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Stimulation of Endothelial Cell Migration by Vascular Permeability Factor/Vascular Endothelial Growth Factor through Cooperative Mechanisms Involving the $\alpha_V\beta_3$ Integrin, Osteopontin, and Thrombin

Donald R. Senger,*
Steven R. Ledbetter,† Kevin P. Claffey,*
Ageliki Papadopoulos-Sergiou,*
Carole A. Perruzzi,* and Michael Detmar*‡

From the Departments of Pathology and Dermatology,‡ Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts, and The Upjohn Company,† Kalamazoo, Michigan*

We have identified several mechanisms by which the angiogenic cytokine vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) likely regulates endothelial cell (EC) migration. VPF/VEGF induced dermal microvascular EC expression of mRNAs encoding the α_v and β_3 integrin subunits resulting in increased levels of the $\alpha_v\beta_3$ heterodimer at the cell surface, and VPF/VEGF also induced mRNA encoding osteopontin (OPN), an $\alpha_v\beta_3$ ligand. OPN promoted EC migration* in vitro*; and VPF/VEGF induction of $\alpha_v\beta_3$ was accompanied by increased EC migration toward OPN. Because thrombin cleavage of OPN results in substantial enhancement of OPN's adhesive properties, and because VPF/VEGF promotes increased microvascular permeability leading to activation of the extrinsic coagulation pathway, we also investigated whether VPF/VEGF facilitates thrombin cleavage of OPN* in vivo*. Consistent with this hypothesis, co-injection of VPF/VEGF together with OPN resulted in rapid cleavage of OPN by endogenous thrombin. Furthermore, in comparison with native OPN, thrombin-cleaved OPN stimulated a greater rate of EC migration* in vitro*, which was additive to the increased migration associated with induction of $\alpha_v\beta_3$. Thus, these data demonstrate cooperative mechanisms for VPF/VEGF regulation of EC migration involving the $\alpha_v\beta_3$ integrin, the $\alpha_v\beta_3$ ligand OPN, and thrombin cleavage of OPN. These findings also illustrate an operational link between VPF/VEGF induction of EC gene expression and VPF/VEGF enhancement of microvascular permeability, suggesting that these distinct biological activities may act coordinately to stimulate EC migration during angiogenesis. (Am J Pathol 1996, 149:293–305)

Angiogenesis, the growth of new blood vessels, is a complex process involving both the proliferation and migration of endothelial cells (ECs). A variety of cytokines have been shown to induce angiogenesis in experimental models, and as determined with *in vitro* assays, some of these, such as the acidic and basic fibroblast growth factors and vascular permeability factor/vascular endothelial growth factor (VPF/VEGF), function through mechanisms involving stimulation of EC growth. However, other angiogenic cytokines do not stimulate EC proliferation *in vitro* and therefore are thought to promote vascularization *in vivo* through indirect mechanisms (reviewed in Ref. 1). Although there are potentially numerous angiogenesis factors, considerable evidence has accumulated indicating that VPF/VEGF may be the angiogenic cytokine of central importance. VPF/VEGF angiogenic activity has been demonstrated in a variety of experimental models including bone grafts,

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Address reprint requests to Dr. Donald R. Senger, Department of Pathology, Research North, Beth Israel Hospital, 99 Brookline Avenue, Boston, MA 02215.

the cornea, chick chorioallantoic membrane, ischemic hind limb, and tumor xenografts.[2–6] Moreover, numerous studies have indicated that expression of VPF/VEGF and its two EC receptors Flt-1[7] and KDR[8] (reviewed in Ref. 9) correlate both temporally and spatially with vascularization during embryogenesis[10–12] and with the angiogenesis associated with cancer,[13–15] wound healing,[16] rheumatoid arthritis,[17] psoriasis,[18] and proliferative retinopathies.[19–21] Additionally, a blocking monoclonal antibody specific for VPF/VEGF was found to inhibit tumor growth and vascularization, thus implicating VPF/VEGF directly as a major tumor angiogenesis factor.[22,23]

In addition to its action as a selective EC growth factor,[2,24,25] VPF/VEGF has other distinct biological activities, and therefore the mechanisms by which VPF/VEGF regulates angiogenesis are likely to be complex. For example, VPF/VEGF also rapidly increases microvascular permeability[26] with a potency, on a molar basis, approximately 50,000 times that of histamine.[27] As a vascular permeability factor, VPF/VEGF promotes extravasation of blood plasma from the microvasculature leading to activation of the extrinsic coagulation pathway, generation of thrombin, and deposition of fibrin.[28,29] Consequently, VPF/VEGF likely modulates the extravascular concentrations of thrombin as well as other plasma-derived proteins. In addition, VPF/VEGF has been shown to induce expression of plasminogen activators and plasminogen activator inhibitor-1 by microvascular ECs *in vitro*,[30] thus suggesting that VPF/VEGF is an important inducer of EC gene expression.

