A Mutant Form of Vascular Endothelial Growth Factor (VEGF) That Lacks VEGF Receptor-2 Activation Retains the Ability to Induce Vascular Permeability*

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Vascular endothelial growth factor (VEGF) is a major mediator of vasculogenesis and angiogenesis both during development and in pathological conditions. VEGF has a variety of effects on vascular endothelium, including the ability to stimulate endothelial cell mitogenesis, and the potent induction of vascular permeability. These activities are at least in part mediated by binding to two high affinity receptors, VEGFR-1 and VEGFR-2.

In this study we have made mutations of mouse VEGF in order to define the regions that are required for VEGFR-2-mediated functions. Development of a bioassay, which responds only to signals generated by cross-linking of VEGFR-2, has allowed evaluation of these mutants for their ability to activate VEGFR-2. One mutant (VEGF0), which had amino acids 83–89 of VEGF substituted with the analogous region of the related placenta growth factor, demonstrated significantly reduced VEGFR-2 binding compared with wild type VEGF, indicating that this region was required for VEGF-VEGFR-2 interaction. Intriguingly, when this mutant was evaluated in a Miles assay for its ability to induce vascular permeability, no difference was found when compared with wild type VEGF. In addition we have shown that the VEGF homology domain of the structurally related growth factor VEGF-D is capable of binding to and activating VEGFR-2 but has no vascular permeability activity, indicating that VEGFR-2 binding does not correlate with permeability activity for all VEGF family members.

These data suggest different mechanisms for VEGF-mediated mitogenesis and vascular permeability and raise the possibility of an alternative receptor mediating vascular permeability.

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen that plays an important role in angiogenesis both in the developing embryo and in the growth and spread of tumors (1–4). VEGF (also called VPF for vascular permeability factor) was originally described as a factor responsible for the accumulation of plasma protein-rich fluid in the ascites of tumor patients due to an ability to induce vascular hyperpermeability (5). Studies of VEGF have demonstrated that it has a permeability enhancing capability, on a molar basis, 50,000 times greater than that of histamine (6).

Structurally, VEGF exists as a dimeric glycoprotein of Mr 34–42,000 and is related to the platelet-derived growth factor (PDGF) family of molecules, having a conserved cystine knot motif in each monomer (7). Although VEGF is the product of a single gene, differential RNA splicing produces at least five isoforms in the human (8–11). Other related but distinct factors have since been described and designated VEGF-B (12), VEGF-C (13), VEGF-D (14), and placenta growth factor (PIGF) (15), which also share the common central region (VEGF homology domain; VHD) containing the cystine knot motif.

VEGF mediates its effects via at least two receptors, Flt-1(VEGFR-1) and Flk-1(VEGFR-2), both of which have been shown to have high affinity binding sites for VEGF (16–19). The responses of these receptors to VEGF are quite different.

VEGF-2 displays ligand-dependent phosphorylation in intact cells and mediates mitogenesis and chemotaxis when transfected into porcine endothelial cells (20). In contrast, VEGFR-1 shows minimal tyrosine phosphorylation in response to VEGF, and binding of VEGF does not lead to a mitogenic signal (20,21). The phenotypes of VEGFR-1- and VEGFR-2-deficient mice are consistent with the critical importance of these receptors to the development of the vascular system (22,23). Mice carrying germ-line mutations in either VEGFR-1 or VEGFR-2 die before embryonic day 10 (E10) due to a failure to organize the vasculature in the case of VEGFR-1 or a complete failure to develop endothelial cells in the case of VEGFR-2. Recent studies have identified neuropilin-1 (NP-1), a receptor of the collapsin/sema-phonin family, as an isoform-specific VEGF receptor (24). NP-1 is structurally unrelated to VEGFR-1 and VEGFR-2 and plays a role in neural cell guidance, suggesting a broader role for VEGF in non-endothelial cells.

