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A Mechanism for Modulation of Cellular Responses to VEGF: Activation of the Integrins

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Summary

Many similarities exist in the cellular responses elicited by VEGF and governed by integrins. Here, we identify a basis for these interrelationships: VEGF activates integrins. VEGF enhanced cell adhesion, migration, soluble ligand binding, and adenovirus gene transfer mediated by $\alpha_v\beta_3$ and also activated other integrins, $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_2\beta_1$, involved in angiogenesis. Certain tumor cells exhibited high spontaneous adhesion and migration, which were attributable to a VEGF-dependent autocrine/paracrine activation of integrins. This activation was mediated by the VEGFR2 receptor and regulated via phosphatidylinositol-3-kinase, Akt, and the PTEN signaling axis. Thus, integrin activation provides a mechanism for VEGF to induce a broad spectrum of cellular responses.

Introduction

Growth factors initiate multiple responses, ranging from induction of cell proliferation and new gene expression to directed cell migration and modulation of cell–cell interactions. Such fundamental changes in cellular properties are often associated with and contingent upon alterations in the adhesive status of the responding cells. Underlying such changes in adhesion are alterations in specific interactions of cells with the extracellular matrix. Therefore, at a molecular level, cellular responses to growth factors are induced by occupancy of the growth factor receptors and are associated with changes in the way in which adhesion receptors on the cell surface engage the extracellular matrix.

The close relationship between occupancy of growth factor and adhesion receptors is evident in the angiogenic response of endothelial cells. Angiogenic growth factors, such as vascular endothelial cell growth factor (VEGF), induce endothelial cell proliferation, new gene expression, and directed cell migration as key steps in the formation of new blood vessels (Ferrara and Alitalo, 1999). These changes are initiated by binding of one the multiple forms of VEGF or its related molecules to members of the VEGF receptor (VEGFR) family. The importance of the VEGF/VEGFR system in angiogenesis is underscored by the lack of vascular development and early embryonic lethality in mice caused by inactivation of the genes for VEGF or its receptors (Carmeliet et al., 1996; Ferrara, 1999). At the same time, inactivation of genes for specific adhesion receptors or blockade of their functions can exert profound effects on the angiogenic response of endothelial cells (Hynes et al., 1999). Indeed, many of the responses in endothelial cells, which are triggered by occupancy of VEGFRs, are also induced upon ligation of endothelial cell adhesion receptors, in particular, members of the integrin family. The mediation of cell adhesion and migration are primary functions of integrins, and gene expression and cell proliferation are two of the consequences of integrin receptor signaling. Of the 22 integrin heterodimers, at least six, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_2\beta_1$, $\alpha_v\beta_1$, and $\alpha_1\beta_1$, have been implicated in angiogenesis (Hynes and Bader, 1997; Hynes et al., 1999). The most extensive body of evidence links $\alpha_v\beta_3$ to blood vessel formation (Eliceiri and Cheresh, 1999). In a variety of in vitro and in vivo models of angiogenesis, $\alpha_v\beta_3$ blockade with monoclonal antibodies (mAbs) or ligand antagonists has resulted in blunted blood vessel formation (Eliceiri and Cheresh, 1999). Despite these observations, vascular development appears generally normal in α_v and β_3 knockout mice (Bader et al., 1998; Hodivala-Dilke et al., 1999), and this may be explained by the functional redundancy provided by other integrins. In this regard, β_1 knockout mice fail to develop a vasculature (Fassler and Meyer, 1995).

The interplay between VEGF and integrins is not restricted to angiogenesis. VEGF and VEGFR are expressed at high levels by many tumor cell lines, elevated levels of VEGF are found in cancer patients (Ferrara, 1999), and inhibition of VEGF can suppress tumor growth (Goldman et al., 1998). Indeed, anti-VEGF is currently being explored as a cancer therapy (Ferrara and Alitalo, 1999). VEGF also has been implicated in bone development. Neutralization of VEGF with monoclonal antibodies in mice leads to inhibition of bone resorption (Gerber et al., 1999), and VEGF can support osteoclast differentiation and bone remodeling (Niida et al., 1999). At the same time, integrins play critical roles in these same processes. Changes in matrix deposition by tumor cells can be traced to changes in integrin surface expression. The metastatic potential of tumor cells is linked to their expression of integrins, particularly to $\alpha_v\beta_3$ (Seftor, 1998). $\alpha_v\beta_3$ also plays a key role in bone resorption, as demonstrated in numerous in vitro models and recently in knockout mice (McHugh et al., 2000).

Overall, the information summarized above suggests a similarity or overlap in the functional responses of cells to engagement of the VEGF/VEGFR and extracellular matrix/integrin systems during a variety of biological responses. Nevertheless, the relationships between

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these two systems are mainly correlative. Mechanisms for direct communication between VEGF and integrins remain undefined and may largely reflect a convergence of their downstream intracellular signaling pathways (Giancotti and Ruoslahti, 1999). One of the general mechanisms for regulation of integrin function involves their activation. As a consequence of such activation, integrins enhance their apparent affinity or avidity for extracellular ligands (Bazzoni and Hemler, 1998). In this study, we have investigated the hypothesis that VEGF acts as an integrin activator and, therefore, can influence physiological and pathophysiological processes, such as angiogenesis, tumor growth and metastasis, and bone development. Our results show that VEGF can induce activation of the key integrins involved in angiogenesis, thereby establishing a mechanism by which the VEGF/VEGFR system can regulate cellular adhesive and migratory responses.

Results

VEGF Regulates Ligand Recognition by $\alpha_v\beta_3$

Given the shared roles of VEGF and integrin $\alpha_v\beta_3$ in angiogenesis, the possibility that VEGF might directly activate $\alpha_v\beta_3$ was considered. The data in Figure 1 show that $VEGF_{165}$, the predominant isoform of VEGF, has a profound effect on the recognition of a series of $\alpha_v\beta_3$ ligands by human umbilical vein endothelial cells (HUVECs). Prothrombin, recently identified as an activation-dependent ligand of $\alpha_v\beta_3$ (Byzova and Plow, 1998), supported limited adhesion of nonstimulated HUVECs; however, when these cells were stimulated with as little as 10 ng/ml $VEGF_{165}$, adhesion to prothrombin increased by more than 10-fold. The increment in adhesion was similar to that induced by PMA and was inhibited by the $\alpha_v\beta_3$ blocking mAb, c7E3 (Figure 1A). A second major angiogenic growth factor, bFGF, at 10 ng/ml induced a slight but significant ($p = 0.00058$ relative to nonstimulated cells) increase in HUVEC adhesion to prothrombin, although this effect was less marked ($p = 0.014$) than that induced by $VEGF_{165}$. Both growth factors were effective at the lower rather that the higher concentration. Such effects are often observed with growth factors (Gerber et al., 1998). VEGF and $\alpha_v\beta_3$ have been implicated in bone formation and metabolism (Gerber et al., 1999; McHugh et al., 2000), and the effects of $VEGF_{165}$ stimulation on HUVEC adhesion to the bone matrix protein, bone sialoprotein (BSP) (Ross et al., 1993; Byzova et al., 2000), were assessed (Figure 1B). The spontaneous adhesion of the cells to BSP was more pronounced than with prothrombin, but stimulation with PMA still induced a substantial increase in cell adhesion. $VEGF_{165}$ also induced a similar increase in adhesion that was suppressed by c7E3 and by an antibody to BSP.

