression of α6 integrin in VEGF-stimulated HBMECs. HBMECs were grown subconfluently onto attachment factor-coated 100-mm dishes. The cells were starved for 4 h and then treated with VEGF (30 ng/ml) or with PBS. After 24 h, HBMECs were lysed, then immunoprecipitated with α6 or β1 antibody. The immune complexes were analyzed by SDS-PAGE under reducing conditions, followed by Western blot analysis using α6 antibody. The graph shows densitometric analysis of the relative expression (%) of α6 integrin. F, surface expression of α6β1 integrin by flow cytometry analysis. HBMECs were starved for 5 h and then untreated or treated with VEGF (30 ng/ml). After incubation for 24 h, cells were detached by incubation with 50 mM EDTA in PBS, centrifuged and resuspended with PBS containing 1% BSA. The cells were next incubated with α6 integrin antibody GoH3 (2 μg/ml) or control Ig-G antibodies for 30 min at 4 °C. After washing with PBS containing 1% BSA, cells were incubated with FITC-conjugated secondary antibodies for 30 min at 4 °C and analyzed by flow cytometry.
of α6 integrin by VEGF is specific, because stimulation of HBMEC with either bFGF or PDGF in various concentrations failed to induce α6 integrin expression in HBMEC (data not shown).

**VEGF Increases α6 Integrin Expression at the Protein Level—**

To determine whether VEGF induces expression of the α6 integrin at the protein level in HBMECs, cells were stimulated with VEGF (30 ng/ml) for the indicated times and subjected to Western blot analysis. Expression of the α6 integrin increased 3 h after treatment with VEGF and was sustained during the examined period, whereas expression of the α6 integrin was not altered by the treatment with VEGF (Fig. 2C). Next, to determine whether VEGF increases the expression of α6β1 integrin at the surface of the HBMECs, cells were stimulated with VEGF for 48 h and labeled by cell surface biotinylation. The lysates were immunoprecipitated with various integrin antibodies and subjected to SDS-PAGE under non-reducing conditions. As shown in Fig. 2D (middle panel), the α6 antibody precipitated increased amounts (3-fold increase compared with the control) of the α6 (160 kDa) and β1 (110 kDa) integrins in the VEGF-stimulated cells. This antibody also precipitated a variant of the α6 integrin that had a small molecular mass of 70 kDa, a result similar to that described previously in other cell types (28). The β1 antibody precipitated β1 integrin (110 kDa), and a protein having a molecular mass of 160 kDa (Fig. 2D, right panel). However, it is not clear whether this protein band corresponds to the α6 or α5 integrin because both molecules have a molecular mass of 160 kDa under non-reducing conditions. Nevertheless, these data showed that VEGF did not significantly increase the expression of β1 integrin at the surface of the HBMECs.

To show that α6 is associated with both β1 and β3 integrins in HBMECs, we performed immunoprecipitation studies using anti-β1 and anti-β3 antibodies followed by immunoblot analysis with α6 integrin antibodies. As shown in Fig. 2E, we observed that VEGF increased the expression of α6β1 integrin, but not α6β3 integrin, in HBMECs. We next confirmed the surface expression of α6β1 integrin using flow cytometry analysis. As shown in Fig. 2F, VEGF stimulation significantly increased the expression of α6 integrin at the surface of the HBMECs.

**α6 siRNA Inhibits the Capillary Morphogenesis as Well as VEGF-induced Adhesion and Migration of HBMECs—**

To examine whether α6 integrin is directly involved in angiogenesis, we studied the loss of function of α6 integrin in angiogenesis using the siRNA approach. HBMECs were preincubated with siRNA oligonucleotides for α6 integrin or with control siRNA for 4 days. Using Western blot analysis, we observed an ~70% knock-down of α6 integrin expression in the HBMECs upon treatment with α6 siRNA (Fig. 3A, upper panel). Biotinylation and immunoprecipitation analyses revealed that α6 siRNA induced an ~70% reduction in α6 integrin expression on the surface of HBMECs (Fig. 3A, lower panel).