Because angiogenesis involves EC migration as well as EC proliferation, a potent angiogenesis factor such as VPF/VEGF likely promotes both of these processes. Therefore, we have sought to identify specific mechanisms by which VPF/VEGF stimulates EC migration. In particular, because extracellular matrix proteins and their receptors, the cell surface integrins, function critically in the process of cell migration,[31–34] we investigated the possibility that VPF/VEGF regulates EC migration through mechanisms involving integrins or their ligands. As a model system we chose to study human dermal microvascular ECs[35] because these cells are derived from small blood vessels that respond both to the permeability-enhancing[26,27] and angiogenic activity of VPF/VEGF *in vivo*[6] and thus they are highly relevant for investigating VPF/VEGF function. We began our studies with dermal microvascular ECs and VPF/VEGF by examining VPF/VEGF-regulated expression of the $\alpha_v\beta_3$ integrin and one of its ligands, the secreted adhesive glycoprotein osteopontin (OPN) (reviewed in Refs. 36,37). We specifically examined expression of the $\alpha_v\beta_3$ integrin because this receptor has been found previously to be expressed at substantially higher levels by the newly formed blood vessels of healing wounds and of the chick chorioallantoic membrane,[38] and furthermore, a blocking monoclonal antibody to $\alpha_v\beta_3$ was shown to inhibit angiogenesis.[38–40] We investigated possible relationships between VPF/VEGF function and OPN because OPN promotes cell adhesion and migration through interactions with $\alpha_v\beta_3$.[41–43] Because OPN function in promoting cell adhesion is substantially increased when OPN is cleaved by thrombin,[41] we also examined the possibility that cleavage and activation of OPN by thrombin occurs as a consequence of VPF/VEGF enhanced microvascular permeability. Our findings identify induction of $\alpha_v\beta_3$ and OPN as a likely mechanism for VPF/VEGF stimulation of EC migration during angiogenesis and, moreover, support the hypothesis that VPF/VEGF enhancement of microvascular permeability further promotes EC migration by promoting thrombin cleavage and activation of OPN.

Materials and Methods

Cells, Cell Culture, and VPF/VEGF

Human dermal microvascular ECs were isolated from neonatal foreskins as described previously.[44,45] Cells were grown on collagen-coated dishes in endothelial cell basal medium (Clonetics, San Diego, CA) with 20% fetal calf serum (Gibco BRL, Grand Island, NY), 50 μmol/L dibutyryl cyclic AMP, 1 μg/ml hydrocortisone acetate, 100 U/ml penicillin, 100 U/ml streptomycin, and 250 μg/ml amphotericin B (Sigma Chemical Co., St. Louis, MO).

ECs at passage 4 to 7 were seeded at 1×10^4 cells/cm^2 into 100-mm plastic dishes (Costar, Cambridge, MA) in fully supplemented growth medium (see above). For experiments involving Northern analysis of integrin subunit mRNAs, cells also were maintained in endothelial cell basal medium supplemented only with 2% fetal calf serum and antibiotics; similar results were obtained with both culture conditions. In all cases, media were replaced every second day until cells were confluent. Purified recombinant VPF/VEGF (165-amino-acid isoform), void of detectable endotoxin, was prepared as described previously[46] and added to cultures as indicated in the figure legends. All experiments were performed at least twice with similar results.

RNA Isolation and Northern Analyses

Total cellular RNA was isolated from cultured cells as previously described,[47] subjected to electrophoresis,[48] and transferred to Biotrans nylon supported membranes as recommended by the manufacturer (ICN Pharmaceuticals, Costa Mesa, CA). ^{32}P-radiolabeled cDNA probes were prepared with a random primed synthesis kit (Multiprime, Amersham, Arlington Heights, IL) employing purified cDNA inserts as templates. The OP-30 plasmid with a 1.4-kb human OPN insert[49] was obtained from the American Type Culture Collection (Rockville, MD). Plasmids containing a 2.3-kb human β_3 insert[50] and a 2.8-kb human α_v insert[51] were kindly provided by Dr. Larry Fitzgerald, University of Utah (Salt Lake City, UT). A plasmid containing a 3.1-kb human α_5 insert was kindly provided by Dr. Lisa Plantefaber, Beth Israel Hospital. A 2.0-kb human β-actin cDNA was purchased from Clontech (Palo Alto, CA). After hybridization, blots were washed multiple times at 55°C in 1X standard saline citrate, 5% sodium dodecyl sulfate (SDS) and exposed to X-OMAT AR film (Kodak, Rochester, NY). Bands were quantitated with a Molecular Dynamics (Sunnyvale, CA) scanning densitometer together with the ImageQuant program.

Cell Surface Iodination and Immunoprecipitation Analyses

Cell surface iodination of confluent monolayers was performed with the Iodobead method[52] (Pierce Chemical Co., Rockford, IL). After 15 minutes, the beads and media containing ^{125}I (Dupont NEN Products, Boston, MA) were removed and the cell layers were washed four times with Puck's saline G. Cells were lysed in immunoprecipitation buffer containing the following detergents and protease inhibitors: 0.02 mol/L Tris-HCl pH 7.5, 0.05 mol/L NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) (Calbiochem, La Jolla, CA), and 1 mmol/L iodoacetic acid. Lysates were clarified by centrifugation at 10,000 × *g* for 30 minutes at 4°C. To measure incorporation of ^{125}I into protein, aliquots were subjected to precipitation with 10% trichloroacetic acid and quantitated in a gamma counter. Clarified cell lysates, normalized for minor differences in incorporation, if any, were subjected to immunoprecipitation with either rabbit antiserum (377) raised to a human β_3 carboxyl-terminal peptide[41] or to a human α_v carboxyl-terminal peptide (AB1930, Chemicon International, Temecula, CA) and protein-A-Sepharose beads (Pharmacia, Piscataway, NJ), as previously described.[41] Precipitates were washed once with wash buffer 1 containing 1 mol/L NaCl, 0.1% NP-40, 0.01 mol/L Tris, pH 7.2 and twice with wash buffer 2 containing 0.15 mol/L NaCl, 1% sodium deoxycholate, 1% Triton X-100, 1 mol/L urea, 0.01 mol/L Tris, pH 7.2, and subjected to SDS-polyacrylamide (8.5%) gel electrophoresis under reducing conditions[53] followed by autoradiography with Kodak X-OMAT AR film. Bands were quantitated as above. Molecular weight markers employed were myosin (200 kd), β-galactosidase (116 kd), phosphorylase b (97 kd), serum albumin (67 kd), ovalbumin (43 kd), and carbonic anhydrase (30 kd).