$VEGF_{165}$ also influenced the interaction of HUVECs with the prototypic ligand for $\alpha_v\beta_3$, vitronectin (Figure 1C). The extent of HUVEC adhesion to vitronectin was dependent on ligand concentration, and $VEGF_{165}$ stimulation resulted in a marked leftward shift in the dose–response curve (Figure 1C). Thus, the effects of stimulation were evident at low concentrations of vitronectin, whereas the extent of adhesion of the $VEGF_{165}$-stimulated and nonstimulated cells was similar at the highest

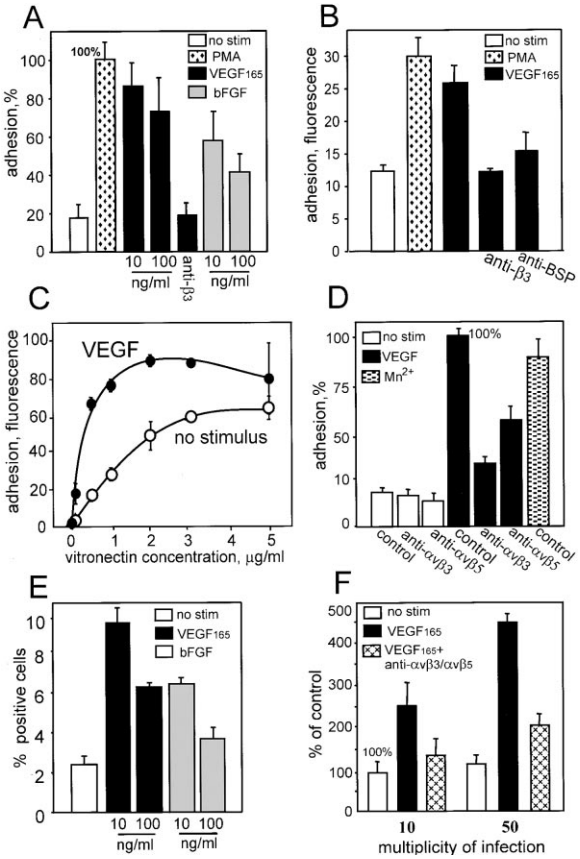

Figure 1. VEGF Activates $\alpha_v\beta_3$ on Endothelial Cells

HUVEC adhesion to (A) immobilized prothrombin (50 μg/ml), BSP (5 μg/ml) (B), or vitronectin at various concentrations (C) or vitronectin at 0.5 μg/ml (D). Cells were stimulated with 200 nM PMA, 10 and 100 ng/ml $VEGF_{165}$, 10 and 100 ng/ml bFGF, or 1 mM Mn^{2+}. Inhibitors used were as follows: c7E3 as an anti-β_3 blocking agent, an anti-BSP mAb, and P1F6 as an anti-$\alpha_v\beta_5$ mAb (20 μg/ml each). Control bars are adhesion in the presence of nonimmune IgG. In (A) and (D), adhesion of PMA- and VEGF-stimulated cells was assigned a value of 100%. (E) shows results of specific binding of WOW-1 Fab to nonstimulated cells and to cells stimulated with $VEGF_{165}$ or bFGF. (F) shows the effect of $VEGF_{165}$ on integrin-mediated gene transfer to HUVECs. Cells were pretreated with anti-$\alpha_v\beta_3$ and $\alpha_v\beta_5$ antibodies (20 μg/ml each), stimulated with 20 ng/ml $VEGF_{165}$ for 5 min or remained unstimulated, and the selected amounts of GFP adenovirus were added. After 24 hr, cells were analyzed by FACS. The MFI of unstimulated cells was assigned a value of 100%. The data shown are the means ± SD of three separate experiments.

ligand concentration. The marked increase in HUVEC adhesion to a low concentration of vitronectin (0.5 μg/ml) induced by $VEGF_{165}$ was similar to that induced by Mn^{2+} (Figure 1D). In addition, $VEGF_{165}$ stimulation also may activate $\alpha_v\beta_5$; mAb P1F6 to this integrin, as well as c7E3 to β_3, partially inhibited the $VEGF_{165}$-enhanced adhesion of the cells to vitronectin.

Recently, the penton-based protein of adenovirus was identified as an activation-dependent ligand for $\alpha_v\beta_3$. When its RGD-containing segment was engineered into the H-CDR3 of a mAb to create WOW-1, the resulting Fab fragment reacted selectively with activated $\alpha_v\beta_3$ (Pampori et al., 1999). As monitored by FACS (Figure 1E), $VEGF_{165}$ induced specific binding of WOW-1 Fab to

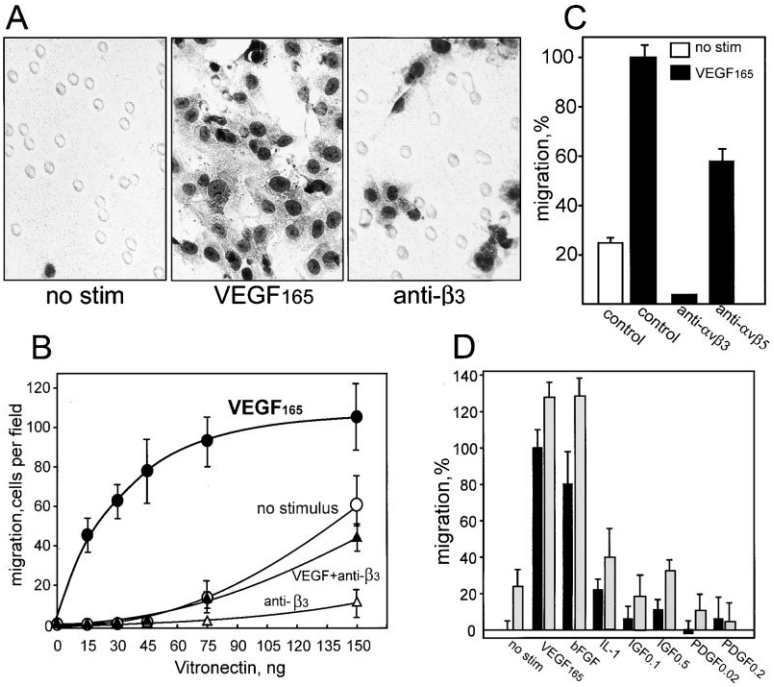

Figure 2. VEGF Enhances $\alpha_v\beta_3$-Mediated Cell Migration

(A) Micrographs of migration of nonstimulated or $VEGF_{165}$ (20 ng/ml) stimulated HUVECs. Inhibition of $VEGF_{165}$-stimulated migration by anti-β_3 antibody c7E3 is also shown.

(B) Cell migration as a function of vitronectin concentration in the absence or presence of $VEGF_{165}$ (20 ng/ml).

