Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) is a polypeptide mediator, elaborated by certain tumors and other cell types, that exerts multiple effects on endothelium via interaction with a class of high-affinity binding sites. In this report, the interaction of VPF/VEGF with human mononuclear phagocytes (MPs) is characterized. Radioligand binding studies at 4°C showed the presence of a single class of binding sites, with a dissociation constant (Kd) of approximately 300 to 500 pmol/L (20 times lower affinity than the high-affinity binding site on endothelial cells [ECs]).

The interaction of VPF/VEGF with ECs resulted in subsequent activation of intracellular signal transduction mechanisms, as shown by an increase in MP intracellular calcium concentration. Cross-linking studies with radioactive VPF/VEGF showed a new high-molecular weight band (corresponding to putative VPF/VEGF-receptor complex), the appearance of which was blocked by excess unlabeled VPF/VEGF. Consistent with these results, immunoprecipitation of P-32PO4-labeled MPs exposed to VPF/VEGF showed a single band of similar mobility, not seen in untreated controls. These results demonstrate that the interaction of VPF/VEGF with MPs, though of lower affinity than that observed with ECs, also results from interaction of the polypeptide with a specific cell-surface protein and leads to activation of intracellular transduction mechanisms.

MATERIALS AND METHODS

Preparation and radiiodination of VPF/VEGF. The 165-amino acid form of VPF/VEGF was used in these studies. A cDNA construct containing the VPF/VEGF cDNA and the herpes simplex virus immediate early promoter was used to transfect BHK cells that express the herpes VP16 trans-activator. The VPF/VEGF that was secreted into the serum-free medium of these cells was purified to homogeneity in three chromatographic steps. Cation-exchange chromatography demonstrated that the secreted polypeptide migrates at an apparent molecular weight of 40 000-50 000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; accounting for the apparent molecular weight of the ligand), suggesting that it may contribute to the modulation of vascular function in a broader context than just in the tumor bed.

Mononuclear phagocytes (MPs) contribute to the host-response in tumor vasculature, as well as serve a pathogenetic role in other types of vascular lesions, such as the development of atherosclerotic lesions. VPF/VEGF has been shown to modulate hemostatic properties of MPs, through induction of tissue factor, and to induce MP chemotaxis. When VPF/VEGF was placed below an endothelial monolayer, it enhanced the migration of MPs across the endothelium. However, the dose-dependency of these effects of VPF/VEGF on MP functions was in the range of 30 to 1000 pmol/L, being half-maximal at approximately 200 to 500 pmol/L. This is quite different from the potency of VPF on ECs, where a class of high-affinity binding sites has been characterized with a Kd of approximately 1 to 50 pmol/L, which appears to mediate the mitogenic and certain other effects of VPF/VEGF on cultured endothelium.

These observations led us to characterize the interaction of VPF/VEGF with MPs to determine if it was due to the specific interaction of the ligand with a receptor that activated signal transduction mechanisms, and to contrast the results with the EC system. The results indicate that VPF/VEGF binds specifically to the surface of MPs, is subjected to cellular processing, and induces an increase in cytosolic calcium and phosphorylation of a high-molecular weight polypeptide. Cross-linking studies indicate that the cell-surface receptor has a similar migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; accounting for the apparent molecular weight of the ligand), suggesting that it becomes phosphorylated as a consequence of ligand-receptor interaction.
was aspirated, MPs were washed two times with Hank's balanced salt solution (HBSS), and then 0.25 mL of incubation buffer, Dulbecco's modified Eagle medium (DMEM) containing HEPES (25 mmol/L; pH 7.4), penicillin/streptomycin (50 U/mL; 50 µg/mL), and fetal calf serum (1%) were added. Cells were incubated at 4°C for 2 hours (this is the time for maximal binding even at lower concentrations of tracer) with different concentrations of 125I-VPF/VEGF. After the incubation period, cultures were washed four times with HBSS, and then lysis buffer containing Tris (10 mmol/L; pH 7.4), NP-40 (1%), pepstatin (1 µg/mL), aprotinin (1 µg/mL), and EDTA (1 mmol/L) was added for 10 minutes at 22°C. In certain experiments, 2% SDS was used in place of NP-40. Specific binding, the difference of binding to the equation of Klotz and Hunston (B = n × KA, where n = specifically bound ligand, A = free ligand concentration) using nonlinear least-squares analysis (Enzfitter Biosoft, Cambridge, UK). Where indicated, chloroquine (80 pmol/L) treatment of MPs was done by preincubating the cells with the drug in binding buffer for 2 hours at 37°C, then 125I-VPF/VEGF was added and the internalization/degradation experiment was performed as above.