The angiogenic process can be divided into a series of sequential events, including focal digestion of the basement membrane, migration, adhesion, proliferation, and tube formation (29). VEGF can mediate most angiogenic processes through activation of its receptor, KDR/Flk-1. α6 integrin may be targeted as a downstream effector of VEGF-induced angiogenesis. Based on this hypothesis, we studied whether α6 integrin is involved in the VEGF-induced migration of HBMECs to Laminin-1, the ligand for α6 integrin.

HBMECs were preincubated with siRNA for 4 days and then stimulated with VEGF for 24 h. As shown in Fig. 3, B and C, α6 siRNA significantly blocked the VEGF-induced adhesion and migration of HBMECs to Laminin-1, the ligand for α6 integrin, but not to the ECM protein fibronectin (data not shown). Furthermore, this effect was specific to VEGF, because α6 siRNA failed to block the PDGF-induced adhesion of HBMEC (data not shown). Next, we studied whether the α6 integrin has the ability to stimulate the morphogenic process of HBMECs.

HBMECs were preincubated with α6 siRNA or control siRNA for 4 days and seeded onto Matrigel-coated plates. Notably, the Matrigel primarily consists of laminin components and induces the spontaneous capillary morphogenesis of endothelial cells without growth factors, such as bFGF and VEGF. From this experiment, we found that α6 siRNA strongly inhibited the capillary morphogenesis of these cells onto Matrigel compared with the control siRNA (Fig. 3D), indicating that α6 integrin mediates the capillary morphogenesis of HBMECs. Taken together, these data indicate that the increased expression of α6 integrin induced by VEGF can be a positive step in angiogenic processes.

Next, we examined the VEGF receptor through which VEGF up-regulates the expression of α6 integrin in HBMECs. The HBMECs were untreated or were preincubated either with Flt-1 blocking antibody (kindly obtained from Dr. Masabumi Shibuya) or with control antibody for 30 min, and then stimulated with VEGF. Total cell lysates were prepared and analyzed by SDS-PAGE followed by Western blot analysis using various antibodies. High expression levels of the α6 integrin were observed in the HBMECs, and Flt-1-blocking antibody inhibited the VEGF-induced expression of α6 integrin in these cells (Fig. 3E). Interestingly, there were no changes in β3 integrin expression in these cells. Thus, VEGF up-regulates α6 integrin expression, at least in part through the Flt-1 receptor.

**VEGF Induces Activation of α6 Integrin in HBMECs—**

In addition to integrin expression, VEGF can stimulate angiogenesis through integrin activation in endothelial cells (13). In fact, when HBMECs were stimulated with VEGF or the integrin activators PMA and Mn2+ for 30 min, the VEGF-stimulated cells showed increased adhesion onto fibronectin or collagen IV-coated plates compared with the unstimulated cells (Fig. 4A). VEGF also induced the increased adhesion of HBMECs onto Laminin-1-coated plates (Fig. 4, C and D), suggesting that laminin receptors, such as α6 integrin, might be activated by VEGF in HBMECs. Consistent with this possibility, the α6 antibody GoH3 (or clone 135–13C) significantly blocked the VEGF-induced adhesion and migration of HBMECs onto Laminin-1-coated plates (Fig. 4, B and C). In addition, we found that β1 antibody also potently blocked the VEGF-induced adhesion and migration of HBMECs to Laminin-1, whereas β3 antibody had no effects (Fig. 4, B and C). Furthermore, the α6 antibody as well as the β1 antibody strongly inhibited the capillary morphogenesis of HBMECs onto Matrigel (Fig. 4D). However, the β3 antibody had no effects (Fig. 4D). These data strongly suggest that α6 integrin is involved in multiple angiogenic processes as is the α6β1 heterodimer.
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**A** parental si-CTL si-α<sub>6</sub> α<sub>6</sub> WB: α<sub>6</sub> Ab Csk WB: Csk Ab si-CTL si-α<sub>6</sub> si-CTL si-α<sub>6</sub> α<sub>6</sub> lysates IP: α<sub>6</sub>Ab