(C) $VEGF_{165}$-induced migration to BSP (35 ng/well).

(D) Cell migration stimulated by PDGF (0.2 and 0.02 μg/ml), IGF (0.5 and 0.1 μg/ml), IL-1 (0.1 μg/ml), bFGF (0.02 μg/ml), or $VEGF_{165}$ (0.02 μg/ml). The data shown were derived at two different concentrations of vitronectin (35 [black bars] and 75 [gray bars] ng/well), but similar results were observed at lower and higher concentrations of the ligand (15 to 100 ng/well). The data are means ± SD of three separate experiments. Migration stimulated by $VEGF_{165}$ was assigned a value of 100%.

HUVECs. The increase in WOW-1 Fab binding was similar to that induced by Mn^{2+} (data not shown), an activator of integrins. As WOW-1 Fab is a monomeric and soluble ligand, these results establish that activation of $\alpha_v\beta_3$ by $VEGF_{165}$ can arise as a consequence of affinity modulation of this integrin. bFGF also induced a significant ($p = 0.004$) increase in WOW-1 Fab binding, but this effect was less marked than with $VEGF_{165}$.

These results suggested a practical implication of $VEGF_{165}$ activation of $\alpha_v\beta_3$. Since adenovirus entry into cells depends upon interaction with $\alpha_v\beta_3$ (Wickham et al., 1993), $VEGF_{165}$ might be useful for enhancing the efficiency of adenovirus-mediated gene transfer. To test this possibility, an adenovirus GFP construct was used to infect a HUVEC monolayer in the presence or absence of $VEGF_{165}$, and FACS was used to quantify the extent of GFP gene transfer. As shown in Figure 1F, $VEGF_{165}$ significantly enhanced the extent of gene transfer at both a lower (10) and higher (50) multiplicity of adenovirus infection. At the higher dose of adenovirus, the increase in infection induced by $VEGF_{165}$ was 3.6-fold. For comparison, treatment of HUVEC monolayers with 1 mM Mn^{2+} to activate $\alpha_v\beta_3$ increased infection by 4- to 5-fold. The increase induced by $VEGF_{165}$ was inhibited substantially by $\alpha_v\beta_3 + \alpha_v\beta_5$ blocking antibodies (Figure 1F).

VEGF Enhances $\alpha_v\beta_3$-Mediated Cell Migration

Cell migration is an essential step in the angiogenic response, and $\alpha_v\beta_3$ is an important mediator of endothelial cell migration (Eliceiri and Cheresh, 1999). Accordingly, the effect of $VEGF_{165}$ on HUVEC migration mediated by $\alpha_v\beta_3$ was assessed. In a transwell system, $VEGF_{165}$ exerted a profound effect on cell migration to vitronectin. As shown in Figure 2A (left), while few cells (1–3 per field) migrated onto the substrate during 4 hr in the absence of $VEGF_{165}$, numerous HUVECs migrated in its presence (Figure 2A, middle). This migration was inhibited substantially by mAb c7E3 (Figure 2A, right). The quantitative aspects of $VEGF_{165}$ stimulation of HUVEC migration were explored by varying the coating concentration of the vitronectin substrate. $VEGF_{165}$ stimulation enhanced migration, particularly at the lower concentrations of vitronectin (Figure 2B). Spontaneous migration of HUVECs to vitronectin was noted, but only at higher concentrations of the substrate. Since migration occurs over a relatively long time course, we considered whether the effects of $VEGF_{165}$ might depend upon changes in $\alpha_v\beta_3$ expression levels. Using LM609 to quantify $\alpha_v\beta_3$ by FACS, a 5 hr incubation of the cells with $VEGF_{165}$ in the same transwell system had no effect on expression levels (MFI of the cells at time 0 was 55.4 ± 4.5, at 1 hr was 56 ± 9, and at 5 hr was 55.5 ± 2.1). We also considered whether the VEGF effects on cell migration might simply reflect increased adhesion of HUVECs to the undersides of the filters. HUVECs were labeled with ^{51}Cr, and the radioactivity was used to quantitate the cells on the filters and in the lower chambers. Under the conditions used, very little radioactivity was recovered from the lower chamber, with and without $VEGF_{165}$, even with incubation times up to 6 hr. The marked influence of $VEGF_{165}$ on cell migration was demonstrable with a second $\alpha_v\beta_3$ ligand. When HUVECs were provided with a BSP substrate, a substantial increase in HUVEC migration was observed when the cells were stimulated with $VEGF_{165}$ (Figure 2C). c7E3 markedly inhibited this response, whereas P1F6 to $\alpha_v\beta_5$ was less effective.

To determine whether the effects of $VEGF_{165}$ stimulation were specific, we tested the ability of selected relevant growth factors to influence HUVEC migration to vitronectin. PGDF and IGF receptors associate with integrins (Schneller et al., 1997; Woodard et al., 1998), but neither of these growth factors at optimal concentra-

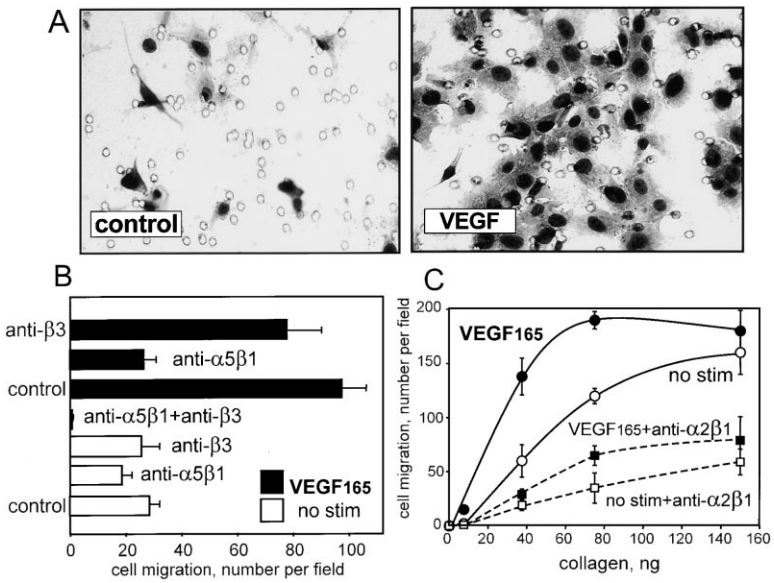

Figure 3. VEGF Activates β_1 Integrins

Migration to fibronectin (50 ng/well) (A and B) or various concentrations of type I collagen (C). Cells were stimulated with 20 ng/ml $VEGF_{165}$ or remained unstimulated. c7E3 was used as a β_3-specific mAb, JBS5 as an $\alpha_5\beta_1$ mAb, and BHA2.1 as an $\alpha_2\beta_1$ mAb (20 μg/ml each). In (B) and (C), migration was quantified by performing microscopic counts of 10–12 random fields at 200× power. The data shown are means ± SD of three separate experiments.

tions was as effective as $VEGF_{165}$ in enhancing cell migration on vitronectin (Figure 2D). The differential activities of $VEGF_{165}$ versus PGDF and IGF were observed with a second $\alpha_V\beta_3$ ligand, BSP (data not shown). The differential activities of the growth factors on cell migration were corroborated using ^{51}Cr-labeled HUVECs to verify that the cells adhered to the filter rather than migrated through. Interestingly, although bFGF was less effective than $VEGF_{165}$ in inducing adhesion to prothrombin and the WOW-1 response (Figure 1), bFGF did augment cell migration onto vitronectin, almost as effectively as $VEGF_{165}$. The bFGF-stimulated migration also was inhibited by c7E3 (data not shown). Thus, both major angiogenic growth factors enhanced HUVEC migration in an $\alpha_V\beta_3$-dependent fashion.