The signaling mechanism by which VEGF mediates vascular permeability remains essentially uncharacterized, although recent work has shown that nitric oxide may play a role (25,26).
Nevertheless, some studies have suggested that VEGFR-2 is the receptor responsible for the permeability activity (25, 27). The permeability induced by VEGF is transient and reversible, is not associated with mast cell degranulation or infiltration of inflammatory cells, and is not inhibited by antihistamines (5, 28). Recently, it has been shown that VEGF increases microvascular permeability within tumors by induction of clusters of small vesicles and vacuoles within the cytoplast. These structures have been termed vesicular-vacuolar organelles (VVO) (29, 30). They span the cytoplast and can form channels to connect the endothelial lumen to the tissue space, thereby allowing a point of exit for plasma and plasma proteins. The receptor signaling pathway that mediates formation of VVOs is at present not clear, nor are the roles of VEGFR-1, VEGFR-2, or NP-1 in this process. Other recent studies have suggested that VEGF can alter the tight junctions of endothelial cells, which may provide an additional mechanism for VEGF permeability (31, 32).

In this study we have used site-directed mutagenesis to determine the residues of mouse VEGF important for mediating functions via VEGFR-2 and to determine their effect on endothelial mitogenesis and vascular permeability. We found that mutations that mapped to the third variable (V3) domain of VEGF had the most profound effect on VEGFR-2 binding. A mutant that had this region substituted with the V3 domain from human placenta growth factor (PIGF) showed reduced binding to VEGFR-2 and an inability to induce mitogenesis or activation of a VEGFR-2 bioassay. Nevertheless, this mutant was found to be equivalent to wild type VEGF when evaluated for its ability to induce vascular permeability in a Miles assay. Furthermore, another member of the VEGF family, VEGF-D, was shown to be incapable of inducing vascular permeability, even though it has the ability to bind and activate VEGFR-2. These data imply that the two biological effects of VEGF, mitogenesis and vascular permeability, are mediated by different mechanisms and that permeability may be signaled through a receptor other than VEGFR-2.

MATERIALS AND METHODS

Cell Culture—Transfected CHO cells were grown in Glasgow modified Eagle’s medium (without L-glutamine and NaHCO3), Cytosystems, Castle Hills, New South Wales, Australia) supplemented with 10% heat-inactivated FBS, 50 μg/ml gentamicin, and was stably transfected into CHO K1 cells. Expressing clones were selected in the presence of 25 μM MSX. The mutant VEGF0 was also subcloned into the pEE6 vector and expressed in CHO cells.

Production of Antisera to Mouse VEGF—Mice were injected subcutaneously with 200 μg of VEGF in 0.5 ml of anhydrous formamide. Two to three weeks later, the mice were bled and the serum was collected. This serum was imprinted onto nitrocellulose, dried, and rehydrated in phosphate-buffered saline. The serum was incubated with blocking buffer (5% milk, 0.1% Tween 20, 1 M NaCl, 100 mM Tris-HCl, pH 7.5) for 1 h at room temperature. The bound material was then washed with washing buffer (5% milk, 0.1% Tween 20, 0.1 M NaCl, 300 mM Na2CO3, pH 9.5) for 3 h at 4°C before being incubated with the baculovirus-expressed recombinant VEGF protein in blocking buffer for 1 h at room temperature. The blots were then washed three times with washing buffer at 4°C before being incubated with secondary antibody for 1 h at room temperature. The secondary antibody was alkaline phosphatase conjugated to anti-mouse IgG. The blots were then washed with washing buffer before being incubated with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium for 1 h at room temperature. The blots were then washed and exposed to X-ray film.

Antibodies—mAbs to the extracellular domain of the tie2 receptor tyrosine kinase are described elsewhere (33). mAb 4H3 to the extracellular domain of VEGF was used in the biolayer interferometry analysis. A rabbit antiserum made to mouse VEGF is described below.

Purification of Mouse VEGF—Mice were sacrificed and blood was collected from the heart. The serum was collected and centrifuged at 10,000g for 20 min. The serum was then dialyzed against PBS and concentrated by ultrafiltration. The concentrated serum was then dialyzed against 2 ml of Buffer A (0.01 M Tris, 0.01 M NaCl, 0.02% Tween 20) for 2 h at room temperature. The dialyzed serum was then loaded onto a protein A column (Bio-Rad, Hercules, CA) and eluted with 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.02% Tween 20. The purified VEGF was then diluted in 0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.02% Tween 20, and incubated with 0.5 μM of 125I-protein A for 1 h at room temperature. After washing, the signal was detected by autoradiography using XAR film (Eastman Kodak Co.). Alternatively, blots were probed with anti-rabbit Ig horseradish peroxidase conjugated to goat anti-mouse IgG (Promega). Protein bands were visualized using the Enhanced Chemiluminescence kit (ECL, Amersham Pharmacia Biotech).
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2-EX-FLAG were identified by immunoprecipitation of [35S]methionine-labeled cells with anti-FLAG monoclonal antibody (M2, IBI/Kodak) and analysis by SDS-PAGE.