**B**

![Graph showing the number of adherent cells](image)

**C**

![Graph showing the number of migrated cells](image)

**D**

![Graph showing the length of capillary morphogenesis](image)

**E**

![Graph showing the effects of VEGF on adherent cells](image)

*FIGURE 3. siRNA oligonucleotides for α<sub>6</sub> integrin inhibit in vitro angiogenesis in HBMECs.***

*V** EGFR Induces the Expression and Activation of α<sub>6</sub> Integrin in HUVECs—Next, we examined the effects of VEGF on another endothelial cell system, using HUVECs. Northern blot analysis revealed that VEGF increased the expression of α<sub>6</sub> integrin mRNA in HUVECs (Fig. 5A). VEGF also induced the adhesion of HUVECs onto Laminin-1-coated plates (Fig. 5B). The α<sub>6</sub> antibody GoH3 and β<sub>1</sub> antibody significantly blocked the VEGF-induced adhesion of HUVECs onto Laminin-1-coated plates, whereas β<sub>4</sub> antibody had no effects. These data indicate that VEGF activates the α<sub>6</sub>β<sub>1</sub> heterodimer in HUVECs, and imply that VEGF can induce the expression and activation of α<sub>6</sub> integrin in a broad range of endothelial cells, including HBMECs and HUVECs.

*The α<sub>6</sub> Integrin Antibody Inhibits VEGF-induced Angiogenesis in Vivo—*To demonstrate the participation of the α<sub>6</sub> integrin in VEGF-driven angiogenesis in vivo, we used monoclonal antibody (clone 135-13C) for the mouse α<sub>6</sub> integrin in an athymic nude mouse model. This model involved the subcutaneous injection of Matrigel containing human recombinant VEGF (20 μg/ml) and MDA-MB-231 cells infected with adenovirus encoding VEGF. Specifically, the 135-13C is similar to the commercially available rat monoclonal antibody for mouse α<sub>6</sub> integrin, GoH3. Furthermore, this antibody significantly inhibited the capillary morphogenesis of HBMECs on Matrigel. Soon after injection of Matrigel, the animals were co-injected peritoneally with 250 μg of the α<sub>6</sub> antibody or control antibody, which was administered repeatedly at an interval of 2 days. After 7 days, mice were sacrificed and the excised implants were photographed under a surgical microscope and processed for immunohistochemical analyses. We observed that VEGF-producing Matrigel showed a much higher density of blood vessels at their surfaces compared with the Matrigel containing the MDA-MB-231 cells infected with control adenovirus alone (Fig. 6A). However, α<sub>6</sub> antibody significantly reduced the density of blood vessels at the surface of the Matrigel producing VEGF compared with control antibody (Fig. 6A, see the arrow). Notably, the α<sub>6</sub> integrin antibody did not alter the secretion of
VEGF in MDA-MB-231 cells infected with VEGF-adenovirus. To identify blood vessels at the interface of the dermis and Matrigel, skin tissues containing Matrigel were cut into 7-μm sections and stained for the endothelial cell marker, von Willebrand factor. Similar to the result shown in Fig. 6A, α6 antibody significantly reduced microvessel density compared with control antibody (Fig. 6B). These data indicate that the α6 integrin mediates VEGF-induced angiogenesis in vivo.

The α6 Integrin Antibody Suppresses Breast Carcinoma Growth in Vivo—Blocking of VEGF potently inhibits tumor growth and induces tumor regression, indicating that VEGF is a strong inducer of tumor angiogenesis (30, 31). Because our data showed that functional blocking of the α6 integrin inhibited VEGF-mediated angiogenesis, we therefore examined the effects of this inhibition on the suppression of tumor growth. We used human MDA-MB-231 breast cancer cells as an in vivo tumor model since these cells express VEGF, and because blocking of VEGF inhibited the growth of these tumor cells when implanted in mice (32). When the tumor cells were implanted subcutaneously into both flanks of the nude mice, we observed that α6 antibody (clone 135-13C) significantly reduced tumor volume and tumor weight compared with the control antibody (Fig. 7, A and B).