OPN, Cell Migration, and Attachment Assays

OPN was purified from human breast milk as described previously[41] and filter sterilized by passing through Millex GV filters (Millipore, Bedford, MA). These preparations were homogeneous as determined by SDS-polyacrylamide gel electrophoresis and as illustrated previously.[41,54] Furthermore, the EC migration-promoting activity associated with our OPN preparations, assayed as described below, was abolished by affinity-purified OPN-specific antibody (25 μg/ml) that was raised to a 20-amino-acid synthetic peptide representing the glycine-arginine-glycine-aspartate-serine (GRGDS) region of human OPN.[41]

To assay cell migration, we employed 8-μm pore size Transwell migration chambers (Costar).[33,34] Undersides of membranes were coated with OPN at the indicated concentrations for 60 minutes at room temperature. Subsequently, remaining protein-binding sites on the undersides of the membranes were blocked by coating with a solution of 100 mg/ml bovine serum albumin (BSA) at room temperature for 60 minutes; next, membranes were washed with Dulbecco's modified Eagle's medium (DME). We also performed experiments in which the undersides of membranes were coated with OPN followed by BSA and then bound OPN was cleaved *in situ* by incubating the underside of the membranes with 1 U of human thrombin (Sigma). As determined with SDS-polyacrylamide gel electrophoresis and autoradiography of radiolabeled OPN tracer (see below), thrombin cleavage of OPN to two M_r 35,000 fragments[54] was ~75% efficient with this method. After incubation with thrombin, membranes were washed extensively with DME. The absence of residual thrombin was verified with the highly sensitive Kabi S2238 chromogenic substrate assay (Kabi Pharma-

cia Diagnostics, Piscataway, NJ), and as an additional precaution, the filters were incubated with 2 μg of the thrombin inhibitor D-phe-L-pro-L-arg-CH_2 Cl (PPACK; Calbiochem, La Jolla, CA), which is a 25-fold excess of inhibitor for 1 U of thrombin. After incubation with PPACK, filters again were washed extensively with DME.

After membranes were prepared as outlined above, 1×10^5 cells were added to the upper chambers in DME containing 10 mg/ml BSA. Cell migration was allowed to proceed at 37°C in a standard tissue culture incubator for the indicated time intervals; cells then were removed from the upper side of the membrane with a cotton swab, and cells that migrated to the undersides were stained with 0.2% crystal violet in 2% ethanol followed by a brief rinse in distilled water. Dried membranes were cut out and mounted on glass slides in immersion oil. At least 10 random high power fields were counted from each membrane and all assays were performed in triplicate. No cell migration was observed when membrane filters were coated with BSA alone, and in no cases did we observe cells in the lower chamber that had traversed the membranes but did not remain attached. Data are presented as mean ± standard deviation of the mean. Statistical significance was calculated using the two-sided Student's *t*-test.

To determine the importance of the $\alpha_v\beta_3$ integrin for cell migration toward OPN and thrombin-cleaved OPN, we employed LM609 $\alpha_v\beta_3$-blocking mouse monoclonal antibody.[55] LM609 ascites was kindly provided by Dr. David Cheresh, Scripps Clinic and Research Foundation (La Jolla, CA), and antibody was purified with the Affigel protein A MAPS II kit (Bio-Rad Laboratories, Richmond, CA). Control mouse IgG was purchased from Sigma and re-purified according to the same procedure.

Quantitative cell attachment assays were performed as previously described,[41] employing 96-well strip plates (Costar 2388). Wells were coated with 5 μg/ml human OPN, and remaining protein binding sites were blocked with 100 mg/ml BSA. Where indicated, bound OPN was cleaved by thrombin *in situ* as described previously.[41]

Analysis of OPN Cleavage in Vivo

Purified human OPN (see above) was radioiodinated with ^{125}I with the Iodobead method (Pierce Chemical) and dialyzed extensively to remove free isotope. Specific activities obtained were ~1 μCi/μg OPN. Aliquots (200 μl) of 3 μg of ^{125}I-labeled OPN in phosphate-buffered saline were injected at multiple intradermal sites into adult Hartley guinea pigs together with VPF/VEGF (1 nmol/L) and PPACK (100 μmol/L), where indicated. Identical samples were injected 30 minutes and 15 minutes before the time that animals were anesthesized and exsanguinated. Skin test sites were harvested immediately thereafter, and skin samples were extracted at 90°C with vortexing in 500 μl of electrophoresis sample buffer[53] containing 2% SDS and supplemented with protease inhibitor (5 mmol/L 4-(2-aminoethyl)-benzenesulfonylfluoride, Calbiochem). Extracts were clarified by centrifugation and aliquots counted in a gamma counter. Extracted samples, normalized for minor differences in recovery, were subjected to electrophoresis and autoradiography as described above.

Results

VPF/VEGF Induces $\alpha_v\beta_3$ Integrin Expression by Microvascular ECs

All studies were performed with the recombinant 165-amino-acid isoform of VPF/VEGF, void of detectable endotoxin; of the several VPF/VEGF isoforms, this isoform is most abundantly expressed.[56,57] As demonstrated by Northern analyses and scanning densitometry, incubation of postconfluent ECs with 10 ng/ml VPF/VEGF for 24 hours resulted in greater than 10-fold increases in the relative levels of both α_v and β_3 mRNAs (Figure 1A). Maximal increases in α_v mRNA were observed at concentrations of ~1 ng/ml VPF/VEGF, whereas ~10 ng/ml VPF/VEGF was required for maximal increases in β_3 mRNA. For comparison, we also probed the blots for α_5 integrin mRNA, and in contrast to α_v and β_3 mRNAs, there were no significant changes in levels of mRNA encoding the α_5 integrin subunit after VPF/VEGF stimulation (Figure 1A).