*Mutants of VEGF*—Mutants of mouse VEGF were generated by oligonucleotide-directed mutagenesis according to the methods of Kunkel. 

*Alignment of VEGF and PlGF Sequences*—VEGF and PlGF sequences were obtained from the EMBL database and analyzed by the Genetics Computer Group (GCG, Madison, WI) package. 

*RESULTS*—Alignment of VEGF and PlGF Sequences—VEGF and PlGF are two related members of the cystine knot family of growth factors. Both bind a common receptor, VEGFR-1, whereas only VEGF binds VEGFR-2. We hypothesized that examination of the sequence differences between VEGF and PlGF could provide details about the regions of VEGF specific for its interaction with VEGFR-2. In addition, knowledge of the related factor PDGF, which has been shown to interact with the PDGF β receptor via sequences within the third variable (V3) domain of brane and cytoplasmic domains of EpoR. This construct was subcloned into the expression vector pBOS and co-transfected into the Ba/F3 cell line with pgk-Neo at a ratio of 20:1. Transfected cells were selected in G418, and VEGFR-2 expressing cell lines selected by FACs analysis using mAb 4H3, which was directed to the VEGFR-2 extracellular domain. The VEGFR-2-Ig cDNA was then ligated to VEGFR-2-EX-FLAG, and 293EBNA cells were transfected with the expression vector pBOS and co-transfected into the Ba/F3 cell line expressing higher levels of VEGFR-2-IgEpoR were selected by growing the cells in either 5 μg/ml mAb 4H3 (an activating mAb) or 25 ng/ml recombinant VEGF.

**Use of VEGFR-2 Bioassay**—Cells expressing the VEGFR-2/EpoR chimera (VEGFR-2-bioassay cells) were washed three times in PBS, and then in complete medium lacking 10% WEHI-3D conditioned medium to remove residual WEHI-3L-2 media. Cells were aliquoted into 96-well microtiter plates containing dilutions of either WEHI-3D conditioned medium, 4H3 mAb, VEGF, VEGF mutants, PiGF, or medium alone. Cells were incubated for 48 h at 37 °C in a humidified atmosphere of 10% CO2. Cell proliferation was quantitated by the addition of 1 μCi of [3H]thymidine (Amersham Pharmacia Biotech) for 4 h prior to harvesting. Incorporated [3H]thymidine was determined using a cell harvester (Tomtec®, Orange, CT) and β counting (LKB 1205 liquid scintillation β counter, Amersham Pharmacia Biotech).

**Binding Assays with Soluble VEGFR Extracellular Domains**— Constructs encoding the extracellular domain of VEGFR-1 and VEGFR-2 fused to the Pc portion of human IgG1 were used for binding assays. VEGFR-1- and VEGFR-2-Ig cDNA (kindly supplied by K. Alitalo, E. Korpelainen & Y. Gunji, Helsinki) were expressed in 293EBNA cells grown in Dulbecco’s modified Eagle’s medium containing either low Ig serum or 0.2% BSA. The fusion proteins were then immunoprecipitated from the conditioned medium using protein A-Sepharose beads. These beads were then combined with 900 μl of medium from CHO-VEGF or CHO-VEGFO cells biosynthetically labeled with [35S]Cys/Met and 100 μl of 5% BSA, 0.2% Tween 20, 10 μg/ml heparin. The Sepharose beads were then washed twice with binding buffer (0.5% BSA, 0.02% Tween 20, 1 μg/ml heparin) at 4 °C, once with PBS, and boiled in SDS-PAGE sample buffer before resolving the proteins by SDS-PAGE.

**Bioassay Analysis**—The purified extracellular domain of VEGFR-2 (VEGFR-2-EX-FLAG) was coupled to the carboxymethylated dextran layer of the sensor chip using standard NHS-EDC chemistry for analysis of binding using an optical biosensor (BIAcore2000) (42). The residual activated ester groups were blocked with treatment with 1 M ethanolamine hydrochloride, pH 8.5, followed by washing with 10 μg/ml diethylamine to remove non-covalently bound material. Samples for analysis were diluted in HBS (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20). The viability of the bound VEGFR-2-EX-FLAG was assessed by binding of the anti-VEGFR-2 extracellular domain 4H3 mAb and purified VEGF.