To examine whether the reduced tumor weight correlated with the reduced density and length of blood vessels within the tumor, excised tumor tissues were cut into 6-μm sections and stained for the endothelial cell marker, CD31 (Fig. 7C). Blood vessels were photographed under a light microscope and counted from three random fields of 200 magnification. The lengths of the blood vessels were measured using Scion Image Beta 4.02 software as described under “Experimental Procedures.” The α6 antibody reduced the density of blood vessels by 19% (Fig. 7D), and caused a much greater reduction (58%) in blood vessel length within the tumors compared with the control antibody (Fig. 7E). To demonstrate whether α6β1 is expressed on neovessels in vivo, frozen sections from these tumors were immunostained with either von Willebrand Factor (vWF) or...
with the α₆ integrin antibody GoH3 and then analyzed by confocal microscopy. As shown in Fig. 7F, we observed that vWF and the α₆ integrin were co-localized on the neovessels, indicating that the newly formed blood vessels in the tumors expressed α₆ integrin. Taken together, these data demonstrate that functional blocking of the α₆ integrin inhibits breast tumor angiogenesis and growth in nude mice.

DISCUSSION

In this study, we examined the effect of VEGF in regulating the expression of integrins in HBMECs by using human cDNA arrays and RNase protection assays. Based on these experiments, we observed that VEGF significantly increased mRNA expression of the α₆ integrin in HBMECs, suggesting that the α₆ integrin might play a role in VEGF-induced angiogenesis. We demonstrated that blocking of α₆ integrin with its specific antibody inhibited the VEGF-induced adhesion and migration as well as in vivo angiogenesis, and markedly suppressed tumor angiogenesis and breast carcinoma growth in vivo.

The α₆ integrin (primarily α₆β₄ integrin) is involved in maintaining the integrity of stratified epithelia by a mechanical connection between laminin and the intermediate filamentous cytoskeleton of the cells (33). Consistent with this role, α₆ integrin-deficient mice have been reported to die at birth, presenting with severe blistering of the skin and other epithelia (21). In addition to epithelial cells, various cell types including fibroblasts and endothelial cells are known to express the α₆ integrin (19, 20, 33, 34). In fibroblasts and carcinoma cells, the α₆ integrin (especially α₆β₃) mediates invasion through the PI3-kinase signaling pathway and through cross-talk with the Met tyrosine kinase, a receptor for hepatocyte growth factor (22, 23, 35). Besides its signaling properties, the α₆ integrin directly induces cell migration by stimulating both the formation of actin-rich protrusions and ECM remodeling in carcinoma cells (36).

To establish whether the α₆ integrin is also required for angiogenesis, we used the siRNA approach to specifically knockdown α₆ integrin expression. Based on these experiments, we observed that α₆ siRNA induced a significant reduction in α₆ integrin expression in HBMECs and inhibited capillary morphogenesis onto Matrigel-coated plates as well as the VEGF-induced adhesion and migration of the cells. Thus, our results indicate a direct role of α₆ integrin in the angiogenic processes of HBMECs and suggest that the α₆ integrin induced by VEGF is a positive regulator of angiogenesis.

Next, we examined whether VEGF activates α₆ integrin in HBMECs. When HBMECs were stimulated with VEGF, cells showed increased adhesion onto Laminin-1-coated plates. The VEGF-induced adhesion of these cells was abolished by the pre-treatment with α₆ integrin antibody, indicating that α₆ integrin is activated by VEGF in HBMECs. Therefore, based on our data, we propose that VEGF can mediate angiogenesis through the

**FIGURE 5.** VEGF induces the expression and activation of α₆ integrin in HUVECs. A, Northern blot analysis. HUVECs were starved in assay medium for 4 h and then stimulated with VEGF (30 ng/ml) for the indicated times. Total RNAs (10 µg) were isolated and analyzed by Northern blotting. The 28 S RNA is shown to verify equal loading. B, adhesion assay. The suspended HUVECs were stimulated with VEGF (30 ng/ml) for 30 min and then loaded onto Laminin-1-coated plates. The antibodies were used at a concentration of 20 µg/ml. After 30 min of incubation, attached cells were stained and counted from 15 random fields of ×400 magnification. p values (*, p < 0.001) indicate significant differences between the effects of integrin antibodies compared with control IgG. Data were analyzed by the Student’s t test and are presented as the means ± S.D. of three individual studies.

