Heterodimers of Placenta Growth Factor/Vascular Endothelial Growth Factor

ENDOTHELIAL ACTIVITY, TUMOR CELL EXPRESSION, AND HIGH AFFINITY BINDING TO Flk-1/KDR*

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Here we show that the Escherichia coli expressed monomers of placenta growth factor (PLGF)129 and vascular endothelial growth factor (VEGF)165 can be refolded in vitro to form PLGF/VEGF heterodimers. The purified recombinant PLGF/VEGF heterodimers and VEGF homodimers have potent mitogenic and chemotactic effects on endothelial cells. However, PLGF/VEGF heterodimers display 20-50-fold less mitogenic activity than VEGF165 homodimers. In contrast, PLGF129 homodimers have little or no effect in these in vitro assays. We also demonstrate the presence of natural PLGF/VEGF heterodimers in the conditioned media of various human tumor cell lines. While PLGF/VEGF heterodimers bind with high affinity to a soluble Flk-1/KDR receptor, PLGF129 homodimers fail to bind to this receptor. Cross-linking of 125I-ligands to human umbilical vein endothelial cells reveals that PLGF/VEGF heterodimers and VEGF165 homodimers, but not PLGF129 homodimers, form complexes with membrane receptors. VEGF165 homodimers and PLGF/VEGF heterodimers stimulate tyrosine phosphorylation of a 220-kDa protein, the expected size for the KDR receptor in human umbilical vein endothelial cells, whereas PLGF129 homodimers are unable to induce tyrosine phosphorylation of this protein. These data indicate that PLGF may modulate VEGF-induced angiogenesis by the formation of PLGF/VEGF heterodimers in cells producing both factors.

Angiogenesis, the growth of new capillary vessels, is a multi-step process that involves enzymatic degradation of the basement membrane of a local venule, capillary endothelial cell proliferation, migration, tissue infiltration, and lumen formation (1, 2). It is required for many physiological and pathological processes such as embryonic development, wound healing, tissue and organ regeneration, diabetic retinopathy, and tumor growth (3). Neovascularization is essential for solid tumor growth and is thought to be regulated by tumor cell-produced factors that have mitogenic and chemotactic effects on vascular endothelial cells (4, 5).

A variety of growth factors can stimulate angiogenesis in vitro and in vivo (6). Among these angiogenic factors, vascular endothelial growth factor (VEGF), also known as vascular permeability factor (7, 8), has been characterized as an endothelial cell-specific growth factor. VEGF/vascular permeability factor is a homodimeric 34–42-kDa glycoprotein with potent endothelial cell mitogenic, chemotactic, and vascular permeability-enhancing activities (9–12). The gene for human VEGF is organized into 8 exons. As a result of alternative splicing, at least four transcripts encoding mature monomeric VEGF containing 121, 165, 189, and 206 amino acid residues (VEGF121, VEGF165, VEGF189, and VEGF206) have been identified (13, 14). While VEGF121 and VEGF165 are diffusible proteins that are secreted into the medium, VEGF189 and VEGF206 are mainly bound to heparan sulfate proteoglycans on the cell surface and in the extracellular matrix (15, 16). VEGF contains a potential N-linked glycosylation site; the natural protein is a glycoprotein. Recombinant VEGF expressed in Escherichia coli is indistinguishable from the natural protein in its in vitro biological actions, suggesting that the carbohydrate moiety may not be required for activity. VEGF is a highly conserved protein that has a cross-species activity.

A cDNA encoded protein having 53% amino acid sequence identity in the PDGF-like region of VEGF has recently been isolated from a human placenta cDNA library (17). This protein, named placenta growth factor (PLGF), is now recognized as a member of the family of vascular endothelial growth factors. Based on its homology with VEGF, PLGF was also proposed to be an angiogenic factor although little is known about its biological functions in vivo (17). As a result of alternative splicing of the same gene, at least two different mRNAs coding for monomeric PLGF precursors with 149 and 170 amino acids have been described similar to VEGF (18). The smaller isoform, also called PLGF-1, has a 20-amino acid signal peptide that is cleaved to yield a 129-residue mature protein. The large isoform (PLGF-2) is identical to PLGF-1 except for the insertion of a highly basic 21-amino acid sequence encoded by exon 6. This positively charged sequence increases the binding affinity of PLGF-2 to heparin or heparin-like molecules (18, 19). Thus, PLGF-1 is readily secreted into the conditioned medium, whereas PLGF-2 is most likely sequestered on the cell surface or in the extracellular matrix.

In normal tissues, VEGF has been found to be expressed in a variety of cell types such as activated macrophages, keratino-

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‡ Supported by the International Human Frontier Science Program.

§ Supported by the National Institutes of Health Grant PO1-CA-45548. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; PLGF, placenta growth factor; PDGF, platelet-derived growth factor; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; AP, alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay.
PLGF and VEGF, we have developed both interchain disulfides are conserved among these growth chains of PDGF. All eight cysteine residues involved in intra- but significant (approximately 18%) homologies with A and B form of Flt-1 has been cloned from a human vascular endothelial kinase domains (28). These receptors are almost exclusively contain seven immunoglobulin-like domains in their intracellular regions and large insert sequences in their intracellular kinase domains (28). These receptors are almost exclusively expressed on endothelial cells (29). In addition to the membrane-spanning Flt-1, a cDNA coding for a truncated soluble form of Flt-1 has been cloned from a human vascular endothelial cell library (30). PLGF bind with high affinity to Flt-1, but not to KDR/Flik-1 (31).

The amino acid sequences of VEGF and PLGF reveal limited but significant (approximately 18%) homologies with A and B chains of PDGF. All eight cysteine residues involved in intra- and interchain disulfides are conserved among these growth factors (14). In order to study the possible interactions between PLGF and VEGF, we have developed both in vitro and in vivo heterodimerization assays of these two factors. We report here that PLGF forms a heterodimer with VEGF. We show that PLGF/VEGF heterodimers exert overlapping but different biological activities on endothelial cells and on the receptor-mediated signal transductions as compared to PLGF and VEGF homodimers.