VEGF Activates β_1 Integrins

The data in Figure 1D suggested that a second α_V integrin, $\alpha_V\beta_5$, which has been implicated in angiogenesis, may also be activated by $VEGF_{165}$. Therefore, we sought to determine if still other integrins implicated in angiogenesis, $\alpha_5\beta_1$, a fibronectin receptor (Bloch et al., 1997), and $\alpha_2\beta_1$, a collagen receptor (Senger et al., 1997), might be activated by $VEGF_{165}$. In cell migration assays, $VEGF_{165}$ markedly stimulated HUVEC movement onto fibronectin (see Figure 3A). The migrating cells exhibited extensive spreading, much more so than for their $\alpha_V\beta_3$-mediated migration onto vitronectin (see Figure 2A). As shown in Figure 3B, $VEGF_{165}$-enhanced migration was only partly dependent upon $\alpha_V\beta_3$ activation (partial inhibition by c7E3) and partly dependent upon $\alpha_5\beta_1$; mAb JBS5 to $\alpha_5\beta_1$ produced marked inhibition. The combination of mAbs c7E3 and JBS5 produced full inhibition of cell migration. Thus, integrin $\alpha_5\beta_1$ also can be activated when HUVECs are stimulated with $VEGF_{165}$. A similar conclusion was reached with regard to $VEGF_{165}$ activation of integrin $\alpha_2\beta_1$ using Type I collagen as a matrix (Figure 3C). The role of $\alpha_2\beta_1$ in this migration was confirmed using blocking mAb BHA2.1. Taken together, these data provide evidence that the $VEGF_{165}$ can activate the major integrins, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_5\beta_1$, and $\alpha_2\beta_1$, implicated in angiogenesis.

Identity of the VEGF Receptors Involved in Integrin Activation

Two major VEGF receptors, VEGFR1 and VEGFR2, are expressed by endothelial cells, and $VEGF_{165}$ is a ligand for both (Ferrara and Alitalo, 1999). By FACS and Western blot analysis, both $VEGF_{165}$ receptors are present at similar levels on the HUVECs used in our studies (data not shown). To determine which VEGFR is responsible for integrin activation, ligands selective for VEGFR1 and VEGFR2 were tested for their effects on $\alpha_V\beta_3$ activation. VEGF-D is a ligand specific for VEGFR2, whereas placental growth factor (PlGF) interacts selectively with VEGFR1 (Ferrara, 1999). As shown in Figure 4A, both of these ligands stimulated [^{3}H]thymidine uptake by the HUVECs to a similar extent, indicating that both VEGFR1 and VEGFR2 are functional on the cells. The effects of these two ligands on HUVEC migration to a low concentration of vitronectin (30 ng/well) are shown in Figure 4B. VEGF-D enhanced HUVEC migration at concentration as low as 0.1 μg/ml, and a maximal effect was observed at 1 μg/ml. At high concentrations of VEGF-D, 10 μg/ml, the migratory response was slightly blunted, an effect often observed with VEGFR2 ligation (Gerber et al., 1998). In contrast, PlGF induced minimal stimulation of HUVEC migration at concentrations, which stimulated a proliferative response.

To verify the role of VEGFR2 in integrin activation, this receptor was expressed in GM1500 B-lymphoblastoid cells. The $\alpha_V\beta_3$ in these cells is normally expressed in a low-affinity state and can be activated by stimulation of the cells with PMA (Bennett et al., 1997). Using a transient transfection system, ~10%–15% of the transfected GM 1500 cells expressed VEGFR2 by FACS (data not shown). When the cells were stimulated with $VEGF_{165}$, the binding of WOW-1 Fab increased markedly compared to the nonstimulated cells (Figure 4C), whereas $VEGF_{165}$ had no effect on WOW-1 binding to mock-transfected

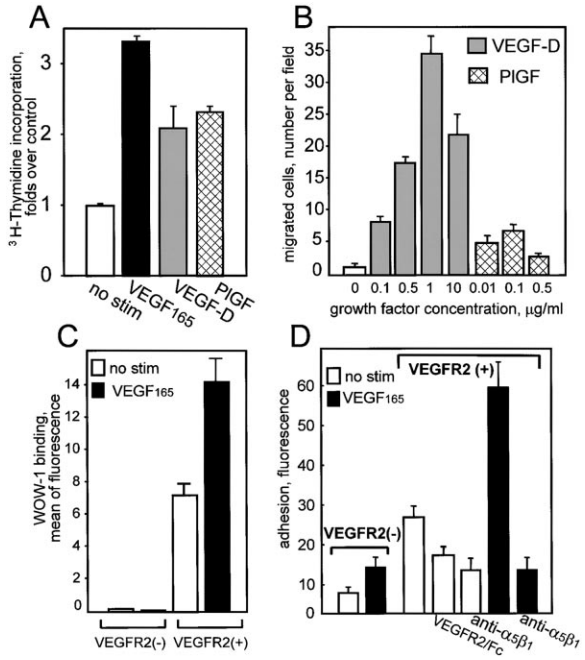

Figure 4. Identification of the VEGF Receptor on Endothelial Cells Involved in $\alpha_v\beta_3$ Activation

(A) HUVEC proliferation in response to $VEGF_{165}$ (20 ng/ml), VEGF-D (100 ng/ml), and PlGF (100 ng/ml). Means ± SD of three separate experiments.

(B) Migration of VEGF-D- or PlGF-stimulated HUVECs to vitronectin (100 ng/well). Assays were performed as described in Figure 2.

(C) Control or VEGFGR2 transfected GM1500 cells were stimulated with $VEGF_{165}$ or remained unstimulated. WOW-1 binding was measured as described in Experimental Procedures.

(D) Adhesion of nontransfected [VEGFR2(−)]or VEGFR2 transfected K562 cells to fibronectin (10 μg/ml) was stimulated with $VEGF_{165}$ (50 ng/ml). Nonspecific adhesion to BSA-coated wells was subtracted. The data are means ± SD of quadruplicates in one experiment and are representative of three separate experiments.

cells. In Figure 4C, the VEGFR2 transfectants showed substantial WOW-1 expression even in the absence of added $VEGF_{165}$. We attribute this to the availability of VEGF, either synthesized by the cells or in the culture medium. Indeed, when anti-VEGF agents were added to these cells, the WOW-1 expression decreased by 1.5- to 2-fold. Communication between VEGFR2 and β_1 integrins also was demonstrable. K562 erythroblastoid cells express low levels of VEGFR2, as detected by FACS analyses, and this level was enhanced ~4-fold by transfection of these cells with cDNA for VEGFR2. Stably transfected cells exhibited a dramatic increase in adhesion to fibronectin, and this adhesion was inhibited by preincubation of these cells with VEGFR2/Fc chimera (Figure 4D), which serves to neutralize endogenous $VEGF_{165}$. The adhesion of these cells was almost entirely dependent upon $\alpha_5\beta_1$ (see Figure 4D).