**FIGURE 6.** Functional blocking of α₆ integrin suppresses VEGF-induced angiogenesis in vivo. A, photographs of skin containing Matrigel by surgical microscopy at a magnification of 10. The arrow indicates reduced blood vessels at the Matrigel surface. This experiment is representative of two individual studies (3 mice per group). B, immunohistochemistry of tumor sections (as described in A) either untreated or following treatment with α₆ integrin antibody or control antibody. The blood vessels were stained with von Willebrand factor antibodies. Vessel density was determined by counting the stained capillary blood vessels. Ten random high-power fields per 5 tumor sections were evaluated (*, p < 0.01).
increased expression and activation of $\alpha_6$ integrin in HBMECs. Notably, our in vitro studies showed that functional blocking of $\alpha_6$ integrin or $\beta_1$ integrin inhibited all stages of the angiogenic process (adhesion, migration, and capillary morphogenesis) in HBMECs, whereas the $\beta_1$ antibody had no effects. This strongly suggests that $\alpha_6$ integrin is involved in multiple angiogenic processes as is the $\alpha_5\beta_1$ heterodimer.

$\alpha_6\beta_1$ integrin has been shown to play a major role in cord formation on the basement membrane matrix (37). Our results indicate that integrin $\alpha_6\beta_1$ participates in the angiogenic process. It is possible that during the early phase of vessel sprouting, activated endothelial cells may utilize integrin $\alpha_6\beta_1$ to mediate the angiogenic response, while in the late phases of angiogenesis, maintenance of vessel integrity may occur via integrin $\alpha_6\beta_4$. The integrin $\alpha_6\beta_4$ was also shown to promote tumor angiogenesis in response to bFGF (38). Hence, integrins such as $\alpha_6\beta_4$, $\alpha_6\beta_3$, and $\alpha_6\beta_1$ may be selectively utilized to play distinct roles in vessel sprouting and maintenance during different phases of angiogenesis upon induction by various signals and/or growth factors. Of note, whereas short-term stimulation of endothelial cells by angiogenic cytokines (such as bFGF) leads to acute activation of integrins (such as $\alpha_6\beta_4$), prolonged treatment with VEGF leads to the up-regulation of integrin $\alpha_6\beta_1$.

By using the mouse Matrigel assay, we examined whether the $\alpha_6$ integrin mediates angiogenesis in vivo. To induce blood vessels in mouse skin, we utilized MDA-MB-231 breast carcinoma cells infected with the VEGF-encoding adenovirus. We found that when Matrigel containing recombinant human VEGF alone was implanted in mouse skin, newly formed blood vessels were minimal at the interface between the dermis and Matrigel, which might result in instability of the protein in vivo. To overcome this problem, we then used MDA-MB-231 cells infected with the VEGF-encoding adenovirus. These cells as well as control cells were implanted under the mouse skin, and analyzed for their potential to induce angiogenesis in vivo. It has been reported previously that when VEGF-encoding adenovirus was injected into mouse ear tissue, the virus constitutively produced VEGF over 10 days (39). Using this experimental system, we observed that unlike control MDA-MB-231 cells and MDA-MB-231 cells transduced with vector alone, the MDA-MB-231 cells transduced with VEGF-adenovirus induced significant amounts of newly formed blood vessels. Furthermore, when monoclonal antibody against $\alpha_6$ integrin was injected intraperitoneally into mice, a significant reduction in the density of blood vessels at the surface of the Matrigel producing VEGF was observed, compared with injection with control antibody.
Regulation of αv Integrin by VEGF

Thus, αv integrin directly mediates VEGF-induced angiogenesis in vivo.