Preparation of MPs, ECs, radioligand binding, and internalization assays. Human monocytes were isolated from the mononuclear fraction of peripheral blood using a discontinuous Ficoll gradient (Histopaque 1077; Sigma, St Louis, MO). Monocyte preparations were assessed morphologically and by trypsin blue exclusion, as described previously. Cells were then dispersed in 24-well plates (~1.5 to 2.5 × 10⁴ cells/well) or 96-well plates (~5 to 6 × 10⁵ cells/well), and cultured in RPMI 1640 with human serum (1%) and cultured in RPMI 1640 with human serum (5%) and penicillin/streptomycin (50 U/mL; 50 µg/mL), and fetal calf serum (1%) as an index of degradation. SDS-PAGE (12.5%) was performed by preincubating the cells with the drug in binding buffer for 2 hours at 37°C, then 125I-VPF/VEGF was added and the internalization/degradation experiment was performed as above.

Studies of VPF/VEGF biologic activity: MP migration and EC proliferation. Chemotaxis assays were performed in 48-well microchemotaxis chambers (Neuro-Probe, Bethesda, MD) containing Nucleopore polycarbonate membrane (5 µm; Nucleopore, Pleasanton, CA). MPs were suspended in RPMI 1640 containing fetal bovine serum (1%) and 10% cells were added per well to the upper chamber. The lower chamber contained the indicated amount of VPF/VEGF. Assays were performed in triplicate over a 3-hour incubation period at 37°C, after which nonmigrating cells were removed, membranes were fixed in methanol, and migrating cells were visualized with Wright's stain. Cells in 10 high-power fields were counted, and the mean ± SEM was determined. This method has been described previously.

Proliferation of human umbilical vein ECs in response to VPF/VEGF was studied using ³H-thymidine incorporation, as described previously.

Cytosolic calcium flux in MPs. Coverslips attached to 35-mm plastic dishes with a hole punched in the bottom were coated with poly-D-lysine and seeded with MPs. Cells were allowed to attach and spread for approximately 1 hour, and intracellular calcium levels were determined as described previously. 100-fold excess of unlabeled material, was analyzed according to the equation of Klotz and Hunston (B = n × KA, where n = specifically bound ligand, A = free ligand concentration) using nonlinear least-squares analysis (Enzfitter Biosoft, Cambridge, UK). Where indicated, after a binding assay, cell-bound 125I-VPF/VEGF was eluted by exposure to acidic buffer (HCl, 0.1 mol/L; NaCl, 0.1 mol/L; final pH 2.0) or by treating the cells with trypsin/EDTA (0.25%/wt:w1/1 mmol/L; Gibco, Grand Island, NY) for 5 to 10 minutes at 22°C. Under these conditions with acidic buffer and trypsin/EDTA, MPs maintained their viability, based on trypan blue exclusion, and could then be eluted with detergent-containing lysis buffer. Radioactivity in the intracellular pool and culture supernatants was subjected to precipitation in trichloroacetic acid (final, 20%) as an index of degradation. SDS-PAGE (12.5%) was performed on the intracellular pool of cell-associated 125I-VPF/VEGF. Where indicated, chloroquine (80 µmol/L) treatment of MPs was performed by preincubating the cells with the drug in binding buffer for 2 hours at 37°C, then 125I-VPF/VEGF was added and the internalization/degradation experiment was performed as above.