**Experimental Procedures**

In Vitro Heterodimerization between PLGF129 and VEGF165—The E. coli expressed homodimeric PLGF129 and VEGF165 were obtained from R&D Systems Inc. An equimolar mixture of PLGF129 and VEGF165 at a total protein concentration of 0.5 mg/ml was incubated in 20 mM Tris-HCl, pH 8.0, 6 mM guanidine-HCl, and 10 mM DTT. The thiol groups in the unfolded and reduced PLGF and VEGF were protected with 5 mM dithiothreitol. After an incubation period of 20 min at 25°C, the protein solution was dialyzed overnight at 4°C against 10 volumes of 25 mM Tris-buffered saline, 0.5% Tween 20, pH 7.4. Subsequently, the protein solution was dialyzed at 4°C overnight against 20 volumes of 25 mM Tris-HCl, pH 8.0. Using this re-forming protocol, a mixture of homodimeric PLGF129 and VEGF165 as well as heterodimeric PLGF/VEGF was generated.

Purification of PLGF/VEGF Heterodimers—The homodimeric and heterodimeric proteins were separated from one another by affinity chromatography using a goat polyclonal anti-VEGF antibody affinity column and a goat anti-PLGF polyclonal antibody column. The antibody columns were prepared by coupling the antigen affinity-purified anti-VEGF antibodies to the commercially available agarose support Affi-Gel 10 (Bio-Rad) at pH 7.5 according to protocols recommended by the manufacturer. The protein solution was applied at a flow rate of 2 ml/min initially on the anti-VEGF antibody-affinity column equilibrated with phosphate-buffered saline. The column was then washed at the same flow rate with phosphate-buffered saline until the absorbance reading at 280 nm reached baseline level. VEGF homodimers and PLGF/VEGF heterodimers, but not PLGF homodimers, were retained by the column and eluted with 0.1 M sodium citrate, 0.3 M NaCl (elution buffer). The VEGF homodimers and PLGF/VEGF heterodimers eluted from the anti-VEGF column were dialyzed against 20 volumes of phosphate-buffered saline at 4°C for 4 h and applied to the anti-VEGF-affinity column under the same conditions. Only PLGF/VEGF heterodimers but not VEGF homodimers bound to the column. The heterodimer was then eluted by the same elution buffer. The purified PLGF/VEGF heterodimeric protein was then dialyzed against phosphate-buffered saline and analyzed by SDS-PAGE, followed by measuring protein concentrations.

Mitogenic Assay—Each well of 96-well plates was coated with 50 μl of solution containing 40 μg/ml type I collagen for 2 h at room temperature or at 4°C overnight. Human umbilical vein endothelial (HUVE) cells (10^4) suspended in 5 × 10^4 cells/ml of medium and incubated for 72 h at 37°C under 5% CO2. Serial dilutions of PLGF and VEGF homodimers or PLGF/VEGF heterodimers were prepared in an assay medium (Medium 199 with 10 mM HEPES, 10% heat-inactivated fetal calf serum, and antibiotics) and 50 μl of each dilution was added in duplicate to each wells. [H]thymidine (0.5 μCi/well) was added to each well and pulse-labeled with [H]thymidine for the last 16–24 h of the total 72-h incubation period. At the end of incubation, cells were harvested onto a glass fiber filter and the amount of [H]thymidine incorporated into DNA was determined by a Packard Matrix-96 β counter.

Chemotaxis Assay—The motility responses of HUVE cells to PLGF129 homo-dimers, VEGF165 homo-dimers, and PLGF/VEGF heterodimers were assayed using a modified Boyden chamber technique (32). Nucleopore polyvinylpyrrolidone-free polycarbonate filters with pore size of 8 μm (25 × 80 mm, Nuclepore Co., Pleasant, CA) were coated with 13.4 μg/ml fibronectin (Organon Teknika Co., West Chester, PA) in PBS for 30 min. The filter was allowed to air-dry and placed on a 48-well chamber plate. Approximately 10,000 cells in 50 μl of serum-free M199 containing different concentrations of PLGF129 homo-dimers or PLGF/VEGF heterodimers, VEGF165 homo-dimers and PLGF/VEGF heterodimers were added to each well. The chamber plate was incubated at 37°C for 5 h. Following incubation, filters were fixed for 40 min with 10% formalin, washed with PBS, and stained overnight with Giil's triple strength hematoxalin (Polysciences, Inc., Warrington, PA). Following destaining with H2O, filters were mounted with Permount (Sigma) on glass plates and total cell numbers were counted. All experiments were performed as triplicates for each concentration of PLGF129 homo-dimers, VEGF165 homo-dimers and PLGF/VEGF heterodimers.

ELISA Immunoassays for the Quantitative Measurement of PLGF Homodimers, VEGF Homodimers, and PLGF/VEGF Heterodimers—A goat polyclonal VEGF antibody at a concentration of 0.2 μg/ml was coated onto each well of 96-well plates in a volume of 200 μl was coated onto each well of 96-well plates. After 2 h incubation at room temperature, the coated plates were washed with Tris-buffered saline, 0.5% Tween 20, pH 7.4. The wells were then blocked overnight at room temperature with phosphate-buffered saline containing 1% bovine serum albumin. After blocking, 200 μl each of VEGF homo-dimer standards or samples were added to the wells and incubated at room temperature for 2 h. The unbound solution was aspirated, followed by three washes with a mixture of Tris-buffered saline and Tween 20, pH 7.4. After the last wash, 200 μl of a solution containing 0.3 μg/ml of horseradish peroxidase-conjugated and affinity-purified anti-VEGF polyclonal antibody was added to each well and incubated at room temperature for 2 h. At the end of incubation, the microtiter plates were washed three times with Tris-buffered saline. To each well of a substrate solution containing 10 mM H2O2 and 0.2 mg/ml of 2 M sulfuric acid was added to each well to stop the color reaction. The optical density of each well was determined at 450 nm using a spectrophotometer.