VEGF has an essential role in promoting vascularization during tumor development. Moreover, inhibition of its function effectively prevents tumor growth via impaired blood vessel formation (30, 40). Thus, tumor growth is highly dependent on the ability of tumors to induce their own vascularization (41). VEGF expression has been reported in a number of cancer cell lines and in several clinical specimens derived from breast, brain, and ovarian cancers (14, 42, 43). For these reasons, it seemed probable that blocking of αv integrin would also inhibit tumor angiogenesis and growth. Consistent with this premise, administration of αv antibody to nude mice with human MDA-MB-231 breast carcinoma xenografts suppressed angiogenesis by 58% and tumor growth by 46%. The reduced blood vessel diameter and density in the tumors of nude mice receiving αv integrin-specific antibody showed that this antibody suppressed breast carcinoma growth in mice through inhibition of tumor neoangiogenesis.

We have previously reported that VEGF modulated the migration of MDA-MB-231 breast cancer cells through the regulation of brain microvascular endothelial cell permeability, and that MDA-MB-231 cells express VEGF abundantly as well as secrete VEGF (18). These results, together with the data from this study, strongly suggest that VEGF secreted from breast cancer cells can contribute breast cancer metastasis to the brain through the increased permeability of the blood-brain barrier and/or through tumor angiogenesis induced by the expression and activation of αv integrin in human brain microvascular endothelial cells.

In summary, we have demonstrated that the αv integrin participated in VEGF-driven angiogenesis in vitro and in vivo and provided insights into the critical steps that occur during angiogenesis and tumorigenesis. Additionally, our data suggest that the αv integrin might be an attractive target for therapeutic approaches in angiogenic diseases, such as neoplastic tumor growth.