Preparation of MPs, ECs, radioligand binding, and internalization assays. Human monocytes were isolated from the mononuclear fraction of peripheral blood using a discontinuous Ficoll gradient (Histopaque 1077; Sigma, St Louis, MO). Monocyte preparations were assessed morphologically and by trypsin blue exclusion, as described previously. Cells were then dispersed in 24-well plates (~1.5 to 2.5 × 10⁴ cells/well) or 96-well plates (~5 to 6 × 10⁵ cells/well), and cultured in RPMI 1640 with human serum (5%) and penicillin/streptomycin (50 U/mL; 50 µg/mL), and fetal calf serum (1%) were added. Cells were incubated at 4°C for 2 hours (this is the time for maximal binding even at lower concentrations of tracer) with different concentrations of 125I-VPF/VEGF. After the incubation period, cultures were washed four times with HBSS, and then lysis buffer containing Tris (10 mmol/L; pH 7.4), NP-40 (1%), pepstatin (1 µg/mL), aprotinin (1 µg/mL), and EDTA (1 mmol/L) was added for 10 minutes at 22°C. In certain experiments, 2% SDS was used in place of NP-40. Specific binding, the difference of binding observed in wells incubated with 125I-VPF/VEGF alone minus binding observed in wells incubated with 125I-VPF/VEGF in the presence of 100-fold excess of unlabeled material, was analyzed according to the equation of Klotz and Hunston (B = n × KA, where n = specifically bound ligand, A = free ligand concentration) using nonlinear least-squares analysis (Enzfitter Biosoft, Cambridge, UK). Where indicated, after a binding assay, cell-bound 125I-VPF/VEGF was eluted by exposure to acidic buffer (HCl, 0.1 mol/L; NaCl, 0.1 mol/L; final pH 2.0) or by treating the cells with trypsin/EDTA (0.25%/wt:w1/1 mmol/L; Gibco, Grand Island, NY) for 5 to 10 minutes at 22°C. Under these conditions with acidic buffer and trypsin/EDTA, MPs maintained their viability, based on trypan blue exclusion, and did not detach from the wells. Radioligand binding assays with confluent monolayers of cultured ECs (passage 3) were performed by the same procedure described above for MPs.

For studies to assess internalization of 125I-VPF/VEGF, cultures were preincubated at 4°C for 2 hours with 125I-VPF/VEGF (100 to 300 pmol/L) alone or in the presence of a 100-fold molar excess of unlabelled VPF/VEGF. At the end of this period, cultures were washed four times with HBSS, fresh binding buffer was added, and cells were warmed to 37°C. After the indicated incubation periods, cell-associated radioactivity and 125I-VPF/VEGF in culture supernatants was characterized. Cell-associated radioactivity was considered surface-bound if it could be eluted with acidic buffer (HCl, 0.1 mol/L; NaCl, 0.1 mol/L; final pH 2.0 exposed to cultures for 5 seconds) or trypsin/EDTA (as used above as described). Cell-associated radioactivity was considered to have entered an intracellular pool if it remained cell-associated after cultures were subjected to treatments that eluted surface-bound material, and could then be eluted with detergent-containing lysis buffer. Radioactivity in the intracellular pool and culture supernatants was subjected to precipitation in trichloroacetic acid (final, 20%) as an index of degradation. SDS-PAGE (12.5%) was performed on the intracellular pool of cell-associated 125I-VPF/VEGF. Where indicated, chloroquine (80 µmol/L) treatment of MPs was performed by preincubating the cells with the drug in binding buffer for 2 hours at 37°C, then 125I-VPF/VEGF was added and the internalization/degradation experiment was performed as above.

Cross-linking experiments. MPs plated in 9.6-cm² wells (1.2 to 1.4 × 10⁴ cells/well) were washed twice with HBSS, and then HBSS containing bovine serum albumin (0.1%; fatty acid-free, Sigma) to a final volume of 0.5 mL. Then, 125I-VPF/VEGF alone or in the presence of an 100-fold molar excess of unlabelled ligand was added, and cultures were incubated at 22°C for 30 minutes. At the end of the incubation period, the cross-linker, disuccinimidyl suberate (0.8 mmol/L; Pierce, Rockford, IL) was added for 15 minutes at 22°C.
Cultures were washed five times with HBSS, and then lysis buffer was added. Lysates were harvested, centrifuged at 13,000g for 10 minutes to remove cellular debris, and supernatants were concentrated by precipitation in trichloroacetic acid (final, 20%). The pellet was containing NP-40 (1%). Lysates were harvested, centrifuged at 10 minutes to remove cellular debris, and supernatants were concentrated (5.0% to 7.5%). Gels were dried and subjected to autoradiography. Molecular weights were estimated, when appropriate, from the migration of standard proteins run simultaneously (Amersham, Arlington Heights, IL): myosin (200 Kd), phosphorylase b (97.5 Kd), bovine serum albumin (69 Kd), ovalbumin (46 Kd), carbonic anhydrase (30 Kd), trypsin inhibitor (21.5 Kd), and lysozyme (14.3 Kd). Cross-linking studies with confluent monolayers of cultured ECs were performed using the same procedure as that described for MPs.