Tumor Cells Express a VEGF-Dependent Autocrine Loop for Integrin Activation

Certain tumor cell lines, exemplified by M21 melanoma cells, exhibit high spontaneous adhesion to $\alpha_v\beta_3$ ligands, and the extent of adhesion is not augmented by PMA stimulation (Pampori et al., 1999; Byzova et al., 2000). Since many tumors synthesize VEGF, we reasoned that the high constitutive activity of $\alpha_v\beta_3$ on these cells might be a consequence of VEGF stimulation via an autocrine/paracrine mechanism. To test this, the effects of VEGF antagonists on the adhesion of the M21 cells to BSP was assessed. As shown in Figures 5A and 5B, the M21 cells exhibited robust adhesion to BSP, and this spontaneous adhesion was mediated by $\alpha_v\beta_3$. Two approaches were utilized to evaluate the role of endogenous VEGF in this adhesion: VEGF-neutralizing antibodies and the chimeric soluble VEGF receptor, VEGFR2/Fc. As shown in Figure 5B, each of these reagents antagonized the adhesion of these cells to BSP to a level similar to that obtained with c7E3. In contrast, nonimmune rabbit antibodies and irrelevant protein additions had no effect on cell adhesion. The effects of the same two VEGF antagonists on migration of the M21 cells onto BSP are shown in Figure 5C. The extent of inhibition was 83% for anti-VEGF and 71% for the VEGFR2/Fc chimera.

An additional approach verified the role of VEGF in the high spontaneous migration of M21 melanoma cells. An adenovirus vector system was used to infect the M21 cells with cDNA encoding a secretable and soluble form of VEGFR1 (s-flt-1). S-flt-1 retains a high affinity for VEGF and been utilized to suppress VEGF-dependent tumor growth in vitro and in vivo (Goldman et al., 1998). As a control, the same adenovirus vector containing a construct for GFP was used to infect the cells. As shown in Figures 6A and 6B, infection of the M21 cells with the GFP virus had no effect on the migration of the cells onto BSP; the spontaneous migration remained high and was inhibited by c7E3. In contrast, cells infected with the sVEGFR1 virus failed to migrate; the reduction in migration produced by the sVEGFR1 virus was similar to that produced by c7E3. Similar results were obtained with a second $\alpha_v\beta_3$ substrate, vitronectin, and with a second integrin, $\alpha_5\beta_1$ (Figure 6D).

Mechanism of Integrin Activation by VEGF

The high spontaneous adhesion and migration of tumor cells to BSP could be suppressed by inhibitors of specific intracellular signaling molecules, calpain by calpeptin, PI 3-kinase by wortmannin, and Src family kinases by geldanamycin, but not by PD98059, an inhibitor of ERK 1/2 (Byzova et al., 2000). Therefore, we predicted that these same inhibitors should block VEGF-mediated activation of $\alpha_v\beta_3$, and this prediction was verified using HUVECs. $VEGF_{165}$ stimulation of adhesion and migration of HUVECs to BSP and vitronectin was suppressed by the same set of inhibitors that blocked the spontaneous adhesive reaction of the tumor cells. As specific examples, the increase in migration of HUVECs onto vitronectin stimulated by $VEGF_{165}$ was inhibited completely (>99%) by calpeptin (100 μg/ml) and wortmannin (20 nM), but only ~20% by PD98059 (10 μM).

The inhibition by wortmannin suggested a prominent role for PI 3-kinase and its 3-phosphoinositide lipid products in the activation of $\alpha_v\beta_3$ by $VEGF_{165}$. To assess this, we focused on Akt, a downstream effector of PI 3-kinase, and PTEN, a lipid phosphatase that removes 3-phosphoinositides, thereby reversing the effects of PI

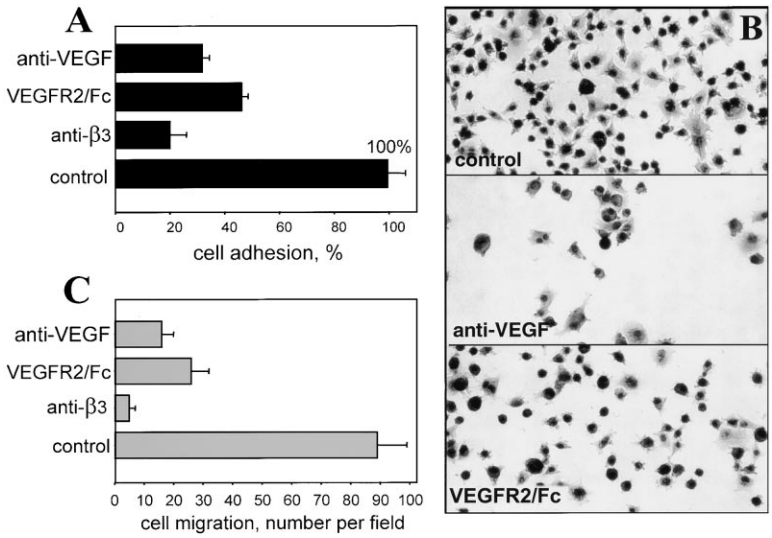

Figure 5. Evidence for a VEGF-Dependent Autocrine Loop on Tumor Cells

(A) and (B) show adhesion of M21 melanoma cells to BSP (5 μg/ml). Cells were harvested, preincubated with c7E3 (anti-β_3), VEGF antibodies, or control IgG (20 μg/ml each) for 30 min and added into wells. After 30–40 min, adhesion was measured.

(A) The data are means ± SD of three separate experiments.

(B) Micrographs (100× power) of cells adherent, fixed, and stained with hematoxylin. Inhibition by anti-VEGF was 78% and by VEGFR2/Fc, 52%.

(C) The effect of anti-VEGF agents on M21 cell migration to BSP (50 ng/well). The control is migration in the presence of nonimmune rabbit serum. The data are means ± SD of triplicates in one experiment and are representative of three separate experiments.