We also performed analyses to follow the time course of induction of α_v and β_3 mRNAs after VPF/VEGF stimulation. As shown in Figure 1B, α_v mRNA levels were increased substantially by 4 hours and relatively high levels persisted for 48 hours after stimulation with 10 ng/ml VPF/VEGF. No increases in β_3 mRNA were evident at 4 hours, but substantial increases were evident by 24 hours, raising the possibility that VPF/VEGF induction of α_v and β_3 mRNAs involves distinct regulatory pathways. The level of β_3 mRNA was further increased by 48 hours (Figure 1B). In contrast, no significant fluctuations in the levels of α_5 integrin subunit mRNA were observed over a 48-hour interval after VPF/VEGF stimulation.

To determine whether induction of α_v and β_3 mRNAs in microvascular ECs translated into induc-

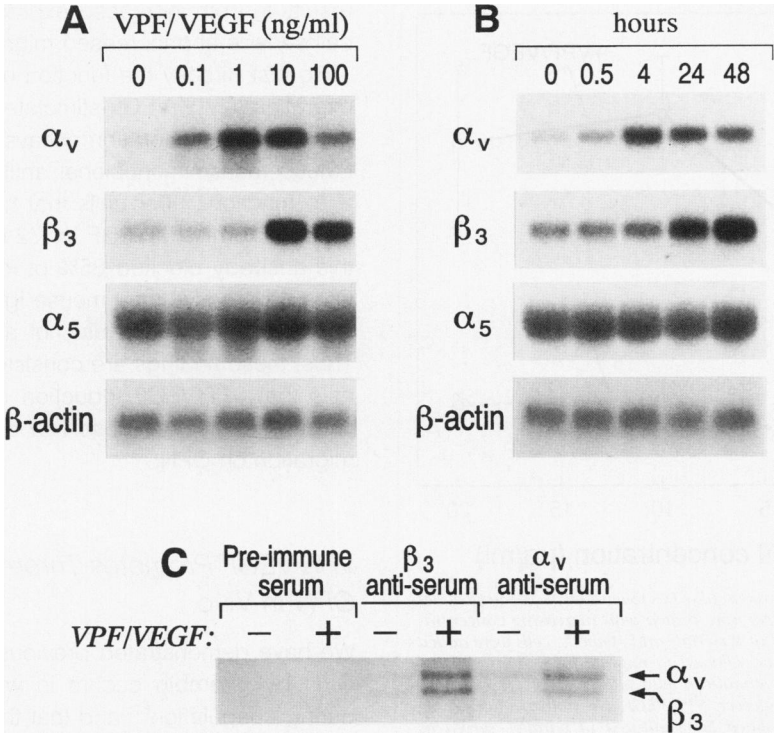

Figure 1. *Time- and dose-dependent induction of α_v and β_3 integrin subunit mRNAs in microvascular ECs by VPF/VEGF and induction of $\alpha v\beta 3$ heterodimers at the cell surface.* **A** *and* **B:** *Northern analysis of total RNA (10 μg) isolated 24 hours after stimulation by VPF/VEGF at the indicated concentrations* (**A**) *or at the indicated times after stimulation by 10 ng/ml VPF/VEGF* (**B**). *Blots were hybridized separately and sequentially with the integrin cDNA probes as indicated and finally with β-actin probe as a control. Note that, in contrast to α_v and β_3 mRNAs, the levels of α_5 mRNA and β-actin mRNA are relatively constant. Sizes of mRNAs corresponded to ~7.5 kb (α_v), ~6.0 kb (β_3), ~5.5 kb(α_5), and ~1.9 kb (β-actin).* **C:** *SDS-polyacrylamide gel electrophoresis autoradiography of cell surface integrins. VPF/VEGF (10 ng/ml) was added to confluent ECs at time zero and again at 48 hours. At 72 hours after initial stimulation, VPF/VEGF (+) and unstimulated (−) cells were subjected to surface protein labeling with [^{125}I]. Incorporation of isotope was identical for VPF/VEGF (+) and VPF/VEGF (−) cultures, and equal volumes of cell lysates were subjected to precipitation with preimmune serum (rabbit 377), β_3 antiserum (rabbit 377), and α_v antiserum (Chemicon). β_3 antiserum* (lanes 3 *and* 4) *precipitated bands corresponding to β_3 (M_r ~110,000; reducing conditions) and α_v (M_r ~125,000), which were absent in precipitates prepared with preimmune serum from the same rabbit* (lanes 1 *and* 2). *Precipitation of both α_v and β_3 subunits by this β_3-specific antibody*[41] *is consistent with the heterodimeric association of these proteins at the cell surface. Antiserum to α_v precipitated these same bands* (lanes 5 *and* 6). *Note that both α_v and β_3 are represented at substantially higher levels (more than fourfold) in VPF/VEGF$^+$ cell lysates in comparison with parallel controls.*

tion of $\alpha_v\beta_3$ at the cell surface, we performed immunoprecipitation analyses of iodinated cell surface proteins and found that VPF/VEGF-stimulated ECs expressed substantially higher quantities of $\alpha_v\beta_3$ heterodimers at the cell surface in comparison with parallel cultures of control ECs. As quantitated with autoradiography and scanning densitometry, greater than twofold induction of $\alpha_v\beta_3$ at the cell surface was evident within 48 hours of continuous VPF/VEGF stimulation and greater than fourfold induction was present by 72 hours (Figure 1C).