Radioligand binding assays on MPs maintained in culture for the indicated times were performed according to the same procedure described in Fig 1B.

Phosphorylation experiments: MPs in 9.6-cm² wells (as above) were washed twice in HBSS, and phosphate-free DMEM (1 mL/well; Gibco) was added. Cells were incubated at 37°C for 4 hours with [32P]-orthophosphoric acid (0.25 mCi/mL; Amersham). Then VPF/VEGF (5 nmol/L) was added to certain wells (no further addition was made to control wells) for 10 minutes at 37°C. Next, cultures were washed twice with ice-cold Tris-buffered saline containing sodium orthovanadate (100 μmol/L), and solubilized in buffer containing Tris (10 mmol/L), NaCl (50 mmol/L), EDTA (5 mmol/L), sodium orthovanadate (100 μmol/L), Triton X-100 (0.1%), sodium pyrophosphate (30 mmol/L), and phenylmethylsulfonyl fluoride (1 mmol/L) (final pH 7.4). Lysates were centrifuged (10 minutes at 13,000g) at 4°C, and supernatants (~1 mL) were subjected to immunoprecipitation with rabbit antiphosphotyrosine antibody (20 μg/mL; generously provided by Dr P. D’Corleto, Cleveland Clinical Research Institute, Cleveland, OH) at 4°C. Formalin-fixed bacteria bearing protein A (IgG Sorb; New England Enzyme Center, Malden, MA) (0.04 mL packed volume) was added for 18 hours at 4°C, and then the beads were pelleted by centrifugation (13,000g). The pellet was suspended in SDS-PAGE sample buffer, boiled for 5 minutes, and the mixture was centrifuged again (13,000g). The supernatant was then applied to SDS-PAGE (5%)..

RESULTS

Radioligand binding and internalization studies with 125I-VPF/VEGF and MPs: When MPs were incubated with 125I-VPF/VEGF at 4°C, specific cell-surface binding occurred that could be eluted with either acidic buffer or detergent-containing lysis buffer (Fig 1A). Over a range of 125I-VPF/VEGF concentrations, binding to MPs was dose-dependent and saturable with a kd of approximately 308 pmol/L and approximately 4.3 × 10^3 molecules bound per cell at saturation (Fig 1B). Representative radioligand binding studies showed a comparable number of 125I-VPF/VEGF binding sites per cell when experiments were performed on MPs in culture from 1 to 22 days (Table 1). In contrast, binding studies with identical preparations of 125I-VPF/VEGF and cultured human umbilical vein ECs demonstrated higher-affinity binding with a kd of approximately 20 pmol/L (data not shown), as well as a lower-affinity site, both in the range of previously reported studies.4 Consistent with these data, the same VPF/VEGF preparations used in the binding studies were about an order of magnitude more effective in stimulating EC growth (Fig 2A) (note that 1 ng/mL corresponds to 25 pmol/L) compared with their capacity to induce MP migration (Fig 2B).

To assess cellular processing of the radioligand, 125I-VPF/VEGF was bound to the cell surface at 4°C, and then cultures were warmed to 37°C. 125I-VPF/VEGF initially bound to the cell surface (elutable with acidic buffer) entered a compartment from which it could only be eluted by solubilization of