**Purification of VEGF-DANaC-FLAG and VEGF-D-FULL-N-FLAG**—The VHD of VEGF-D, VEGF-DANaC, VEGF-D-FULL was purified using M2 affinity chromatography as described previously (14). Material used for the Miles assay was evaluated in the VEGFR-2 bioassay and shown to be active for binding VEGFR-2. VEGF-D-FULL-N-FLAG was produced in 293EBNA cells and purified by M2 chromatography as described elsewhere (43).

**Miles Vascular Permeability Assay**—The Miles vascular permeability assay (38) was performed using anesthetized guinea pigs as described previously (44). The amount of dye extracted from the skin with formamide was quantitated by spectrophotometry at 620 nm.

**Human Microvascular Endothelial Cell (HMVEC) Proliferation Assay**—HMVECs were grown in EBM-2, 5% FBS, and growth supplements. For the assay, cells were removed with trypsin, washed, and resuspended in complete medium and aliquoted at 104 cells/well in a 24-well plate. Cells were allowed to adhere for 16 h at 37 °C after which the appropriate dilution of VEGF, VEGF mutant, or control sample was added in EBM-2, 22% FBS plus supplements but without growth factors. After 96 h of growth at 37 °C the cellular proliferation was quantitated by cell counting.

**Alignment of VEGF and PiGF Sequences**—VEGF and PiGF are two related members of the cystine knot family of growth factors. Both bind a common receptor, VEGFR-1, whereas only VEGF binds VEGFR-2. We hypothesized that examination of the sequence differences between VEGF and PiGF could provide details about the regions of VEGF specific for its interaction with VEGFR-2. In addition, knowledge of the related factor PDGF, which has been shown to interact with the PDGF β receptor via sequences within the third variable (V3) domain of...
Fig. 1. Alignment of the predicted amino acid sequences of mouse VEGF, human VEGF, mouse PlGF, and human PlGF in the region of the cystine knot motif. The mouse VEGF sequence is numbered from the first residue of the secreted protein, whereas the PlGF sequences are numbered from their initiation methionines. The sequences are reported elsewhere (15, 37, 56). β strands (β1–7), α helices (α1–2), and variable domains (V1–3) are indicated. The shaded sequence represents the region of mouse VEGF that was replaced by the corresponding region of human PlGF in the VEGF0 mutant.

Fig. 2. Western blotting analysis of VEGF mutants. Wild type VEGF or mutant VEGF cDNA was expressed in COS cells, and the conditioned media were concentrated by heparin-Sepharose chromatography. The resulting preparations were then quantitated by a competitive radioimmunoassay and Western blotting. The VEGF0 mutant was also expressed in CHO cells and purified by affinity chromatography. As mutations did not change the number of amino acids in the polypeptides, there was little detectable difference in the relative migration of the mutants by SDS-PAGE under non-reducing conditions the mutants migrated in the same size of the biologically active VEGF homodimer (Fig. 2A). Unreduced samples were then electrophoresed on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes that were probed with polyclonal antisera raised to purified mouse VEGF. Blots were then probed with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and the gel developed using the ECL detection system. Dimeric VEGF polypeptides are indicated by large arrowheads, and the smaller arrowheads indicate the VEGF monomer.
Binding of VEGF Mutants to VEGFR-2—
To evaluate whether the VEGF0 mutant, which failed to activate the VEGFR-2 bioassay, lacked the ability to bind VEGFR-2, binding studies were performed with soluble receptor extracellular domains and with immobilized VEGFR-2-EX-FLAG using the BIAcore. VEGF0 was able to bind the extracellular domain of VEGFR-1 (human) to the same level as wild type VEGF; however, the mutant displayed a significantly reduced level of binding to the dimeric human VEGFR-2-Ig construct indicating that its binding sites for VEGFR-2 had been substantially altered (Fig. 5A). This result was confirmed by analysis of purified VEGF and VEGF0 for binding to immobilized VEGFR-2-EX-FLAG (monomeric) on the optical biosensor (Fig. 5B). VEGF bound to the VEGFR-2 domain in a dose-dependent manner, whereas the non-binding but structurally related factor PlGF fails to induce proliferation (Fig. 3D). Some VEGF mutants (Q36D, D40S, E43S, Y44H, K47S, E66H, and E72T) had little or no effect on the ability of the molecule to induce proliferation in the VEGFR-2 bioassay, presumably because these mutations are not critical for the interaction of VEGF with VEGFR-2 (data not shown). However, a subset of the VEGF mutants, in particular those that mapped to the V3 domain of VEGF, exhibited reduced ability to stimulate proliferation in the VEGFR-2 bioassay (Fig. 4). The VEGF0 mutant, which had part of the VEGF V3 domain substituted for the V3 domain of PlGF, had almost a 100-fold reduction in its ability to stimulate proliferation in the bioassay, which indicated that the VEGF binding determinants had been disrupted in this mutant (or it lacked the ability to cross-link the receptors). When VEGF mutants which contained single amino acid substitutions in the V3 domain were evaluated for their ability to induce proliferation, most showed some reduction in their ability to stimulate the bioassay cell line, when compared with VEGF itself. The most significant were K83A, H85G, and I90Y, whereas G91V gave only slight reduction in activity.