For quantitation of PLGF homo-dimers, 200 μl of an affinity-purified goat anti-human PLGF polyclonal antibody at a concentration of 2 μg/ml was coated onto each well of 96-well microtiter plates. After an overnight incubation, the solution was removed and wells were washed and blocked as mentioned above. At the end of incubation, wells were washed and color development was performed as described above.

For quantitation of PLGF/VEGF heterodimers, 96-well microtiter plates identical to those used in the PLGF homodimer-ELISA were used. However, instead of the horseradish peroxidase-conjugated anti-PLGF polyclonal antibody, an enzyme-conjugated anti-VEGF polyclonal antibody was used for detection. This ELISA assay is specific for detection of PLGF/VEGF heterodimers and shows no cross-reactivity with PLGF or VEGF homodimers.

Iodination Labeling—For 125I labeling, 5 μg of IODO-GEN (Pierce) was dissolved in chloroform in a volume of 100 μl. The IODO-GEN was dried in a glass tube by evaporating the solution with nitrogen gas. The reagents were dissolved in 0.2 M phosphate buffer (pH 7.2) containing a recombining human PLGF129 homo-dimers, VEGF165 homo-dimers, and PLGF/VEGF heterodimers, respectively. Na2125I (DuPont NEN) of 500 μCi was added to each reaction and incubated for 15 min at room temperature. The reactions were terminated by addition of 50 μl of 10
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Discuscinimylidate cross-linker and incubated at room temperature for 30 min. Cells were washed three times with PBS and then lysed with 0.5% Nonidet P-40, 1 mg/ml EDTA, 1 mg/ml phenylmethylsulfonyl fluoride in PBS. After centrifugation, the soluble fraction of each lysate was analyzed by a 12% SDS gel.

Tyrosine Phosphorylation Assay—Monolayers of HUVE cells were grown in M199 medium supplemented with 20% fetal calf serum and growth factors (bovine pituitary extracts) to confluence in 25-cm² flasks. Cells were incubated with M199 medium containing 20% fetal calf serum without added growth factors for 24 h. Confluent cells were incubated with growth factor-deprived M199 medium containing 0.5% serum for 24 h. Media were then removed, and cells were incubated for 5 min at 37°C in the presence of deprived medium containing 100 ng/ml PLGF129 homodimers, VEGF165 homodimers, and PLGF/VEGF heterodimers, respectively. At the end of incubation, media were aspirated and cells were washed twice with cold PBS containing 1 mg/ml sodium vanadate. Cells were then lysed at 4°C with 200 μl of lysis buffer containing 1% Triton X-100, 37 mM NaCl, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10% glycerol, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 0.15 mM aprotinin, and 20 μg/ml leupeptin. The cell extracts were centrifuged, and the supernatants were mixed with a double-strength Laemmli sample buffer and subjected to electrophoresis on a 12% SDS-polyacrylamide gel. The gel was electroblotted to a nitrocellulose membrane. Following incubation with 4G10 anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at 4°C overnight, immunoreactive bands were visualized with a Western blotting detection kit (Amersham Corp.).

RESULTS

Generation of PLGF/VEGF Heterodimers in Vitro—To study the heterodimerization between VEGF and PLGF, we expressed recombinant human PLGF129 and VEGF165 monomers that would give different mobilities on SDS gels. Using protocols described under “Experimental Procedures,” recombinant monomers of PLGF129 and VEGF165 expressed from E. coli could be readily dimerized into a mixture of PLGF129 homodimers, VEGF165 homodimers and PLGF/VEGF heterodimers after removal of reducing reagent, DTT (Fig. 1A). As shown in Fig. 1A, SDS-PAGE analysis under reducing (lanes 5–8) and non-reducing (lanes 1–4) conditions revealed both monomeric and dimeric forms of PLGF129, VEGF165, and PLGF129/VEGF165. Each of these dimers was purified to homogeneity as detected by silver staining (lanes 1, 2, 4, and 5). The PLGF129 and VEGF165 monomers (lanes 6 and 5, respectively) have predicted molecular masses of 14.5 and 19.5 kDa, respectively. Thus, PLGF129 homodimers (lane 2), VEGF165 homodimers (lane 4) and PLGF129/VEGF165 heterodimers (lanes 3 and 4), each with equal weight and volume of the extracellular domains of each, were harvested at 4°C with an equal volume of the conditioned medium containing 2.5 μg/ml of the ligand-Flk-AP receptor complexes were then immunoprecipitated by protein A-Sepharose (Pharmacia). For co-immunoprecipitation, the 35S-labeled materials precipitated by the anti-VEGF antibody were eluted by the SDS Laemmli sample buffer (36). The eluted materials were further immunoprecipitated with the anti-PLGF specific antibody in the solubilization buffer containing a final concentration of 0.003% SDS. The precipitated proteins were harvested by protein A-Sepharose and were analyzed on a 15% SDS-polyacrylamide gel as described previously.

Co-immunoprecipitation Using the Flk-AP Receptor—Thirty nanograms of the 125I-labeled PLGF129 homodimer, VEGF165 homodimer, and PLGF/VEGF heterodimer (30,000 cpm) in 200 μl of solubilization buffer were incubated for 12 h at 4°C with a 12% polyacrylamide gel containing 2.5 μg/ml of Flk-AP fusion protein. The ligand-FK-AP receptor complexes were then immunoprecipitated by incubation on a rotator for 60 min with CNBr-Sepharose beads coupled to an excess amount of monoclonal antibody against human placental alkaline phosphatase (Medix Biotech Inc., Foster City, CA). The beads were then washed 6 times in a modified TNE buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl). The radiolabeled proteins were separated on a 15% SDS gel.