VPF/VEGF Both Induces OPN mRNA in Microvascular ECs and Regulates OPN-Directed EC Migration

OPN mRNA was not detectable in control cultures of microvascular ECs, but within 4 hours of stimulation by VPF/VEGF, OPN mRNA was induced (Figure 2). Moreover, induction of OPN mRNA persisted for at least 48 hours after stimulation at VPF/VEGF concentrations of 10 ng/ml. With metabolic labeling and immunoprecipitation, we did not detect soluble OPN

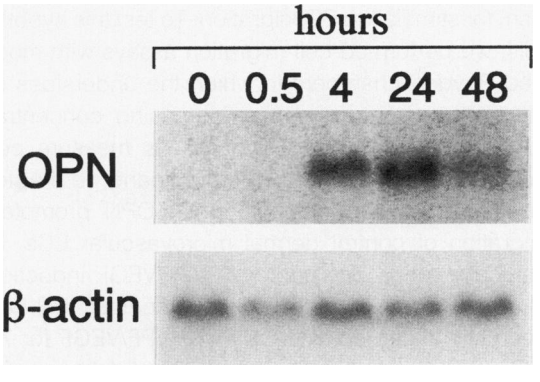

Figure 2. *Northern analysis of OPN mRNA in microvascular ECs after stimulation by VPF/VEGF. Each track represents 10 μg of total RNA isolated from cells stimulated with 10 ng/ml VPF/VEGF for the indicated times; 0 hours = no VPF/VEGF added. The blot was probed for OPN mRNA (~1.5 kb) and then stripped and re-probed for β-actin mRNA (~1.9 kb) as a control.*

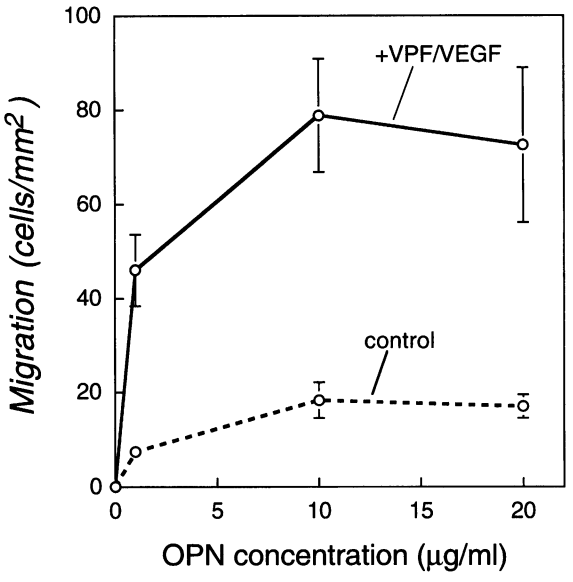

Figure 3. *Migration of microvascular ECs toward OPN. The undersides of migration chamber filters were coated with increasing concentrations of OPN, as described in Materials and Methods; cells were added to the upper chambers and allowed to migrate for 4 hours. Before assay, confluent ECs were stimulated with VPF/VEGF (10 ng/ml) continuously for 72 hours; however, VPF/VEGF was not included in the migration assay. Unstimulated ECs, cultured in parallel, served as controls.*

protein in the medium of VPF/VEGF-stimulated ECs, possibly because OPN was retained at the cell surface by its receptors or because OPN was incorporated into the extracellular matrix. OPN is a substrate for transglutaminases,[58,59] enzymes that covalently cross-link proteins through isopeptide bonds involving reactive lysines and glutamines, and ECs express a cell-associated transglutaminase.[60,61] Therefore, an intriguing possibility is that OPN expressed by ECs is covalently bound to the matrix.

Because OPN is an $\alpha_v\beta_3$ ligand, co-induction of OPN and $\alpha_v\beta_3$ by VPF/VEGF suggested a mechanism for stimulating EC migration. To test this hypothesis, we performed cell migration assays with modified Boyden chambers in which the undersides of the filters were coated with increasing concentrations of purified OPN. Such assays measure cell migration in a gradient of bound ligand, ie, haptotaxis, and as shown in Figure 3, OPN promoted migration of control dermal microvascular ECs. In particular, and consistent with VPF/VEGF induction of $\alpha_v\beta_3$ expression at the cell surface, ECs that had been stimulated continuously with VPF/VEGF for 72 hours migrated toward OPN with 4.5-fold greater efficiency than controls even though VPF/VEGF was not included in the migration assay. Thus, OPN promotes migration of dermal microvascular ECs, and chronic pretreatment of these cells with VPF/VEGF results in both increased expression of $\alpha_v\beta_3$ at the cell surface and increased migration toward OPN.

To test directly the function of $\alpha_v\beta_3$ in promoting migration of VPF/VEGF-stimulated cells toward OPN, we performed migration assays in the presence of LM609 mouse monoclonal antibody, which blocks $\alpha_v\beta_3$ function.[55] For cells that had been stimulated with 10 ng/ml VPF/VEGF for 72 hours before assay, this antibody blocked 85% of the migration toward OPN, whereas control mouse IgG at the same concentration (10 μg/ml) did not affect cell migration. Thus, these findings are consistent with the hypothesis that VPF/VEGF induction of $\alpha_v\beta_3$ is primarily responsible for the observed enhancement of cell migration on OPN.

VPF/VEGF Promotes Thrombin Cleavage of OPN in Vivo

We have demonstrated previously that cleavage of OPN by thrombin occurs in whole blood plasma during coagulation[62] and that this cleavage occurs at a single site in close proximity to the GRGDS domain.[54,63] We also determined that cleavage of OPN by thrombin results in substantial activation of OPN's adhesive properties.[41] Consequently, activation of blood coagulation with generation of active thrombin from prothrombin is likely an important mechanism for regulating OPN function. Because VPF/VEGF increases microvascular permeability with concomitant extravasation of plasma proteins, activation of the extrinsic blood coagulation pathway, and extravascular deposition of fibrin,[28,29] we hypothesized that co-administration of VPF/VEGF and OPN would result in thrombin cleavage of OPN *in vivo.* To test this hypothesis, we radiolabeled OPN with ^{125}I and performed intradermal injections with labeled OPN together with VPF/VEGF or with vehicle alone. As shown in Figure 4 (lane 2), within 15 minutes of co-injection of OPN and VPF/VEGF into guinea pig skin, there was detectable conversion of OPN to its thrombin-cleaved form, which consists of two M_r ~35,000 fragments with indistinguishable electrophoretic mobilities.[54] In addition, we found evidence that VPF/VEGF also promoted generation of an additional OPN fragment of yet unknown origin (M_r ~30,000). In contrast, we observed little or no cleavage of OPN at skin test sites where VPF/VEGF was omitted (lane 1).