![Fig 1. Binding of 125I-VPF/VEGF to MPs at 4°C. (A) Elution of bound 125I-VPF/VEGF with buffer containing NP-40 (1%), trypsin/EDTA (0.25%/1 mmol/L), or acidic buffer (pH 2.0). MPs (2 × 10^6 cells/well) were incubated for 2 hours at 4°C with 125I-VPF/VEGF (0.7 mmol/L); cells were then washed to remove bound tracer, and monolayers were eluted at room temperature with buffer containing NP-40 (1%, 10 minutes), trypsin/EDTA (0.25%/1 mmol/L; 5 minutes), or acidic buffer (pH 2.0; 5 seconds). The data shown represent the mean ± SEM of triplicate determinations. Similar results were observed when cell-bound 125I-VPF/VEGF was eluted by exposure of monolayers to buffer containing SDS (2%). (B) Dose-dependence of the specific binding of 125I-VPF/VEGF to MPs. MPs (2 × 10^6 cells/well) were incubated at 4°C for 2 hours with the indicated concentration of 125I-VPF/VEGF alone or in the presence of a 100-fold molar excess of unlabeled VPF/VEGF. Unbound tracer was removed by washing, and cell-bound 125I-VPF/VEGF was eluted with buffer containing SDS (2%). Details of the procedure are described in the text. Data represent the mean of at least duplicate determinations, and the inset shows Scatchard analysis. Binding parameters are kd = 308 ± 22 pmol/L and N = 0.22 ± 0.005 fmol bound per well at saturation.](image1.png)

<table>
<thead>
<tr>
<th>Time in Culture</th>
<th>kd (pmol/L)</th>
<th>Capacity (sites/cell)</th>
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<tbody>
<tr>
<td>Day 1</td>
<td>387 ± 37</td>
<td>4.7 × 10^3</td>
</tr>
<tr>
<td>Day 2</td>
<td>308 ± 22</td>
<td>4.3 × 10^3</td>
</tr>
<tr>
<td>Day 22</td>
<td>495 ± 94</td>
<td>4.2 × 10^3</td>
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Radialigand binding experiments on MPs showed a comparable number of 125I-VPF/VEGF binding sites per cell when experiments were performed on MPs in culture from 1 to 22 days (Table 1). In contrast, binding studies with identical preparations of 125I-VPF/VEGF and cultured human umbilical vein ECs demonstrated higher-affinity binding with a kd of approximately 20 pmol/L (data not shown), as well as a lower-affinity site, both in the range of previously reported studies.4 Consistent with these data, the same VPF/VEGF preparations used in the binding studies were about an order of magnitude more effective in stimulating EC growth (Fig 2A) (note that 1 ng/mL corresponds to 25 pmol/L) compared with their capacity to induce MP migration (Fig 2B).
Fig 2. Effect of VPF/VEGF on (A) endothelial proliferation and (B) MP migration. (A) Cultured ECs were incubated with the indicated concentration of VPF/VEGF, and incorporation of \(^{3}H\)-thymidine was determined as an index of cellular proliferation. (B) MPs were added to the upper compartment of modified chemotaxis chambers and migration was initiated by placing VPF/VEGF at the indicated concentration, in the lower well. In each case, details of experimental methods are described in the text, and data shown represent the mean ± SEM. Note that 1 ng/mL corresponds to ~25 pmol/L.

the cells with detergent (1% NP-40) (Fig 3). This was not due to formation of a covalent complex between \(^{125}I\)-VPF/VEGF, as no higher–molecular weight band was evident on SDS-PAGE compared with the initial tracer (data not shown). Furthermore, \(^{125}I\)-VPF/VEGF was not present on the cell surface, as it could not be removed by either acidic buffer or trypsin treatment of the MPs. These data suggested that VPF/VEGF entered an intracellular pool in a time-dependent manner as the cells were warmed to 37°C (Fig 3). Formation of this internal pool of \(^{125}I\)-VPF/VEGF was followed closely by the production of \(^{125}I\)-labeled trichloroacetic acid–soluble degradation products within MPs and in culture supernatants (Fig 3, inset I). Consistent with the hypothesis that \(^{125}I\)-VPF/VEGF degradation was due to the interaction of ligand with specific cell-surface receptors, addition of excess unlabeled VPF/VEGF during the initial binding period blocked formation of the internal (detergent elutable) pool and degradation of the tracer (Fig 3, inset II). In addition, chloroquine,
a weak base that prevents intracellular acidification, also blocked degradation of $^{125}$I-VPF/VEGF (Fig 3, inset II).