Cross-linking Ligand-Receptor Complexes—125I-PLGF129 homodimers, 125I-VEGF165 homodimers, and 125I-PLGF/VEGF heterodimers (30 ng/ml) were incubated at 4°C with 0.5 μl of conditioned medium containing 2.5 μg/ml of the soluble Flk-AP chimeric receptor for 12 h. The final volume of the reaction was 500 μl. Bis(sulfosuccinimidyl) suberate cross-linker was added at the end of incubation to a final concentration of 1 μM for 2 min at 4°C. The cross-linking reaction was terminated by the addition of glycerol to a final concentration of 100 μl. The cross-linked complexes were immunoprecipitated by the anti-AP antibody and were analyzed by a 12% SDS gel.

For chemical cross-linking of iodinated factors to endothelial cells, monolayers of HUVE cells were grown to confluence in six-well plates. The conditioned medium was removed, and cells were washed twice with phosphate-buffered saline. Twenty nanograms of 125I-PLGF129 homodimers, 125I-VEGF165 homodimers, and 125I-PLGF/VEGF heterodimers were added to each well in a total volume of 1 ml of Dulbecco’s modified Eagle’s medium without serum. The mixture was incubated at 4°C for 1 h, followed by addition of 20 μl of 10 μm discuscinimylidate cross-linker and incubated at room temperature for 30 min. Cells were washed three times with PBS and then lysed with 0.5% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride in PBS. After centrifugation, the soluble fraction of each lysate was analyzed by a 12% SDS gel.

Western immunoblotting analysis showed that a polyclonal antibody raised against PLGF specifically recognized both monomers (Fig. 1B, lane 6) and homodimers of PLGF129 (lane 2), but not VEGF (lanes 1 an 5). Similarly, an anti-VEGF polyclonal antibody specifically reacted with VEGF165 homodimers (Fig. 1C, lane 1) or monomers (lane 5), but not with PLGF (lanes 2 and 6). The heterodimeric form of PLGF/VEGF could be detected by the anti-PLGF antibody (Fig. 1B, lanes 3 and 4) and the anti-VEGF antibody (Fig. 1C, lanes 3 and 4). From these results, we conclude that the PLGF129 and VEGF165 monomers expressed from E. coli, similarly to PDGF A chain and B chain monomers, can be re-folded in vitro to form PLGF/VEGF heterodimers in addition to their homodimers.

Mitogenic Activity on Human Umbilical Vein Endothelial Cells—The mitogenic effects of PLGF129 homodimers, VEGF165 homodimers and PLGF/VEGF heterodimers on cultured HUVE cells were examined. The VEGF165 homodimer expressed by E. coli was a potent endothelial cell mitogen, which stimulated DNA synthesis of HUVE cells at nanogram levels (Fig. 2). The concentration of VEGF homodimer that elicited a half-maximal...
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Fig. 1. Generation of PLGF/VEGF heterodimers in vitro. A, SDS-PAGE analysis of dimers (lanes 1–4) and monomers (lanes 5–8) of recombinant PLGF129 (lanes 2 and 6), VEGF165 (lanes 1 and 5), and PLGF129/VEGF165 under reducing (lanes 1–4) and non-reducing (lanes 5–8) conditions. The PLGF/VEGF heterodimers (lanes 3 and 4) were prepared from a mixture of equimolar amounts of PLGF129 and VEGF165 monomers in the presence of DTT followed by dialysis (see "Experimental Procedures"). About 300 ng of each protein purified by affinity chromatographies was loaded onto a 12% SDS gel, followed by silver staining. B, Western immunoblot analysis of monomers (lanes 1–4) of recombinant PLGF129, VEGF165, and PLGF129/VEGF165 using a goat anti-human PLGF polyclonal antibody. The antibody specifically recognized PLGF129 homodimers (lanes 2 and 3), PLGF129 monomers (lanes 6–8), and PLGF/VEGF heterodimers (lanes 3 and 4). No cross-reactivities with either VEGF165 homodimers (lane 1) or monomers (lane 5) were detected by the antibody. C, Western blot analysis using a goat anti-human VEGF antibody. The antibody reacted with either VEGF165 homodimers (lanes 1 and 3) or monomers (lanes 5, 7, and 8) and PLGF/VEGF heterodimers (lanes 3 and 4), but failed to recognize PLGF homodimers (lane 2) and monomers (lane 6). VV, VEGF165 homodimers; PP, PLGF129 homodimers; M, mixture of PLGF129 homodimers and VEGF165 homodimers and PLGF129/VEGF165 heterodimers; VP, VEGF165/PLGF129 heterodimers. Molecular markers in kilodaltons (kDa) are indicated on the left side of each panel.

Fig. 2. Mitogenic activity of recombinant PLGF129 homodimers, VEGF165 homodimers, and PLGF/VEGF heterodimers on HUVE cells. Pure PLGF129 homodimers, VEGF165 homodimers, and PLGF/VEGF heterodimers were assayed for their abilities to stimulate DNA synthesis by incorporation of [3H]thymidine. These factors were serially diluted and added to duplicate wells of 96-well plates, which were seeded with 5 × 10⁴ HUVE cells/well. Forty hours later, [3H]thymidine was added to each well, and radioactivity incorporated into DNA was measured 16–24 h later. ○ PLGF129 homodimers; ● VEGF165 homodimers; ▲ VEGF165/PLGF129 heterodimers. Data represent (means ± S.D.) of duplicate of each concentration.

Fig. 3. Chemotactic effects of recombinant PLGF129 homodimers, VEGF165 homodimers, and PLGF/VEGF heterodimers on HUVE cells. HUVE cells (1 × 10⁴) were seeded in the upper wells of a 48-well plate (1 × 10⁴ cells/well) of a Boyden chamber and incubated for 5 h at 37 °C in M199 medium containing different concentrations of PLGF129 homodimers (○), VEGF165 homodimers (●), and PLGF/VEGF heterodimer (▲) in the lower wells. Cells migrating through a polycarbonate filter with pore size of 8 μm were scored. Media without added growth factors (□) serve as controls. Data represent (means ± S.E.) of triplicate for each concentration.

mitogenic response (ED₅₀) for HUVE cells was determined to be typically 2.0–6.0 ng/ml. The PLGF/VEGF heterodimer was approximately 20–50-fold less potent, with ED₅₀ of 100–150 ng/ml. In contrast, pure PLGF129 homodimer appeared to be inactive in this assay, even at a high concentration of 10,000 ng/ml.