Similarly, skin test sites injected with OPN alone and harvested 30 minutes later showed minimal proteolysis (lane 4), but sites injected with both OPN and VPF/VEGF provided clear evidence of OPN

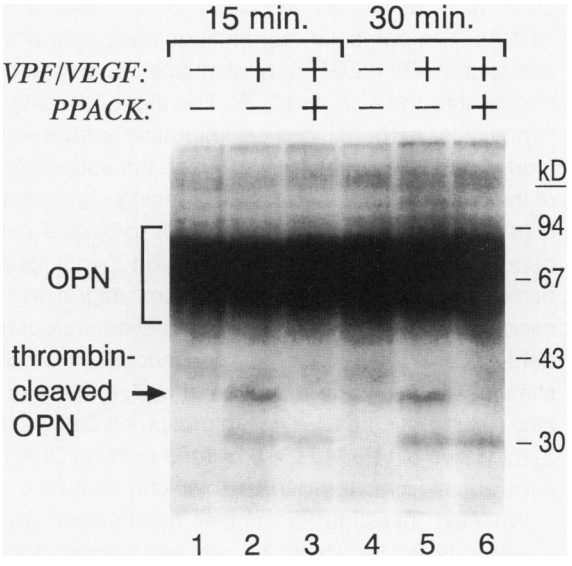

Figure 4. *VPF/VEGF promotes thrombin cleavage of OPN* in vivo. *^{125}I-labeled OPN was injected into the dermis of guinea pigs with (+) or without (−) VPF/VEGF (1 nmol/L) and with or without the thrombin inhibitor PPACK. Sites were injected 30 minutes* (lanes 4 *to* 6) *and 15 minutes* (lanes 1 *to* 3) *before harvest and extraction (see Materials and Methods). Purified thrombin cleaves OPN* in vitro *to generate two principal fragments of* M_r *35,000 with electrophoretic mobility as indicated. VPF/VEGF promoted the generation of a band* (lanes 2 *and* 5) *corresponding identically with thrombin-cleaved OPN, and generation of this band was inhibited by PPACK, a specific irreversible inhibitor of thrombin* (lanes 3 *and* 6).

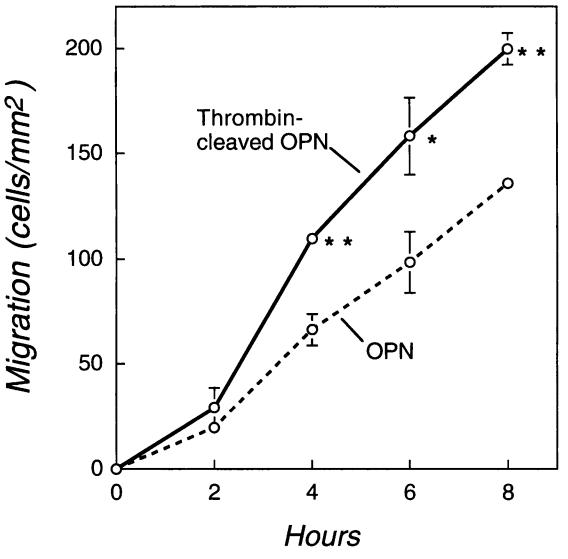

Figure 5. *In comparison with native OPN, thrombin-cleaved OPN promotes a greater rate of microvascular EC migration. The undersides of migration chamber filters were coated with 10 μg/ml OPN or thrombin-cleaved OPN (see Materials and Methods). Before assay, cells were stimulated with VPF/VEGF for 72 hours as in Figures 1C and 3. VPF/VEGF was not included in the migration assay.* *P < *0.05;* **P < *0.001.*

cleavage by thrombin (lane 5). Generation of the thrombin-cleaved OPN fragments was inhibited when the specific and irreversible thrombin inhibitor PPACK was included (lanes 3 and 6), thus establishing conclusively that these fragments were generated from OPN by thrombin. However, production of the lower molecular weight fragment (M_r ~30,000) was not inhibited by PPACK. Thus the appearance of this lower molecular weight fragment, although promoted by VPF/VEGF, apparently is not dependent on active thrombin. This OPN fragment may be generated by another protease derived from blood plasma that extravasates as a consequence of VPF/VEGF-enhanced microvascular permeability, and we are currently investigating this possibility.

Thrombin-Cleaved OPN Promotes a Greater Rate of Microvascular EC Migration through Interactions with the $\alpha_v\beta_3$ Integrin

Consistent with activation of OPN's adhesive properties by thrombin,[41] we found that, in comparison with native OPN, thrombin-cleaved OPN promoted a greater rate of EC migration (Figure 5). For these experiments we employed microvascular ECs that had been exposed before assay with VPF/VEGF (10 ng/ml) for 72 hours. As described above, these cells expressed substantially more $\alpha_v\beta_3$ cell surface integrin than unstimulated controls (Figure 1C), and in comparison with unstimulated controls, VPF/VEGF-stimulated ECs migrated more efficiently on native OPN (Figure 3). Therefore, these data indicate that thrombin cleavage of OPN provides a mechanism for further enhancement of OPN-directed EC migration that is additive to the increased cell migration associated with induction of $\alpha_v\beta_3$. The greater rate of EC migration toward thrombin-cleaved OPN is likely a direct consequence of the enhanced adhesive properties associated with this form of OPN because, as determined with quantitative attachment assays with these ECs, thrombin-cleaved OPN also promoted greater cell attachment than native OPN (19% *versus* 10% after 30 minutes; 42% *versus* 28% after 45 minutes).