**VPF/VEGF-induced cytosolic calcium flux in MPs.** In view of the correlation between the binding of $^{125}$I-VPF/VEGF to the surface of MPs (Fig 1) and VPF/VEGF-induced modulation of MP migration and tissue factor expression, it was logical to consider whether VPF/VEGF receptor interaction would set in motion intracellular signal transduction mechanisms. In ECs, VPF/VEGF has been shown to mobilize intracellular calcium, which led us to test if a similar phenomenon would occur in MPs. Substrate-attached MPs loaded with the calcium-sensitive dye fura-2 were exposed to VPF/VEGF, and intracellular calcium was measured in individual cells by microspectrofluorometry. Although some cells (~30%) demonstrated spontaneous fluctuations in resting intracellular calcium ion concentration in a variety of cell types, the majority of cells (60% to 70%) showed stable resting $[\text{Ca}^{2+}]$. The latter were selected for study. Figure 4 shows the response of a representative MP exposed to VPF/VEGF. Resting $[\text{Ca}^{2+}]$ (25 nmol/L) increased approximately sixfold within 30 seconds of addition of VPF/VEGF (4 nmol/L) to a value more than 150 nmol/L. Following attainment of the peak, $[\text{Ca}^{2+}]$, decreased rapidly to a value approximately 10% greater than the initial resting level, which then decayed more slowly. Approximately 75% of the cell population studied showed a comparable calcium response to that in the Fig 4; the remaining cells, 25%, showed a slightly exaggerated or diminished calcium response. This general pattern of cellular response to VPF/VEGF was observed in four separate experiments, and is representative of agonist-stimulated changes in intracellular calcium ion concentration in a variety of cell types. This has been attributed to an initial release of sequestered calcium, followed by influx from the extracellular space.

**Cross-linking and phosphorylation studies with VPF/VEGF and MPs.** To determine if the interaction of VPF/VEGF with MPs involved its association with a particular cell surface protein, cross-linking studies were performed (Fig 5A). $^{125}$I-VPF/VEGF was incubated with MPs at 22°C to allow binding, and then the bifunctional cross-linker disuccinimidyl suberate was added. Following an incubation period of 15 minutes to allow cross-linking, MPs were solubilized, the lysate subjected to nonreduced SDS-PAGE, and then autoradiography performed. Following cross-linking of cell-bound $^{125}$I-VPF/VEGF, a new more slowly migrating band (see arrow) was observed (lane 2), which was not present when excess unlabeled VPF/VEGF was added (lane 1). A band with the same molecular weight was observed in EC cultures subjected to the same cross-linking procedure with cell-bound $^{125}$I-VPF/VEGF (lane 3). The latter band disappeared when excess unlabeled ligand was added during the binding of $^{125}$I-VPF/VEGF to ECs (lane 4). The high molecular weight band on the MPs was not due to aggregates/contaminants present in the initial tracer (lane 5) or cross-linking of the tracer to itself, regardless of the presence of cells (lane 6), as it was not observed when either cross-linker or cells were omitted (5% SDS-PAGE was used for lanes 5 and 6 to be certain no higher-molecular weight material was present that did not enter the gel). $^{125}$I-VPF/VEGF not exposed to MPs or cross-linker was homogeneous on nonreduced SDS-PAGE, migrating with a molecular weight of approximately 40 Kd. Taken together, these results suggested that the material with slower mobility on SDS-PAGE (lane 2) represented association of $^{125}$I-VPF/VEGF with a cell-surface polypeptide, presumably a ligand-receptor complex. A complex with similar molecular weight was observed on cultured ECs.

Because VPF/VEGF is in the PDGF family of growth factors, we considered whether binding VPF/VEGF to the cell surface would be followed by phosphorylation of cellular proteins. MPs were incubated with VPF/VEGF in the presence of $^{32}$P-containing medium, and immunoprecipitation was performed with antiphosphotyrosine antibody. The immunoprecipitate was then subjected to SDS-PAGE and autoradiography. In the presence of VPF/VEGF, a new high-molecular weight band was observed (Fig 5B, lane 1). No phosphorylated band with comparable mobility was observed in lysates of MPs not exposed to VPF/VEGF (Fig 5B, lane 2). Two migration of the high molecular weight phosphorylated band seen in the presence of VPF/VEGF was similar to that of the VPF/VEGF ligand-receptor complex observed in the cross-linking study, after subtracting the molecular weight of VPF/VEGF. This suggests that VPF/VEGF might be inducing phosphorylation of tyrosine residues on the receptor, although other proteins could be involved.