Chemotactic Activity—Chemotactic response of HUVE cells to PLGF129 homodimers, VEGF165 homodimers, and PLGF/VEGF heterodimers was examined by a modified Boyden chamber assay (32). The migration of HUVE cells through an 8 μm polycarbonate filter toward chemotactants was scored. As shown in Fig. 3, both VEGF165 homodimers and PLGF/VEGF heterodimers were sufficiently potent to induce HUVE cell chemotaxis, whereas PLGF129 homodimers failed to induce such responses at the same concentrations. The chemotactic activities of VEGF165 homodimers and PLGF/VEGF heterodimers were observed at two different doses of concentrations of 2.5 and 10 ng/ml.

Natural Occurrence and Secretion of PLGF/VEGF Heterodimers in Tumor Cells—To detect and quantitate the amounts of PLGF homodimers, VEGF homodimers, and PLGF/VEGF heterodimers secreted in conditioned media of tumor cell lines, sensitive ELISA immunosays were developed using specific antibodies against PLGF and VEGF (Fig. 1, B and C). To further study the specificity of these antibodies for their reactions with the native forms of human PLGF and VEGF, immunoprecipitation analysis was performed. The ¹²⁵I-labeled PLGF129 homodimers and PLGF/VEGF heterodimers could be
and 125I-VEGF165 homodimers (lane 3). This antibody failed to recognize the same amount of 125I-PLGF129 homodimers (lane 1). Similarly, the anti-VEGF monoclonal antibody reacted with 125I-VEGF165 homodimers (lane 6) and 125I-PLGF/VEGF heterodimers (lane 4) but failed to recognize the same amount of 125I-PLGF129 homodimers (lane 5).

“Sandwich” enzyme detection methods were established with these specific antibodies, and each assay was designed to detect a specific dimer. Typical standard curves of the sensitive immunoassays for VEGF homodimers (B), PLGF homodimers (C), and PLGF/VEGF heterodimers (D) are shown in Fig. 4. As described under “Experimental Procedures,” VEGF homodimer and PLGF homodimer detection assays revealed less than 10% cross-reactivity with either VEGF homodimers or PLGF homodimers, while the PLGF/VEGF heterodimer immunoassay exhibited no cross-reactivity with PLGF homodimers or VEGF homodimers. PLGF homodimers but undetectable levels of VEGF homodimers and PLGF/VEGF heterodimers. In addition, an overlapping but distinct pattern of multiple bands with molecular masses ranging from 36 to 54 kDa was immunoprecipitated by the anti-VEGF monoclonal antibody, the anti-PLGF antibody, and anti-VEGF/anti-PLGF antibodies. These results are consistent with previous findings that both VEGF and PLGF exist as various homodimers as well as heterodimers via interchain disulfide bridge formations (14, 18, 19). From these data, we conclude that PLGF and VEGF proteins exist as homodimers and PLGF/VEGF heterodimers in tumor cells that produce both factors.

**TABLE I**

| Quantitation of PLGF homodimers, VEGF homodimers, and PLGF/VEGF heterodimers secreted in the conditioned media of tumor cell lines |
| --- |
| **Cell type** | **VEGF/VEGF** | **PLGF/PLGF** | **PLGF/VeGF** |
| J AR | 0.63 | 4.4 | 4.0 |
| J E-3 | 0.82 | 7.1 | 8.9 |
| A431 | 6.0 | 0.11 | 0.025 |
| HeLa | 0.5 | 0.06 | 0.025 |
| Hep G2 | 4.0 | 0.67 | 0.025 |
| 4MBr | 1.5 | 9.5 | 1.0 |
| HUVE | 0.55 | | |

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Specifically immunoprecipitated by the polyclonal antibody raised against recombinant human PLGF129 (Fig. 4A, lanes 2 and 1). This antibody failed to recognize the same amount of 125I-VEGF165 homodimers (lane 3). Similarly, the anti-VEGF monoclonal antibody reacted with 125I-VEGF165 homodimers (lane 6) and 125I-PLGF/VEGF heterodimers (lane 4) but failed to recognize the same amount of 125I-PLGF129 homodimers (lane 5).

Recent studies have shown that VEGF homodimers and PLGF homodimers, and PLGF/VEGF heterodimers secreted into the conditioned media of various normal and tumor cell lines (Table I). Of note, values reported for VEGF and PLGF homodimers in Table I have been adjusted for the contributions of heterodimers as a result of their cross-reactivities in the VEGF homodimer immunoassay. High concentrations (4.0–8.9 ng/ml) of PLGF/VEGF heterodimers were detected in conditioned media derived from 2-day cultured J AR and J E-3 choriocarcinoma cell lines (Table I). Equivalent quantities of PLGF homodimers (4.4–7.1 ng/ml) were also found in the conditioned media of these cell lines. Approximately, 10-fold lower quantities of VEGF homodimers were detected in the conditioned media derived from these cell lines. These findings are consistent with our data from Northern blot analysis, namely that the amount of VEGF mRNA synthesized was 10–20-fold smaller than that of PLGF. Based on relative concentrations of PLGF/VEGF heterodimers and VEGF homodimers in the conditioned media, it appeared that, at least in these cell lines, there was preference for VEGF to exist as PLGF/VEGF heterodimers and the excess amount of PLGF protein is present as homodimers.