Fig. 3. VEGFR-2/EpoR bioassay cells express a VEGFR-2/EpoR chimeric molecule and respond to purified VEGF and mAb 4H3. A, mouse VEGF (164 form) was purified from conditioned medium of CHO-VEGF cells using the protocol described under “Materials and Methods.” VEGF (2 μg) was combined with 2× SDS-PAGE sample buffer, under either non-reducing (lane 1) or reducing conditions (lane 2), and analyzed by SDS-PAGE and silver staining. Monomeric and dimeric forms of VEGF are indicated by arrowheads. B, flow cytometric analysis of VEGFR-2/EpoR chimeric molecules expressed on Ba/F3 cells. VEGFR-2-EpoR-Ba/F3 cells were stained with either anti-VEGFR-2 mAb (4H3, shaded) or control anti-α2 mAb (3 g1, unshaded) as described under “Materials and Methods.” C, mouse VEGF, human PlGF, or WEHI-3D-conditioned medium (containing IL-3) were tested for their ability to induce proliferation of the VEGFR-2/EpoR cells in a [3H]thymidine assay. Washed cells (10⁴) were plated in a 96-well plate with dilutions of VEGF (starting concentration at 10% dilution: 100 ng/ml), human PlGF (starting concentration 100 ng/ml), WEHI-3D (10% conditioned medium). D, mouse VEGF, anti-VEGFR-2 mAb 4H3 or medium control were tested for their ability to induce proliferation of the VEGFR-2/EpoR cells in a [3H]thymidine assay. Washed cells (10⁴) were plated in a 96-well plate with dilutions of VEGF (starting concentration at 10% dilution: 100 ng/ml) and anti-VEGFR-2 mAb 4H3 (500 ng/ml). Cells plated in “Medium” alone were also assessed in the bioassay, to establish a baseline level of response. C and D, the graphs represent the concentration of stimulating factor versus incorporated counts (cpm). The values represent means ± S.D.

Fig. 4. Evaluation of VEGF and VEGF mutants in the VEGFR-2-EpoR bioassay. Washed VEGFR-2 bioassay cells (10⁴) were incubated with dilutions of VEGF or mutant VEGF molecules with mutations in the V3 domain. Preparations of VEGF, K83A, H85G, Q86D, I90Y, G91V, VEGF0, and vector control were diluted from a starting concentration of 5 pM and incubated with cells for 48 h at 37 °C in a humidified atmosphere of 10% CO₂. Cell proliferation was quantitated by the addition of 1 μCi of [3H]thymidine and counting the amount incorporated over a period of 4 h. The graph shows the mean of at least three assays for each mutant. Error bars represent one S.D.
manner, whereas VEGF0 demonstrated no detectable binding even at 10 μg/ml. These data suggest that the binding sites for VEGFR-2 have been significantly disrupted in VEGF0 leading to reduced binding. The minor binding component seen in the immunoprecipitation experiment may be due to the use of dimeric constructs.