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REFERENCES

1. Ferrara, N., and Davis-Smyth, T. (1997) Endocr. Rev. 18, 4–25
2. Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999) Faseb J. 13, 9–22
3. Ferrara, N. (2002) Nat. Rev. Cancer 2, 795–803
4. Hynes, R. O. (1992) Cell 69, 11–25
5. Hynes, R. O. (2002) Cell 110, 673–687
6. Clark, E. A., and Brugge, J. S. (1995) Science 268, 233–239
7. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) Annu. Rev. Cell Dev. Biol. 11, 549–599
8. Parsons, J. T. (1996) Curr. Opin. Cell Biol. 8, 146–152
9. Fassler, R., and Meyer, M. (1995) Genes Dev. 9, 1896–1908
10. Hynes, R. O., and Bader, B. L. (1997) Thromb. Haemost. 78, 83–87
11. Eliceiri, B. P., and Cheresh, D. A. (1999) J. Clin. Investig. 103, 1227–1230
12. Eliceiri, B. P., and Cheresh, D. A. (2001) Curr. Opin. Cell Biol. 13, 563–568
13. Byzova, T. V., Goldman, C. K., Pampori, N., Thomas, K. A., Bett, A., Shat, S. J., and Plo, E. F. (2000) Mol. Cell 6, 851–860
14. Senger, D. R., Ledbetter, S. R., Claffey, K. P., Papadopoulos-Sergiou, A., Peruzzi, C. A., and Detmar, M. (1996) Am. J. Pathol. 149, 293–305
15. Senger, D. R., Claffey, K. P., Benes, J. E., Peruzzi, C. A., Sergiou, A. P., and Detmar, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13612–13617
16. Senger, D. R., Peruzzi, C. A., Streit, M., Koteliansky, V. E., de Fougerolles, A. R., and Detmar, M. (2002) Am. J. Pathol. 160, 195–204
17. Lee, T. H., Avraham, H., Lee, S. H., and Avraham, S. (2002) J. Biol. Chem. 277, 10445–10451
18. Lee, T. H., Avraham, H. K., Jiang, S., and Avraham, S. (2003) J. Biol. Chem. 278, 5277–5284
19. Cress, A. E., Rabinovitz, I., Zhu, W., and Nagle, R. B. (1995) Cancer Metastasis Rev. 14, 219–228
20. Lotz, M. M., Korzelius, C. A., and Mercurio, A. M. (1990) Cell Regul. 1, 249–257
21. Georges-Labouesse, E., Messaddeq, N., Yehia, G., Gadalbert, L., Dierich, A., and Le Meur, M. (1996) Nat. Genet. 13, 370–373
22. Gambarotta, D., Marchetti, A., Benedetti, L., Mercurio, A. M., Sacchi, A., and Falconi, R. (2000) J. Biol. Chem. 275, 10604–10610
23. Trusolino, L., Bertotti, A., and Comoglio, P. M. (2001) Cell 107, 643–654
24. Colognato, H., Baron, W., Avellana-Adalid, V., Relvas, J. B., Baron-Van Evercooren, A., Georges-Labouesse, E., and French-Constant, C. (2002) Nat. Cell Biol. 4, 833–841
25. Falconi, R., Kennel, S. J., Giaconomi, P., Zupi, G., and Sacchi, A. (1986) Cancer Res. 46, 5772–5778
26. Costantini, R. M., Falconi, R., Battista, P., Zupi, G., Kennel, S. J., Colasante, A., Venturo, I., Curio, C. G., and Sacchi, A. (1990) Cancer Res. 50, 6107–6112
27. Byzova, T. V., and Plo, E. F. (1998) J. Cell Biol. 143, 2081–2092
28. Davis, T. L., Rabinovitz, I., Futscher, B. W., Schnolzer, M., Burger, F., Liu, Y., Kulesz-Martin, M., and Cress, A. E. (2001) J. Biol. Chem. 276, 26099–26106
29. Folkman, J., and D’Amore, P. A. (1996) Cell 87, 1153–1155
30. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. (1993) Nature 362, 841–844
31. Plate, K. H., Breier, G., Weich, H. A., and Risau, W. (1992) Nature 359, 845–848
32. Zhang, W., Ran, S., Sambade, M., Huang, X., and Thorpe, P. E. (2002) Angiogenesis 5, 35–44
33. Stepp, M. A., Spurr-Michaud, S., Tisdale, A., Elwell, J., and Gipson, I. K. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8970–8974
34. Klein, S., Giancotti, F. G., Presta, M., Albeda, S. M., Buck, C. A., and Rifkin, D. B. (1993) Mol. Biol. Cell 4, 973–982
35. Shaw, L. M., Rabinovitz, I., Wang, H. H., Toker, A., and Mercurio, A. M. (1997) Cell 91, 949–960
36. Rabinovitz, I., Gipson, I. K., and Mercurio, A. M. (2001) Mol. Biol. Cell 12, 4030–4043
37. Davis, G. E., and Camarillo, C. W. (1995) Exp. Cell Res. 216, 113–123
38. Nikolopoulos, S. N., Blakie, P., Yoshioka, T., Guo, W., and Giancotti, F. G. (2004) Cancer Cell 6, 471–483
39. Sundberg, C., Nagy, J. A., Brown, L. F., Feng, D., Eckelhoefer, I. A., Manseau, E. J., Dvorak, A. M., and Dvorak, H. F. (2001) Am. J. Pathol. 158, 1145–1160
40. Brekken, R. A., Overholser, J. P., Stauny, V. A., Waltenberger, J., Minna, J. D., and Thorpe, P. E. (2000) Cancer Res. 60, 5117–5124
41. Cai, J., Ahmad, S., Jiang, W. G., Huang, J., Kontos, C. D., Boulton, M., and Ahmed, A. (2003) Diabetes 52, 2959–2968
42. Brown, L. F., Olbricht, S. M., Berse, B., Jackman, R. W., Matsueda, G., Tognazzi, K. A., Manseau, E. J., Dvorak, H. F., and Van de Water, L. (1995) J. Immunol. 154, 2801–2807
43. Boocock, C. A., Charnock-Jones, D. S., Sharkey, A. M., McLaren, J., Barker, P. I., Wright, K. A., Twemlay, P. R., and Smith, S. K. (1995) J. Natl. Cancer Inst. 87, 506–516