A high concentration of PLGF/VEGF heterodimers (1 ng/ml) was also detected in the conditioned medium of 4MBr Rhesus monkey bronchus epithelial cells. Smaller amounts of PLGF/VEGF heterodimers (25 pg/ml) were found in the conditioned media of human HepG2 hepatoma and A431 squamous carcinoma cells. It seems that the amount of PLGF/VEGF heterodimers produced by a cell line directly correlates with the ratio of expression levels between PLGF and VEGF. As a control, we also analyzed the conditioned medium derived from HUVE cells, which contained a small amount of PLGF homodimers but undetectable levels of VEGF homodimers and PLGF/VEGF heterodimers. In addition, an overlapping but distinct pattern of multiple bands with molecular masses ranging from 36 to 54 kDa was immunoprecipitated by the anti-VEGF monoclonal antibody, the anti-PLGF antibody, and anti-VEGF/anti-PLGF antibodies. These results are consistent with previous findings that both VEGF and PLGF exist as multiple, alternatively spliced isoforms that may constitute various homodimers as well as heterodimers via interchain disulfide bridge formations (14, 18, 19). From these data, we conclude that PLGF and VEGF proteins exist as homodimers and PLGF/VEGF heterodimers in tumor cells that produce both factors.

Binding of PLGF129 Homodimers, VEGF165 Homodimers, and PLGF/VEGF Heterodimers to a Soluble Flk-1/KDR Receptor—Recent studies have shown that VEGF homodimers bind to both Flk-1/KDR and Flt-1 high affinity tyrosine kinase receptors (29). While PLGF homodimers bind with high affinity to Flt-1, they fail to bind to the Flk-1/KDR receptor (31). Transfection experiments show that the Flk-1/KDR-expressing endothelial cells undergo striking changes in cell morphology, mitogenesis, and chemotaxis upon VEGF stimulation, whereas Flt-1-expressing cells lack such responses (28). To determine if PLGF/VEGF heterodimers bind to the Flk-1/KDR receptor, a soluble receptor consisting of the extracellular domain of Flk-1 fused to an alkaline phosphatase tag (AD) was constructed and expressed (Fig. 5A). This soluble receptor, named Flk-1-AP, binds with high affinity to recombinant VEGF and to the cell surface associated VEGF (37). When 125I-labeled PLGF129 ho-
modimers, VEGF165 homodimers and PLGF/VEGF heterodimers were incubated with the Flk-1-AP receptor,125I-VEGF-Flk-1-AP (Fig. 5c, VP) and 125I-PLGF/VEGF-Flk-1-AP complexes were readily precipitated by the anti-alkaline phosphatase (anti-AP) antibody, whereas no 125I-PLGF129 homodimers were recovered by the antibody (PP). To exclude the possibility that the anti-AP antibody cross-reacted with these dimeric growth factors,125I-labeled PLGF129 homodimers, VEGF165 homodimers, and PLGF/VEGF heterodimers were precipitated with the anti-AP antibody in the absence of soluble Flk-1-AP receptor. As shown in Fig. 5b, the anti-AP antibody failed to react with these factors (lanes 4–6). These results suggest that PLGF/VEGF heterodimers, similar to VEGF homodimers, bind to the soluble Flk-1 receptor.

To further investigate the interaction between VEGF/PLGF heterodimers and Flk-1 receptor, cross-linking experiments were performed using 125I-labeled factors and the soluble Flk-1-AP receptor. Cross-linked complexes migrating at positions of 220 and 215 kDa, the expected sizes of VEGF-Flk-1-AP and PLGF/VEGF-Flk-1-AP complexes, were detected in the 125I-PLGF/VEGF heterodimer (Fig. 6a, VP) and 125I-VEGF165 homodimer (VV) reactions, but not in the 125I-PLGF129 homodimer reaction (PP). The band of 125I-PLGF/VEGF-Flk-1-AP complexes on the SDS gel was less intensive than that of 125I-VEGF-Flk-1-AP complex, which perhaps is due to various amounts of complexes recovered during experimental procedures. These results are consistent with immunoprecipitation data showing that PLGF/VEGF heterodimers bind to Flk-1 receptor (Fig. 5). Our preliminary results using the soluble Flk-1-AP receptor suggest that PLGF/VEGF heterodimers and VEGF homodimers bind to KDR/Flk-1 receptor with a similar high affinity as described previously (23).2

Binding of PLGF129 Homodimers, VEGF165 Homodimers, and PLGF/VEGF Heterodimers to Membrane Receptors on HUVE Cells—Cross-linking of the 125I-labeled PLGF129 homodimers, VEGF165 homodimers, and PLGF/VEGF heterodimers to the soluble Flk-1-AP receptor and to the membrane receptors of HUVE cells, a,125I-PLGF homodimers (PP), 125I-VEGF homodimers (VV), and 125I-PLGF/VEGF heterodimers (VP) were incubated with 5 μg of Flk-1-AP in the presence of bis(sulfosuccinimidyl) suberate chemical cross-linker. The cross-linked complexes were immunoprecipitated using the anti-AP antibody and were analyzed by a 12% SDS gel. The arrow indicates the position of cross-linked complexes. b, HUVE cells were incubated in Dulbecco's modified Eagle's medium containing 125I-PLGF129 homodimers (PP), 125I-VEGF165 homodimers (VV), and 125I-PLGF/VEGF heterodimers (VP) for 2 h. Cells were washed and incubated with 10 mM disuccinimidyl suberate cross-linker. Following cross-linking, the reaction was quenched, washed with PBS, and extracted with 1% Triton X-100. The extracts corresponding to 10⁵ cells were loaded on a 12% SDS gel.