Evaluation of Mutants in an Endothelial Cell Proliferation Assay—VEGF0 was tested in an endothelial cell proliferation assay to determine if the reduction in activation of VEGFR-2 in the bioassay was seen with full-length VEGFR-2. Studies by others have demonstrated that the mitogenic activity of endothelial cells is via VEGFR-2 and not VEGFR-1 (48). VEGF and VEGF0 were evaluated for their ability to induce proliferation of HMVEC cells after 96 h stimulation using high concentrations (100–10,000 ng/ml) in the Miles assay. Control VEGF was also tested and, as expected, induced strong permeability at concentrations below 100 ng/ml. In contrast VEGF-D (VEGF-D-FULL-N-FLAG) also binds to VEGFR-2 but at a substantially reduced affinity (~300-fold less than for VEGF-DNAC (43)). To determine whether these VEGFR-2 ligands also induced vascular permeability, we assayed the purified VEGF-DNAC-FLAG and VEGF-D-FULL-N-FLAG over a range of concentrations (100–10,000 ng/ml) in the Miles assay. Control VEGF was also tested and, as expected, induced strong permeability at concentrations below 100 ng/ml. In contrast VEGF-DNAC-FLAG and VEGF-D-FULL-N-FLAG produced no significant induction of vascular permeability over a range of concentrations (Fig. 8). In order to confirm that the purified VEGF-DNAC-FLAG and VEGF-D-FULL-N-FLAG bound to VEGFR-2, samples were tested in the bioassay and in binding assays using the biosensor (data not shown). This study indicates that not all VEGFR-2 binding growth factors induce vascular permeability.

DISCUSSION

The molecular mechanisms whereby VEGF induces vascular permeability are not well understood. In particular the cell-surface receptor(s) responsible for coordinating this rapid extravasation of plasma proteins are essentially uncharacterized. In this study we have examined the structure-function relationship of mouse VEGF using site-directed mutagenesis. Re-
gions in and around the V3 domain of the VHD were found to be critical for binding to the major mitogenic receptor VEGFR-2. Interestingly, mutants altered in the V3 region that did not bind VEGFR-2 were still capable of inducing vascular permeability, thus demonstrating that the two biological functions of VEGF, induction of mitogenesis and vascular permeability, can be distinguished by mutations within the V3 domain. These results imply that vascular permeability and mitogenesis induced by VEGF are mediated through the recruitment of different receptors.

In order to evaluate VEGF mutants in an assay system that would be responsive to the binding and cross-linking of VEGFR-2 only, we avoided the use of in vitro cell lines that may express other VEGF receptors. This approach excludes the possibility that other receptors could initiate signaling alone or as part of a heterodimeric complex with VEGFR-2. The approach we followed, of developing a factor-dependent cell line expressing a chimeric RTK/cytokine receptor, was initially described by others (47) as a means of detecting the cross-linking/activation of the ligand binding domains of orphan receptors. The system has been shown to work effectively for other receptors that rely on dimerization for signal transduction (e.g. EGFR (47) and Tie2 in our own laboratory). 2

Screening of the mutant VEGF molecules in the bioassay demonstrated that the V3 domain between β strands 5 and 6 is critical for binding to and activation of VEGFR-2. In particular, the point mutations H85G and I90Y caused dramatic reductions in the level of activity seen in the bioassay. When the loop containing residues 83–89 was replaced with the equivalent loop from human PlGF, to generate VEGF0, a similar reduction in activity was observed, suggesting that key substitutions had been made. The VEGF0 mutant contains substitutions at each position throughout the amino acid sequence 83–89 and appears to be one of the most severely affected of the mutants consistently giving low stimulation in the bioassay. Our results are consistent with studies from other laboratories that have examined the binding determinants of human VEGF for VEGFR-2 (48, 49). In particular, they are also consistent with the large “hot spot” for interaction with VEGFR-2 on the crystal structure of human VEGF, which includes residues Ile-83, Lys-84, and Pro-85 (82–84 in the mouse) of the V3 region (50).