3-kinase (Stambolic et al., 1998). M21 cells were transfected either with the cDNAs for a kinase-inactive form of Akt or for PTEN, stable cell lines were established, and the capacity of $VEGF_{165}$ to activate $\alpha_v\beta_3$ in these cells was examined. Overexpression of PTEN suppressed the proliferative response of the transfected cells to $VEGF_{165}$ or to serum (Figure 7A). The increase in [^{3}H]thymidine incorporation induced by $VEGF_{165}$ was suppressed by anti-VEGFR2. Western blot analyses of the PTEN transfectants showed about a 4-fold increase in PTEN levels relative to control cells tranfected with the same vector expressing GFP instead of PTEN (data not shown). As shown in Figure 7B, overexpression of PTEN or Akt (kin-) mutant markedly suppressed $VEGF_{165}$-induced activation of $\alpha_v\beta_3$, as monitored by WOW-1 Fab binding. In addition, as shown in Figure 7C, the migration of the PTEN transfected cells to BSP also was suppressed; even at BSP concentrations of 100 ng/well, the PTEN transfected M21 cells showed limited migration. Similar results were obtained with M21 cells transiently transfected with a bicistronic vector expressing PTEN and GFP. When these cells were analyzed for $\alpha_v\beta_3$ activation using WOW-1 Fab, PTEN in a sense but not an antisense orientation markedly suppressed WOW-1 binding (Figure 7D). This is seen by comparing either the MFI or the GeoMFI from FACS analyses. Taken together, these data provide clear evidence for the role of PI 3-kinase and its lipid products in $\alpha_v\beta_3$ activation, thereby identifying important participants in the integrin activation pathway by VEGF.

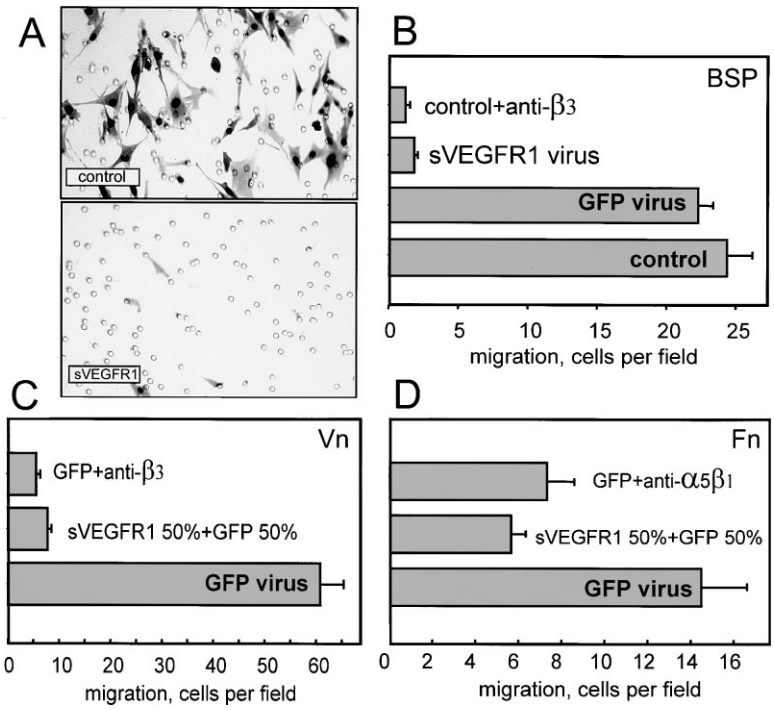

Figure 6. sVEGFR1 Adenovirus Infection "Inactivates" Integrins on M21 Cells

M21 cells were infected with GFP or sVEGFR1 adenovirus at a multiplicity of 300 or remained untreated (control). Twenty hours after infection, cells were harvested, and migration experiments were performed as in Figure 2.

(A) Micrographs of cells migrated onto BSP (50 ng/well) at 200× power.

(B) The quantification of cell migration. Anti-β_3 mAb c7E3 is used at 20 μg/ml.

(C and D) Migration of M21 cells to vitronectin (75 ng/well) and fibronectin (50 ng/well), respectively. Cells were infected with control (GFP) or with 50% of control and 50% of sVEGFR1 adenoviruses. The data are means ± SD of quadruplicates in one experiment and are representative of five separate experiments.

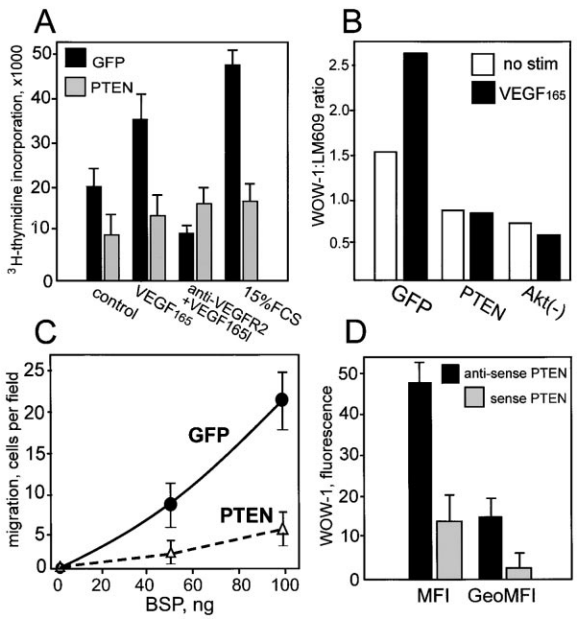

Figure 7. PTEN and Akt Regulate the Function of $\alpha_v\beta_3$ Integrin

(A) The effect of PTEN overexpression on proliferation of M21 cells. Cells were transfected with PcDNA3-PTEN or PcDNA3-GFP. $VEGF_{165}$ (20 ng/ml) or fetal calf serum (FCS, 15%) were used as stimulators of proliferation. The blocking VEGFR2 antibody was added at 20 μg/ml prior to $VEGF_{165}$.

(B) Activation of $\alpha_v\beta_3$ in GFP, PTEN, and Akt (kin-) transfected cells. Samples were incubated in parallel with anti-$\alpha_v\beta_3$ antibodies LM609 (15 μg/ml) and WOW-1 Fab (30 μg/ml), and then with R-phycoerythrin-labeled second antibodies. MFI in the presence of only the second antibody was subtracted from total MFI for each sample. The ratio of MFI of cells stained with WOW-1 Fab and LM609 is shown.

(C) Migration of GFP and PTEN transfected cells to BSP.

(D) Effect of transfection with a bicistronic vector containing GFP and PTEN, either in a sense or antisense orientation, on WOW-1 Fab binding. The mean fluorescence intensity (MFI) and geometric mean of fluorescence intensity (GeoMFI) are shown. The cells were analyzed by FACS as described in the Experimental Procedures.

Discussion

Of the numerous growth factors, VEGF is particularly adept in eliciting an extremely broad spectrum of physiological and pathophysiological responses (Ferrara, 1999). Our studies suggest a specific molecular mechanism by which VEGF may exert many of its diverse functions, i.e., by activation of the ligand binding activities of integrins. This direct modulation of integrin function provides a potential explanation for the many parallelisms between cellular behaviors induced by VEGF and involving integrins. Based upon our data, we must now consider that these correlations may be a direct consequence of the communication between VEGF and integrins: VEGF functions as the inducer and activated integrins as efficient effectors of cellular responses.