![Fig. 5. Co-immunoprecipitation of the 125I-labeled PLGF129 homodimers, VEGF165 homodimers, and PLGF/VEGF heterodimers by a soluble Flk-1-AP receptor.](image1)

![Fig. 6. Cross-linking of 125I-labeled PLGF129 homodimers, VEGF165 homodimers, and PLGF/VEGF heterodimers to the soluble Flk-1-AP receptor and to the membrane receptors of HUVE cells.](image2)
PLGF129 homodimer was found in the reaction (also associated with the cell surface. However, no detectable PLGF/VEGF heterodimers (receptors were also found to form cross-linked complexes with cated form of Flk-1/KDR or Flt-1 receptor. These membrane receptor is unclear, although it may represent a trun-

heterodimers and VEGF 165 homodimers, PLGF129 homodimers (arrow in Fig. 7, PP and VP), which was not seen in the reaction stimulated by VEGF165 homodimers (VV).

**DISCUSSION**

VEGF has been identified as a specific mitogen and chemotactic factor for endothelial cells and also induces blood vessel permeability (12, 14, 39). Other effects of VEGF include induction of plasminogen activator, synthesis of urokinase receptor and plasminogen activator inhibitor-1, stimulation of hexose transport in endothelial cells, mobilization of intracellular Ca++, and stimulation of endothelial cell and monocyte migration in vitro (23). The recently identified PLGF shares some of the structural and functional properties of VEGF. For example, PLGF has significant sequence homology to VEGF including eight conserved cysteine residues in the PDGF-like domain. Both PLGF and VEGF can exist as alternatively spliced multi-

isoforms (18, 39). In addition, PLGF was originally reported as a potent endothelial cell-specific mitogen in in vitro studies (17), although subsequent reports showed that PLGF was only a weak mitogen for endothelial cells (31, 40). While biological activities of VEGF have been well characterized, little is known about functions of PLGF on angiogenesis both in vitro and in vivo.

In the present study, we demonstrate that PLGF and VEGF, similar to the A and B chains of PDGF, can be readily dimerized into homodimers as well as heterodimers both in cell-free and intracellular systems. The PLGF129/VEGF165 heterodimers obtained from in vitro assays were purified to homogeneity using specific antibody-coupled affinity chromatogra-

phies. Similar to VEGF homodimers, PLGF/VEGF heterodimers display a mitogenic response for vascular endothelial cells in vitro. However, the mitogenic activity of PLGF/ VEGF heterodimers with an ED50 of approximately 100 ng/ml is 20–50-fold lower than that of VEGF165 homodimers (ED50 of 2–6 ng/ml). DiSalvo et al. (40) have shown that PLGF/VEGF heterodimers are nearly as potent endothelial cell mitogens as are VEGF homodimers. The discrepancies between these findings and our data may reflect the differences between endothelial cells used in each assay, which may express different types and/or levels of VEGF receptors (see below). In contrast, PLGF129 homodimers even at high concentrations (≥1 μg/ml) have little or no effect on stimulation of DNA synthesis of endothelial cells. Likewise, while both VEGF165 homodimers and PLGF/VEGF heterodimers are potent chemotactic factors for vascular endothelial cells, PLGF homodimers failed to in-

duce a chemotactic response in these cells. These findings indicate that PLGF by formation of heterodimers with VEGF may down-regulate the mitogenic activities but up-regulate the chemotactic effects of VEGF on endothelial cells. Thus, it is possible that PLGF and VEGF interact with each other in vivo by a similar pathway, especially in cells producing both growth factors. This interaction may be important in the regulation of in vivo angiogenesis, a process that is involved in many phys-

iological and pathological events such as embryonic development, tumor growth, wound healing, and diabetic retinopathy (2). A large body of work obtained from recent studies has demonstrated that VEGF is a key regulator of in vivo angiogenesis (41).

PLGF is expressed in tissues switched to angiogenic phenotypes such as the placenta and some tumor tissues, but not in

**Stimulation of Tyrosine Phosphorylation in HUVE Cells by PLGF129 Homodimers, VEGF165 Homodimers, and VEGF/PLGF Heterodimers—To study the membrane and intracellular signal transduction pathways, we tested PLGF129 homodimers, VEGF165 homodimers, and PLGF/VEGF heterodimers for their ability to induce tyrosine phosphorylation of membrane and intracellular proteins in HUVE cells. In agreement with a previous report (31), VEGF165 homodimers stimulated tyrosine phosphorylation of an approximately 220-kDa membrane protein (Fig. 7, VV, arrowhead). PLGF/VEGF heterodimers also stimulated tyrosine phosphorylation of the same protein (VP).

![Fig. 7. Tyrosine phosphorylation of the membrane and intracellular proteins of HUVE cells. HUVE cells grown to confluence were stimulated at 37°C for 5 min with 100 ng/ml PLGF129 homodimers (PP), VEGF165 homodimers (VV), and PLGF/VEGF heterodimers (VP). Cells incubated with M199 medium alone were used as controls (no factor). Cell lysates were separated by a 12% SDS gel and transferred to a nitrocellulose membrane. The membrane was immu-

mobilized with an anti-phosphotyrosine monoclonal antibody. The band corresponding to the position of a phosphorylated membrane protein was cut from the nitrocellulose membrane and its radioactivity was estimated by a β-counter (arrowhead).](image-url)
normal adult tissues (18, 21, 42). To investigate if PLGF and VEGF form heterodimers in vivo, we have developed two specific antibodies that react with both monomeric and dimeric forms of PLGF and VEGF. These specific antibodies allow us to quantitatively detect PLGF and VEGF homodimers as well as heterodimers secreted by various tumor cell lines. Interestingly, PLGF and VEGF polypeptides preferentially form heterodimers in tumor cells such as JAR and JE-3 choriocarcinoma and 4MBr bronchus cell lines expressing both VEGF and PLGF proteins. In addition, a recent study reported that PLGF/VEGF heterodimers were also present in the conditioned medium derived from rat GS-9L glioma cells (40). These findings suggest that PLGF/VEGF heterodimers may play an important role in this tumor cell-induced angiogenesis. Our recent study has shown that JE-3 choriocarcinoma cells develop highly angiogenic tumors when implanted subcutaneously in immunodeficient mice, although these cells produce low quantities of VEGF homodimers (Table I).