**DISCUSSION**

Previous studies have shown that incubation of VPF/VEGF with MPs induces directed migration, even across an EC monolayer, and activation, based on expression of tissue factor procoagulant activity. Each of these VPF/VEGF-mediated alterations in MP function occurred in a dose-dependent manner, being half-maximal at approximately 200 to 500 pmol/L (eg, VPF/VEGF-induced migration of MPs, Fig 2B). These data contrast with the considerably greater...
Cross-linking experiment was then performed as described in the text. Lane 1, $^{125}$I-VPF/VEGF bound to MPs in the presence of excess unlabeled VPF/VEGF, followed by cross-linking and phosphorylation study with VPF/VEGF. A cross-linking experiment was then performed as described in the text. Lane 1, $^{125}$I-VPF/VEGF bound to MPs in the presence of excess unlabeled VPF/VEGF, followed by cross-linking (7.5% SDS-PAGE); lane 2, $^{125}$I-VPF/VEGF bound to MPs, followed by cross-linking (7.5% SDS-PAGE); lane 3, $^{125}$I-VPF/VEGF bound to ECs, followed by cross-linking (7.5% SDS-PAGE); lane 4, $^{125}$I-VPF/VEGF bound to ECs in the presence of excess unlabeled VPF/VEGF, followed by cross-linking (7.5% SDS-PAGE); lane 5, $^{125}$I-VPF/VEGF bound to cells in the absence of cross-linker (5% SDS-PAGE); lane 6, $^{125}$I-VPF/VEGF at same concentration as above incubated with cross-linker as lane 3, but in the absence of cells (5% SDS-PAGE); lane 7, $^{125}$I-VPF/VEGF not exposed to cells or cross-linker. All gels are nonreduced. Marked arrows designate the migration of standard proteins run simultaneously (in kilodaltons). The unlabeled arrow in lanes 2 and 3 represents a new slowly migrating band. Note the absence of slowly migrating bands in lanes 5 to 7, where cells and/or cross-linker have been omitted. Electrophoresis in lanes 5 and 6 was performed for longer times until the free $^{125}$I-VPF/VEGF was almost at the bottom of the gel to be certain that no higher--molecular weight material was present in these samples (thus, the migration of VPF/VEGF here is not an accurate representation of its true molecular weight as in lane 5). (B) Phosphorylation of MP proteins in the presence of VPF/VEGF. MPs were incubated with VPF/VEGF (5 nmol/L; lane 1) or without (lane 2) for 10 minutes at 37°C in medium supplemented with $^{32}$P$_0$O$_4$. Samples were then obtained for nonreduced SDS-PAGE (7.5%) as described in the text. The unlabeled arrow designates a slowly migrating band observed in MPs incubated with VPF (the molecular weight is similar to that in lanes 2 and 3 of [A], above).

Fig 5. Cross-linking and phosphorylation study with VPF/VEGF bound to MPs and ECs. (A) Cross-linking. MPs or confluent ECs were incubated at 22°C with $^{125}$I-VPF/VEGF (200 pmol/L) alone or in the presence of a 100-fold molar excess of unlabeled VPF/VEGF. A cross-linking experiment was then performed as described in the text. Lane 1, $^{125}$I-VPF/VEGF bound to MPs in the presence of excess unlabeled VPF/VEGF, followed by cross-linking (7.5% SDS-PAGE); lane 2, $^{125}$I-VPF/VEGF bound to MPs, followed by cross-linking (7.5% SDS-PAGE); lane 3, $^{125}$I-VPF/VEGF bound to ECs, followed by cross-linking (7.5% SDS-PAGE); lane 4, $^{125}$I-VPF/VEGF bound to ECs in the presence of excess unlabeled VPF/VEGF, followed by cross-linking (7.5% SDS-PAGE); lane 5, $^{125}$I-VPF/VEGF bound to cells in the absence of cross-linker (5% SDS-PAGE); lane 6, $^{125}$I-VPF/VEGF at same concentration as above incubated with cross-linker as lane 3, but in the absence of cells (5% SDS-PAGE); lane 7, $^{125}$I-VPF/VEGF not exposed to cells or cross-linker. All gels are nonreduced. Marked arrows designate the migration of standard proteins run simultaneously (in kilodaltons). The unlabeled arrow in lanes 2 and 3 represents a new slowly migrating band. Note the absence of slowly migrating bands in lanes 5 to 7, where cells and/or cross-linker have been omitted. Electrophoresis in lanes 5 and 6 was performed for longer times until the free $^{125}$I-VPF/VEGF was almost at the bottom of the gel to be certain that no higher--molecular weight material was present in these samples (thus, the migration of VPF/VEGF here is not an accurate representation of its true molecular weight as in lane 5). (B) Phosphorylation of MP proteins in the presence of VPF/VEGF. MPs were incubated with VPF/VEGF (5 nmol/L; lane 1) or without (lane 2) for 10 minutes at 37°C in medium supplemented with $^{32}$P$_0$O$_4$. Samples were then obtained for nonreduced SDS-PAGE (7.5%) as described in the text. The unlabeled arrow designates a slowly migrating band observed in MPs incubated with VPF (the molecular weight is similar to that in lanes 2 and 3 of [A], above).