Finally, and as shown in Figure 6, we also found ECs that were stimulated continuously with VPF/VEGF for 72 hours before assay migrated on thrombin-cleaved OPN more efficiently than control ECs. Consistent with the hypothesis that increased $\alpha_v\beta_3$ expression was responsible for the observed increase in cell migration, we found that the $\alpha_v\beta_3$ blocking antibody (LM609) substantially inhibited migration of VPF/VEGF-stimulated ECs toward thrombin-cleaved OPN (Figure 6). Lack of complete inhibition of cell migration toward thrombin-cleaved OPN by LM609 antibody (Figure 6) leaves open the possibility that other integrins are also involved, but

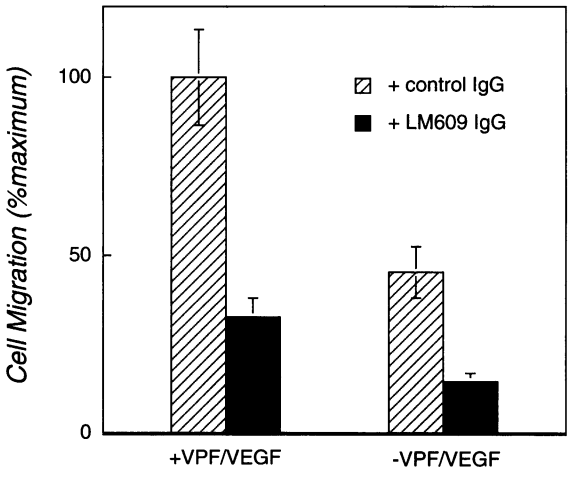

Figure 6. *Microvascular EC migration toward thrombin-cleaved OPN: inhibition by $\alpha_v\beta_3$ monoclonal antibody LM609. ECs stimulated with 10 ng/ml VPF/VEGF continuously for 72 hours before assay and control ECs from parallel cultures were compared for cell migration (4 hours) toward thrombin-cleaved OPN (see Materials and Methods) in the presence of 10 μg/ml control mouse IgG or 10 μg/ml LM609 IgG, which blocks $\alpha_v\beta_3$ function.[55] Consistent with increased $\alpha_v\beta_3$ expression at the cell surface, VPF/VEGF-stimulated cells migrated more efficiently than control (without VPF/VEGF) cells, and moreover, cell migration was substantially inhibited by LM609 IgG.*

these experiments indicate that for dermal microvascular ECs chronically stimulated with VPF/VEGF, $\alpha_v\beta_3$ is an important OPN receptor.

Discussion

In this study we have demonstrated that VPF/VEGF induces dermal microvascular ECs to express increased levels of mRNAs encoding the α_v and β_3 integrin subunits leading to increased expression of the $\alpha_v\beta_3$ heterodimer at the cell surface. The $\alpha_v\beta_3$ integrin is a receptor for multiple ligands that contain the GRGDS cell-binding sequence including OPN, vitronectin, thrombospondin, von Willebrand factor, fibrinogen, fibronectin (reviewed in Refs. 64 and 65), tenascin,[66] and denatured collagen.[67] Consequently, increased expression of $\alpha_v\beta_3$ by microvascular ECs suggests the potential for increased cellular interactions with all of these ligands. Moreover, such interactions likely would have broad consequences for ECs, including not only regulation of cell adhesion but also activation of signal transduction pathways[68,69] and regulation of apoptosis.[39]

A specific functional consequence for $\alpha_v\beta_3$ induction by VPF/VEGF was suggested by our findings that VPF/VEGF also induced expression of mRNA encoding OPN, an $\alpha_v\beta_3$ ligand.[41,43] Co-induction of $\alpha_v\beta_3$ and OPN may prove to be relevant to several cell types as others have shown that induction of differentiation in HL-60 monocytic cells by phorbol ester also results in induction of α_v and OPN mRNAs.[70] In particular, our findings raised the possibility that VPF/VEGF may stimulate EC migration directly through $\alpha_v\beta_3$ and OPN. Therefore, to test this hypothesis, we performed cell migration assays with modified Boyden chambers in which the undersides of the filters were coated with increasing concentrations of purified OPN. These assays provided evidence consistent with the foregoing hypothesis, demonstrating that 1) OPN promotes migration of microvascular ECs and that cell migration is dependent on OPN concentration, 2) continuous VPF/VEGF stimulation, leading to induction of $\alpha_v\beta_3$ expression, also results in increased cell migration on OPN, and 3) migration of VPF/VEGF-stimulated cells on OPN is substantially inhibited by $\alpha_v\beta_3$ blocking antibody.

We next investigated another mechanism, also involving $\alpha_v\beta_3$, by which the vascular permeability-enhancing activity of VPF/VEGF might contribute to increased EC migration on OPN. Previous findings indicated that VPF/VEGF rapidly increases microvascular permeability, resulting in extravasation of plasma proteins including blood coagulation factors. In turn, this leads to activation of the extrinsic coagulation pathway with generation of active thrombin from prothrombin.[26–28] Thrombin not only cleaves fibrinogen and factor XIII to generate a cross-linked fibrin clot, but thrombin also cleaves OPN, resulting in further activation of OPN's adhesive properties.[41] Therefore, we began by testing the possibility that VPF/VEGF could promote thrombin cleavage of OPN *in vivo*. We found that intradermal injection of OPN together with VPF/VEGF resulted in cleavage of OPN by endogenous thrombin but that little or no cleavage of OPN occurred in the absence of VPF/VEGF. Next, we determined that thrombin-cleaved OPN *in vitro* promoted EC attachment more efficiently than intact OPN and, in particular, that thrombin-cleaved OPN promoted a greater rate of EC migration. Moreover, this migration was substantially inhibited by $\alpha_v\beta_3$ antibody. The greater rate of EC migration on thrombin-cleaved OPN, relative to intact OPN, was observed even with cells that had been stimulated continuously with VPF/VEGF for induction of $\alpha_v\beta_3$, indicating that the enhanced cell migration associated with thrombin cleavage of OPN is complementary to the enhanced migration that accompanies elevated expression of $\alpha_v\beta_3$. Thus, our data suggest several distinct but cooperative mechanisms involving $\alpha_v\beta_3$ and OPN by which VPF/VEGF can stimulate EC migration.