Four VEGF and two PLGF isoforms encoded by distinct mRNA splice variants may all participate in various combinatorial homo- and heterodimerizations. These combinations could increase the complexity of secretions, heparin binding affinities, receptor interactions and biological functions of PLGF homodimers, VEGF homodimers, and PLGF/VEGF heterodimers. Coinmunoprecipitation of PLGF/VEGF heterodimers in the conditioned medium of JAR tumor cells with the anti-VEGF monoclonal antibody and the anti-PLGF polyclonal antibody reveals multiple bands with molecular masses ranging from 39 to 55 kDa (data not shown). These experiments support the hypothesis that various isoforms of PLGF and VEGF may be involved in the formation of multiple homo- and heterodimers.

Two high affinity receptors for VEGF have been characterized, Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2) (23). Recently, generation of transgenic mice deficient in Flk-1 by disruption of the gene indicates that the Flk-1 receptor-mediated signals are essential for yolk sac blood island formation and vasculogenesis in the mouse embryo (43). Transfection and signal transduction studies have shown that Flk-1 mediates signals causing striking changes in cell morphology, ligand-induced mitogenicity, and chemotaxis, whereas Flt-1 transfected cells lack such responses to VEGF (28). To date, the cellular events that signal through Flt-1 receptor have not been characterized. However, the fact that Flt-1 knockout mice are embryonic lethal due to a malformation of the vascular system suggests that Flt-1 is an important angiogenic factor for endothelial cell differentiation and vascular organization during embryonic development (44). In the present paper, we demonstrate that the PLGF/VEGF heterodimer binds to a soluble Flk-1 receptor fused to the alkaline phosphatase. The binding affinity of PLGF/VEGF heterodimers to Flk-1 is similar to that of VEGF. As VEGF4b homodimers, 125I-PLGF/VEGF heterodimers can be cross-linked to the membrane of HUVE cells, which express both Flk-1 and Flt-1 receptors. These cross-linked complexes are probably due to KDR/Flk-1 receptor because 125I-PLGF129 known to bind to Flt-1 receptor is unable to be cross-linked to these cells. One possible explanation for these observations could be that the HUVE cells used in our experiments do not express significant amounts of Flt-1 receptor.

Both VEGF homodimers and PLGF/VEGF heterodimers stimulated tyrosine phosphorylation of a 220-kDa protein, the expected size for the KDR receptor in HUVE cells. Interestingly, PLGF/VEGF heterodimers and PLGF homodimers, but not VEGF homodimers induce phosphorylation of a 38-kDa protein, although PLGF homodimers failed to form cross-linked complexes with membrane proteins of HUVE cells. These observations suggest that although VEGF homodimers and PLGF/VEGF heterodimers share the same KDR-mediated signal pathways, the latter is also able to induce signals mediated by PLGF homodimers. It is, however, also possible that PLGF/VEGF heterodimers may interact with an unknown receptor. Our recent studies have shown that the PLGF/VEGF heterodimer does not bind to Flt-4 receptor whose ligand has not yet been reported.

The presence of PLGF homodimers, VEGF homodimers, and PLGF/VEGF heterodimers is analogous to the AA, BB, and AB dimers of the PDGF family (45). Interestingly, PDGF-BB homodimers have also been characterized as an angiogenic factor, which especially induces a potent chemotactic response from endothelial cells (46). Because the eight cysteine residues involving intra- and interchain disulfide bond formations are all conserved within PDGF and VEGF/PLGF families, it is possible that PLGF and VEGF may also form various heterodimers with PDGF-AA and PDGF-BB. The similarity between PDGF and PLGF/VEGF systems also extends to their high affinity membrane receptors. Two types of PDGF receptors, α and β, have been well characterized (47). All PDGF isoforms can induce the formation of αα receptor homodimers, whereas AB and BB, but not AA, are able to induce the dimerization of αβ receptor, and only BB is able to induce ββ receptor dimerization. Similar to the PDGF receptor system, Flt-1 and Flk-1 receptors also form three types of homo- and heterodimeric receptors upon binding to their corresponding ligands as represented in Fig. 8. So far, many of these possible interactions are not known and remain to be characterized. From the PDGF system, one can speculate that PLGF/VEGF heterodimers are able not only to induce the homodimerization of Flk-1/Flk-1, but also to induce the formation of Flk-1/Flt-1 and Flk-1/Flt-1.

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3 Y. Cao, unpublished data.

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**Fig. 8.** Schematic representation of possible interactions of PLGF homodimers, VEGF homodimers, and PLGF/VEGF heterodimers with their membrane receptors. VEGF homodimers bind to both Flt-1 (VEGFR-1) and Flk-1 (VEGFR-2) receptors. PLGF/VEGF heterodimers bind to the Flk-1 receptor that mediates mitogenic, chemotactic, and membrane ruffling signals for endothelial cells. The Flt-3 receptor does not mediate such signaling pathways. PLGF homodimers bind only to Flt-1 receptors. Solid arrows represent the characterized interactions between these ligands with their receptors. Dashed arrows indicate the unknown possible interactions. Upon ligand binding, VEGF receptors may form Flk-1/Flk-1 homodimers, Flt-1/Flt-1 homodimers, and Flt-1/Flt-1 heterodimers. Whether the PLGF/VEGF heterodimer is the only ligand for the Flk-1/Flt-1 heterodimeric receptor and what type of biological signals are transduced by the Flk-1/Flt-1 receptor heterodimers by the Flt-1/Flt-1 homodimers remain to be studied.
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receptor homodimers and heterodimers. Thus, further functional characterization of these receptor dimers that mediate various angiogenic responses would help us to better understand their roles in the regulation of angiogenesis in vivo.

Acknowledgments—We thank Dr. Judah Folkman for support and helpful discussions. We also thank Drs. Gregory Blatch, David Shima, Harvey Jaylord, Ivana Kim, Shay Soker, Jack Arbiser, Michael Klagsbrun, and Bruce Zetter for valuable discussions.

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