Our results with the VEGF0 mutant are also in agreement with a previous suggestion that residues in the loop between β strands 5 and 6 of human VEGF, specifically residues 85, 86 and 89, might influence the conformation of the loop and thus determine the difference in binding to VEGFR-2 between VEGF and PlGF. One could argue that flexibility in this region may therefore be important for binding and activation of VEGF-2 for mitogenic signaling but have less significance for

Fig. 7. Permeability induced by wild type VEGF, mutant VEGFs, and controls in the Miles assay. The figure represents skin patches taken from guinea pigs in which the Miles assay has been performed. Guinea pigs are given an intracardiac injection of Evans blue dye followed by intradermal injections of aliquots of expressed, purified, and quantitated protein (100 µL). The amount (ng) of VEGF/VEGFR mutant protein (quantitated by the competitive radioimmunoassay) injected is indicated above the sample area. After 20 min the animals are sacrificed and the skin patches taken. The Vector sample represents the protein affinity purified from the equivalent amount of conditioned medium from cells transfected with vector lacking VEGF sequence.

Fig. 8. Vascular permeability induced by VEGF-D and controls in the Miles assay. Aliquots of purified VEGF, VEGF-DANAC-FLAG, and VEGF-D-FULL-N-FLAG were injected intradermally into guinea pigs that had Evans blue dye circulating in their bloodstream. The patch of skin at the site of each injection was excised, and dye was extracted from the skin with formamide and the absorbance of the solution quantitated at 620 nm using spectrophotometry. The values are expressed as mean ± S.D. The horizontal axis represents the concentration of growth factor injected per site in ng/ml.
VEGF Mutant with Permeability but Not Mitogenic Activity

The receptor(s) involved in transducing the signal for vascular permeability are as yet uncharacterized. Both VEGFR-1 and VEGFR-2 bind VEGF, and recent data indicate that neuropilin-1 present on endothelial cells and other non-endothelial cell populations can also specifically bind VEGF (24). Given that the induction of vascular permeability occurs readily and extremely rapidly (in less than 5 min) in the peripheral vessels of mature animals, a receptor with constitutive expression on resting endothelium is likely to be involved. However, VEGFR-1 and VEGFR-2 appear to be down-regulated on adult quiescent endothelium compared with fetal endothelium (19, 52, 53) suggesting that these receptors may not be involved in the induction of vascular permeability. Recent experiments describing a mutant of VEGF-C (27) which has lost VEGF binding and permeability activity were suggestive of a role for VEGFR-2 in vascular permeability. Furthermore, we and others (44, 54) have recently characterized viral VEGF molecules from the orf virus family which bind VEGFR-2 and neuropilin-1, or in some cases VEGFR-2 alone, but not VEGFR-1 and VEGFR-3, and induce vascular permeability. In combination these results are suggestive of a role for VEGFR-2 in vascular permeability, although they do not rule out the involvement of an alternative receptor that can bind these mutants and the viral VEGF molecule. VEGFR-1 which binds in addition to PI GF and VEGF-B lack significant permeability (25, 43). NP-1 would also appear not to play a role in vascular permeability as it binds only VEGF

whereas the non-binding VEGF_121 has been shown to induce vascular permeability (55). Our findings with the VEGF mutants and the VHD of VEGF-D suggest that VEGFR-2 by itself is not sufficient for inducing vascular permeability. This leads to two possible conclusions. First, the use of an alternative receptor that has high affinity binding for VEGF and that itself induces the signal for permeability. An alternative explanation would be the use of a heterodimeric complex involving VEGFR-2 in which only low affinity binding of VEGF to VEGFR-2 is required, with the other receptor providing the high affinity interaction. Both of these alternatives are not inconsistent with the results generated by the VEGF mutant.

Recent work (25, 26) has shown a link between the nitric oxide/prostaglandin pathway and vascular permeability induced by VEGF. These findings are not inconsistent with the findings presented here and may suggest that the receptor-mediating permeability acts via nitric oxide or that they both converge on the same downstream signaling molecules.

In summary, the data presented here show that two of the biological functions of VEGF, namely induction of mitogenesis and of vascular permeability, can be distinguished by mutations that alter the binding of VEGF to VEGFR-2. A VEGF mutant that induces permeability but not mitogenesis would be of practical value to examine its effects on the morphology changes of endothelial cells during vascular permeability as distinct from changes that may occur due to cell division. The data presented here also suggest the possibility that one or more as yet unidentified VEGF receptors may exist that are responsible for vascular permeability. The recent unexpected description of NP-1 as a receptor for VEGF demonstrates that other VEGF receptors may indeed exist.

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A Mutant Form of Vascular Endothelial Growth Factor (VEGF) That Lacks VEGF Receptor-2 Activation Retains the Ability to Induce Vascular Permeability

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