Thrombin cleaves OPN principally at a single site, six residues carboxy-terminal to the GRGDS sequence (reviewed in Ref. 71). Antibody to a synthetic

peptide representing the GRGDS region inhibited cell adhesion to thrombin-cleaved OPN, implicating the GRGDS-containing fragment as important for the enhanced adhesive properties associated with this form of the protein.[41] Consequently, a likely hypothesis is that thrombin further activates OPN's adhesive and cell migration promoting activity by further exposing the GRGDS region of the protein to $\alpha_v\beta_3$ and possibly other integrins as well.

Although it seems likely that VPF/VEGF induction of $\alpha_v\beta_3$ expression is largely responsible for the observed increases in EC migration toward OPN, there are other potential mechanisms that might also contribute, including induction of expression of other OPN receptors. In addition, integrin adhesiveness and cell motility both can be regulated by intracellular signal transduction pathways.[72,73] Consequently, VPF/VEGF might further enhance EC migration on OPN by regulating the adhesiveness of OPN receptors or by regulating OPN receptor function in cell motility. At present, however, we have no evidence to support either of these possibilities. Moreover, the increased expression of $\alpha_v\beta_3$ we observed following VPF/VEGF stimulation of EC (Figure 1C) appears sufficient to account for the observed increases in cell migration towards OPN (Figure 3).

Expression of the $\alpha_v\beta_3$ integrin has been shown previously to be induced in blood vessels of human wound granulation tissue and during angiogenesis on the chick chorioallantoic membrane.[38] Moreover, $\alpha_v\beta_3$ monoclonal antibody LM609 was found to suppress angiogenesis.[38–40] Therefore, induction of $\alpha_v\beta_3$ expression in ECs is likely an important element of the mechanism by which VPF/VEGF promotes angiogenesis. By analogy, it might be expected that other angiogenic cytokines also induce $\alpha_v\beta_3$ expression in ECs, and consistent with this possibility, others have reported that basic fibroblast growth factor stimulation of human dermal microvascular ECs results in increased $\alpha_v\beta_3$ expression.[74] However, and in contrast, basic fibroblast growth factor was not found to increase $\alpha_v\beta_3$ expression in microvascular ECs isolated from adrenal cortex.[75] Conceivably, the disparate findings from these two studies might relate to the different origins of the ECs employed or to different culture conditions.

The evidence that $\alpha_v\beta_3$ functions importantly in angiogenesis[38–40] indirectly implicates the $\alpha_v\beta_3$ ligands as critical elements in neovascularization. In particular, data presented here specifically identify OPN as an $\alpha_v\beta_3$ ligand that is likely significant for promoting endothelial cell migration during VPF/VEGF-directed neovascularization. Evidence obtained previously from studies on OPN expression are consistent with a function for OPN in the angiogenesis associated with tissue and bone remodeling (reviewed in Ref. 37). Moreover, OPN and VPF/VEGF are often expressed in close proximity to each other during angiogenesis. For example, OPN is expressed at relatively high levels by some tumor cells and more generally by activated macrophages in tumors, particularly at the stromal interface and in regions of necrosis.[76] VPF/VEGF is highly expressed by tumor cells,[13,14] particularly in necrotic areas.[77] In addition, OPN is highly expressed by infiltrating macrophages associated with myocardial injury and necrosis,[78] and VPF/VEGF expression by myocardial cells is induced in these same regions.[79] VPF/VEGF is also overexpressed by epidermal keratinocytes in dermal wounds and in delayed hypersensitivity reactions, and in these settings OPN mRNA is abundantly expressed by activated macrophages[16,80] (L. Van De Water, personal communication).

Finally, the VPF/VEGF concentrations employed in experiments described here are relevant to a variety of important pathologies. The concentration at which we observed maximal induction of α_v, β_3, and OPN mRNAs was 10 ng/ml (240 pmol/L), and we employed VPF/VEGF at a concentration of 1 nmol/L in experiments demonstrating that VPF/VEGF promotes thrombin cleavage of OPN *in vivo*. Although such concentrations are higher than that required to promote EC growth,[24,35] we have observed VPF/VEGF concentrations estimated to be greater than 10 nmol/L in experimental tumor ascites.[26,27] Moreover, VPF/VEGF concentrations greater than 500, 200, 100, and 50 pmol/L have been reported, respectively, to be present in ocular fluid from patients with proliferative retinopathies,[19] in blister fluid obtained from patients with bullous dermatoses,[81] in human malignant effusions,[46,82] and in synovial fluids associated with rheumatoid arthritis.[17] VPF/VEGF concentrations in fluids are undoubtedly underestimates of the local concentration in adjacent tissues where VPF/VEGF is expressed, and therefore, it is most probable that local tissue concentrations greater than or equal to those employed in this study are generally present in a variety of pathologies.

In summary, data presented here predict that VPF/VEGF stimulates EC migration during angiogenesis, at least in part, through mechanisms involving induction of the cell surface integrin $\alpha_v\beta_3$ and its ligand OPN. In addition, they demonstrate that OPN's migration-promoting activity can be further enhanced by thrombin cleavage, a process that VPF/VEGF promotes through its action as a vascular permeability factor. Our data indicate that these mechanisms can act cooperatively leading to substantial stimulation of

EC migration. More generally, these findings illustrate specific mechanisms by which two apparently distinct biological activities of VPF/VEGF, ie, enhancement of microvascular permeability and induction of EC gene expression, may function coordinately in promoting EC migration during angiogenesis.

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