potency of VPF/VEGF for modulation of EC functions, such as enhancement of cell growth (half-maximal at 25 pmol/L; Fig 2A), and induction of cytosolic calcium flux (half-maximal at 0.4 pmol/L). In addition, ECs display a lower-affinity binding site with a kd of approximately 1.5 nmol/L. These considerations led us to characterize further VPF/VEGF-MP interaction.

Based on radioligand binding experiments, MPs express a single class of VPF/VEGF binding sites with a kd of approximately 300 to 500 pmol/L (Fig 1, Table 1). Occupancy of VPF/VEGF binding sites on the MP surface correlates with previously reported VPF/VEGF-mediated alterations in monocyte functions. Further evidence that these MP binding sites function as receptors for VPF/VEGF derives from their role in cellular processing of the ligand: they appear to mediate internalization and degradation of $^{125}$I-VPF/VEGF (Fig 3). In this context, the potential significance of VPF/VEGF for modulation of multiple properties of MPs is illustrated by its ability to increase cytosolic calcium (Fig 4).

Cross-linking studies demonstrated that $^{125}$I-VPF/VEGF bound to the MP surface becomes associated with slowly migrating polypeptide(s) (Fig 5A), potentially representing the VPF/VEGF receptor. Experiments with ECs and $^{125}$I-VPF/VEGF showed a high--molecular weight band with approximately the same molecular weight (Fig 5A). These observations are consistent with the results of previous cross-linking studies on ECs, although some of the latter also identified bands with lower molecular weights, and suggest that the EC and MP receptors for VPF/VEGF may be identical. In this context, a recent study has identified an fms-like tyrosine kinase (flt) as a receptor for VPF/VEGF. Transfection of COS cells with the flt cDNA led to high-affinity binding of $^{125}$I-VPF/VEGF, and cross-linking studies with $^{125}$I-VPF/VEGF showed bands corresponding to approximately 160 Kd and approximately 260 Kd for the receptor, speculated to represent monomer and dimer forms. These data would appear to explain the high-affinity interaction of VPF/VEGF with the EC surface, and extend the analogy of VPF/VEGF with PDGF by placing its receptor in the class of transmembrane-spanning tyrosine kinase receptors. However, the reason for the lower affinity of VPF/VEGF-MP interaction, especially if the putative receptor is similar on both cell types, is a central issue and will require further studies to elucidate.

Taken together, these data indicate that the VPF/VEGF receptor on MPs bears certain similarities to the EC receptor, including ligand-induced stimulation of tyrosine phosphorylation and cytosolic calcium increase. However, there is also a clear difference between the MP and EC VPF/VEGF binding sites, namely the much lower affinity of VPF/VEGF for the MP compared with the EC. These studies provide a starting point for further work to understand the nature of VPF/VEGF interaction with receptors on MPs, and to compare their properties with VPF/VEGF receptors on ECs. In view of the broad distribution of VPF/VEGF and the ability of this cytokine-like polypeptide to induce MP migration and tissue factor, understanding the basis of VPF/VEGF-monocyte interaction will be important in delineating the contribution of this molecule to the host-response.
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REFERENCES

Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes

H Shen, M Clauss, J Ryan, AM Schmidt, P Tijburg, L Borden, D Connolly, D Stern and J Kao