The major findings of our studies are as follows. First, $VEGF_{165}$ can activate integrin $\alpha_v\beta_3$, leading to enhanced adhesion of endothelial cells to a variety of ligands. Among a panel of tested growth factors and cytokines, $VEGF_{165}$ was the most efficient in activating integrin $\alpha_v\beta_3$. Second, such activation appears to be the result of affinity modulation because $VEGF_{165}$-stimulated cells acquired the capacity to bind the soluble monovalent ligand-mimetic anti-$\alpha_v\beta_3$ antibody, WOW-1 Fab. Third, one of the intriguing and potentially practical implications of the VEGF–integrin link is the possibility of enhancing the efficiency or targeting of adenovirus-mediated gene transfer to tumor cells or vascular cells that coexpress VEGFR2 and $\alpha_v\beta_3$. Fourth, a particularly relevant functional consequence of $VEGF_{165}$ activation of $\alpha_v\beta_3$ is the augmentation of cell migration. Fifth, $VEGF_{165}$ activation is not targeted to a single integrin; β_1 integrins, as exemplified by $\alpha_5\beta_1$ and $\alpha_2\beta_1$, also are affected by $VEGF_{165}$ stimulation. In addition, our data suggest that $\alpha_v\beta_5$ can be activated by the growth factor. Sixth, based upon the activity of different forms of VEGF ligands, VEGFR2 but not VEGFR1 ligation was capable of activating integrins, and this conclusion was confirmed by overexpression of VEGFR2 in cells. Seventh, the adhesive and migratory phenotype of certain tumor cells can be traced to their expression of constitutively active integrins, and this state is sustained by an autocrine loop initiated by VEGF. Eighth, the signaling pathway from VEGFR to integrin activation involves PI3-kinase and Akt and is negatively regulated by PTEN.

Activation of $\alpha_v\beta_3$ in HUVECs was induced by $VEGF_{165}$; adhesion was enhanced to ligands that react poorly with $\alpha_v\beta_3$ unless the integrin is activated. Even ligands such as vitronectin, which can react well with the low-affinity form of $\alpha_v\beta_3$, can support adhesion at much lower ligand concentrations upon cell activation with VEGF. Thus, activation of $\alpha_v\beta_3$ by VEGF increases the affinity of the receptor for all ligands tested, and it is their relative affinity for the receptor in its basal state that may vary. Affinity modulation by $VEGF_{165}$ was directly demonstrated as stimulated HUVECs bound WOW-1 Fab. Integrin activation is often dependent upon a combination of affinity and avidity modulation (Bazzoni and Hemler, 1998) and VEGF may increase receptor clustering as well. However, increases in integrin activity were not dependent upon increases in the levels of $\alpha_v\beta_3$ expressed at the cell surface. We found no evidence for such changes during the 4–5 hr incubations in cell migration assays. Senger et al. (1996) have reported increased β_3 mRNA levels upon VEGF stimulation, but such changes required 24–72 hr.

Cell migration, a vital step in the angiogenic response, also was enhanced substantially by $VEGF_{165}$ stimulation. Of note, while bFGF was not as effective as $VEGF_{165}$ in inducing $\alpha_v\beta_3$-dependent HUVEC adhesion and WOW-1 expression, it did increase these responses somewhat and was as effective as $VEGF_{165}$ in inducing the cell migration mediated by this integrin. Indeed, bFGF-induced angiogenesis has been shown to be particularly dependent upon $\alpha_v\beta_3$ (Eliceiri and Cheresh, 1999), and the effects of bFGF on $\alpha_v\beta_3$-dependent cell migration may be the basis for this relationship. Thus, both of the major angiogenic growth factors can enhance specific $\alpha_v\beta_3$-dependent functions. The differential effects of $VEGF_{165}$ and bFGF on $\alpha_v\beta_3$-mediated functions are complex, and detailed comparative studies will be necessary to determine whether bFGF can directly activate integrins.

Of note, our data suggest that $VEGF_{165}$ can activate

several integrins implicated in angiogenesis, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_2\beta_1$. Thus, the functional redundancy among integrins in angiogenesis may reside in their capacity to be activated to enhance their recognition of available ligands within a particular microenvironment. Similarly, the constitutive activity of integrins on certain tumor cell lines, induced by a VEGF-dependent autocrine loop, was not restricted to $\alpha_v\beta_3$. The constitutive activity of integrins on tumor cell lines can be suppressed by neutralization of VEGF, either using anti-VEGF or a high-affinity soluble form of a VEGFR1 or by cell-penetrating inhibitors of specific intracellular signaling molecules (Byzova et al., 2000). These same inhibitors also block $\alpha_v\beta_3$ activation by $VEGF_{165}$ in HUVECs. Among these, wortmannin, a specific inhibitor of PI 3-kinase, was particularly effective. Numerous studies have implicated PI 3-kinase in the activation of other integrins (Giancotti and Ruoslahti, 1999). Since PI 3-kinase activation is an early downstream event in the signaling from occupied VEGFR (Gerber et al., 1998), we focused on this axis for further exploration. Akt is a downstream effector of PI 3-kinase; conversely, PTEN inhibits the cellular effects of PI 3-kinase by virtue of its phosphatidylinositol 3-phosphatase activity (Stambolic et al., 1998). Very recent studies have suggested possible connections between Akt, PTEN, and integrin biology. For example, integrin-linked kinase (ILK) activation is inhibited by blockade of Akt function (Persad et al., 2000), and PTEN interacts with focal adhesion kinase (FAK) and influences cell migration (Tamura et al., 1999). These interactions affect cell survival, an event influenced by both VEGF and integrins. Also, cell migration and angiogenesis are regulated by Akt and PTEN, and these cellular responses are clearly mediated by integrins (Jiang et al., 2000). By overexpression approaches, we have directly implicated Akt and PTEN in the pathway from VEGF to integrin activation. Thus, some of the functions of PI 3-kinase, Akt, and PTEN in cell migration may depend upon their role in integrin activation, and engagement of VEGFR2 initiates this process. Occupancy of VEGFR1 was not effective in eliciting $\alpha_v\beta_3$ activation. Differences in the functional responses elicited by these two major VEGF receptors have been previous described (Gerber et al., 1998). However, we do not exclude that VEGFR1 could also activate integrins under different experimental conditions.

Several studies have demonstrated physical or functional relationships between integrins and growth factor receptors. The PGDF receptor coimmunoprecipitates with $\alpha_v\beta_3$ (Schneller et al., 1997), and ligation of the IGF receptor leads to its association with integrins (Jones et al., 1996). Nevertheless, neither of these growth factors activated $\alpha_v\beta_3$ in a manner analogous to VEGF. Particularly relevant to this study is the recent demonstration that VEGF and $\alpha_v\beta_3$ coimmunoprecipitate from endothelial cells (Soldi et al., 1999). Coassociations between other receptors, for example, the urokinase receptor and integrins, alter integrin functions (Eliceiri and Cheresh, 1999) and complex formation provides one mechanism by which VEGFR2 could activate the angiogenic integrins. However, the suppression of the VEGF-induced autocrine loop of integrin activation in M21 cells by intracellular antagonists and the effects of overexpression of PTEN and the kinase-dead mutant of Akt indicate that signaling events are required for communication between the two receptor systems. Many of the signaling molecules and events induced by VEGFR ligation and associated with integrin activation and ligation are shared (Giancotti and Ruoslahti, 1999). The present study provides ready functional readouts, adhesion, migration, and integrin affinity modulation that will allow for fuller delineation of this activation pathway.

Experimental Procedures

Cell Adhesion Assays

These were performed as previously described (Byzova and Plow, 1998). HUVECs and M21 melanoma cells were harvested and labeled with Calcein AM (Molecular Probes, Eugene, OR). Cells were preincubated with or without blocking antibodies or RGD peptides, and then stimulated with $VEGF_{165}$ or other selected growth factors. The cell suspensions were then added immediately to the ligand-coated wells. At selected times (40–60 min), the wells were gently washed three times with DMEM/F12 by inversion of the plates. Adherent cells were quantitated in a Fluorescence Multi-Well Plate Reader (PerSeptive Biosystems, Framingham, MA) and examined microscopically.

Cell Migration Assays

Cell migration assays were performed as previously described (Byzova et al., 2000) using Transwell plates (8 μm pore size). Ligand was diluted to a selected concentration, and 10 μl of this solution was placed on the lower surface of a polycarbonate filter and air-dried. Migration was quantified by performing microscopic cell counts at 200 power on 10–12 random fields in each well. Alternatively, 2×10^7 cells were labeled with ^{51}Cr (1 μCi), washed by centrifugation, and used in migration assays. At the end of incubation, the media from the lower chamber and the membrane were collected, the chamber was rinsed with SDS, which was also used to lyse the cells, and the radioactivity was quantified in β counter.

FACS Analysis

The binding of WOW-1 Fab was assessed by FACS as previously described (Pampori et al., 1999). Cells were incubated for 5 min in the absence of an agonist or stimulated with 10–100 ng/ml $VEGF_{165}$ or bFGF. WOW-1 Fab was then added at a final concentration of 30 μg/ml, followed by addition of R-phycoerythrin-goat anti-mouse IgG (Molecular Probes) at 15 μg/ml. After 30 min, the cells were washed and analyzed by FACS. Specific binding of WOW-1 Fab was defined as that inhibitable by 10 mM EDTA. To analyze cell surface integrin expression, two protocols were used. In the first protocol, HUVECs were incubated with LM609 (15 μg/ml) or with control mouse IgG for 60 min on ice. The cells were washed by centrifugation, incubated with Alexa 488- or R-phycoerythrin-goat anti-mouse IgG, and then analyzed by FACS. Alternatively, HUVECs were recovered at selected time points from the transwell chambers used in migration assays, fixed with 4% paraformaldehyde, and then subjected to the same staining protocol. FACS was performed using a FACScan instrument, and the data were analyzed using the CellQuest software program (version 1.2).

HUVEC Proliferation Assays

These assays were performed as described (Gardiner and D'Souza, 1997). Briefly, cells were maintained in 1% serum for 20 hr prior to experiments. Trypsinized HUVECs were distributed at 2×10^5 into 96-well microtiter plates in the presence or absence of growth factors. The cells were labeled with 1 μCi [^{3}H]thymidine per well. After 24–48 hr, the cells were washed, and the radioactivity was precipitated with TCA and quantitated by scintillation.

Adenoviral Gene Transfer into HUVEC Monolayers and M21 Cells

The recombinant adenovirus expressing green fluorescent protein (GFP) has been previously described (de Martin et al., 1997). Adenovirus encoding soluble form of VEGFR1 (sVEGFR1) was generated using standard recombinant DNA techniques for constructing ade-

novirus vectors (Hitt et al., 1995). HUVECs were cultured overnight in plates, coated with 0.1% gelatin in DMEM/F12, containing 1% serum. M21 cells were seeded into 100 cm^2 dishes. HUVECs were pretreated with $\alpha_v\beta_3$ (LM609) and $\alpha_v\beta_5$ (P1F6) mAbs (20 μg/ml each) and stimulated by $VEGF_{165}$ (20 ng/ml) for 5 min. Cells were rinsed twice with DMEM/F12 and selected amounts of adenovirus expressing GFP or sVEGFR1 were added at a multiplicity of infection of 10, 50, 100, or 300 in DMEM/F12 without serum. After 60–90 min, wells were washed and media with 10% FCS was added. After 24 or 48 hr, HUVECs were analyzed microscopically and by FACS.

VEGF-D

The cDNA encoding the VEGF-D homology domain was kindly provided by Dr. Achen (Royal Melbourne Hospital, Australia). Recombinant FLAG tag VEGF-D baculovirus was generated according to the manufacturer's instructions (Clontech, Palo Alto, CA). The expressed protein was purified using the FLAG M1 antibody affinity column. In some migration experiments, VEGF-D from R&D Systems (Minneapolis, MN) was utilized and yielded similar results.

Expression of VEGFR2 in GM1500 and K562 Cells

The cDNA encoding human VEGFR2, inserted into the pCR 3 plasmid, was kindly provided by Dr. Cam Patterson (University of Texas, Galveston, TX). The pCR 3 vector was introduced into GM1500 and K562 cells using GenePorter (Gene Therapy Systems Inc.). The expression of VEGFR2 was analyzed by FACS and Western blot using anti-VEGFR2 polyclonal antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) or from Sigma Chemical Co. (St. Louis, MO). GM1500 lymphocytes expressing VEGFR2 were utilized 40 hr after transfection for binding studies with WOW-1 Fab. Stable K562 transfectants expressing VEGFR2 were selected by growth in media containing G418, and the expression level of VEGFR2 was detected by Western blot and FACS. Although nontransfected K562 exhibited low levels of VEGFR2, the level was increased $>$4-fold in the stably transfected cells as assessed by FACS. In experiments utilizing these VEGFR2 transfectants, optimal results were obtained if the cells were cultured overnight in 1% serum, presumably due to reduction of VEGF in the system.

Expression of PTEN and Akt in M21 Cells

The cDNA encoding human PTEN, inserted into PcDNA3, was kindly provided by Dr. C. Eng (Ohio State University, Columbus, OH). The dominant-negative form of Akt (K179M) in a pLSXN vector was provided by Nissam Hat (Cleveland Clinic Foundation). PcDNA3-GFP was used as a control. The constructs were introduced into M21 cells using Effectin (Quagen, Valencia, CA). The efficiency of transfection was 40%–60% as assessed by fluorescence microscopy for GFP. Transfected cells were grown in 300 μg/ml G418 for 10–14 days, and $>$ 90% were GFP positive. Twenty-four hours prior to experiments, the G418 concentration was increased to 600 μg/ml, and this amount of antibiotic was maintained in all proliferation and migration experiments. PTEN expression was analyzed by immunoprecipitation and Western blot. For selected experiments, PTEN was recloned into pIRES2-EGFP bicistronic vector from Clontech either in a sense or in antisense orientation. PTEN/EGFP-expressing cells ($\sim$15% of total) were identified by FACS and further analyzed for WOW-1 expression. Cells with a mean of fluorescence intensity (MFI) of $>$40 (green channel) were gated, and the MFI of WOW-1 expression in the red channel was quantified from five independent transfections.

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