Vascular Permeability Factor/Vascular Endothelial Growth Factor-mediated Signaling In Mouse Mesentery Vascular Endothelium

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ABSTRACT

Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) is a multifunctional cytokine and growth factor that has important roles in both pathological and physiological angiogenesis. VPF/VEGF induces vascular hyperpermeability, cell division, and other activities by interacting with two specific receptor tyrosine kinases, KDR/Flik-1 and Flt-1, that are selectively expressed on vascular endothelium. The signaling cascade that follows VPF/VEGF interaction with cultured endothelium is only partially understood but is known to result in increased intracellular calcium, activation of protein kinase C, and tyrosine phosphorylations of both receptors, phosphatidylinositol 3'-kinase and phosphatidylinositol 3'-kinase. For many reasons, signaling events elicited in cultured endothelium may not mimic mediator effects on intact normal or tumor-induced microvessels in vivo. Therefore, we developed a system that would allow measurement of VPF/VEGF-induced signaling on intact microvessels. We used mouse mesentery, a tissue whose numerous microvessels are highly responsive to VPF/VEGF and that we found to express Flk-1 and Flt-1 selectively. At intervals after injecting VPF/VEGF into mesenteries, harvested and extracted, and immunoprecipitated. Immunoblots confirmed that VPF/VEGF induced tyrosine phosphorylation of several proteins in mesenteric microvessels as in cultured endothelium: Flk-1, PLC-γ, and mitogen-activated protein kinase. Similar phosphorylations were observed when mesentery was exposed to VPF/VEGF in vitro, or when mesenteries were harvested from mice bearing the mouse ovarian tumor ascites tumor, which itself secretes abundant VPF/VEGF. Other experiments further elucidated the VPF/VEGF signaling pathway, demonstrating phosphorylation of both PYK2 and focal adhesion kinase, activation of c-jun-NH2-kinase with phosphorylation of c-Jun, and an association between Flk-1 and PLC-γ. In addition, we demonstrated translocation of mitogen-activated protein kinase to the cell nucleus in cultured endothelium. Taken together, these experiments describe a new model system with the potential for investigating signaling events in response to diverse mediators on intact microvessels in vivo and have further elucidated the VPF/VEGF signaling cascade.

INTRODUCTION

VPF, also known as VEGF, is a multifunctional cytokine that is secreted in large amounts by many different tumor cells and is also expressed by certain normal cells during development and in the adult. Among other activities, VPF/VEGF potently increases the permeability of venules and small veins to plasma proteins. Microvascular hyperpermeability is one of the earliest steps in angiogenesis, leading to extravasation of fibrinogen and other plasma proteins, activation of the clotting system, and deposition of a fibrin gel in the extravascular space. This fibrin gel, in turn, provides a "pro-angiogenic" provisional matrix that favors and supports the inward migration of fibroblasts and new blood vessels, resulting in generation of mature stroma. This sequence of events has been well documented in many solid and ascites tumors and in other pathologies characterized by angiogenesis. In addition to permeabilizing microvessels, VPF/VEGF is also a selective mitogen for ECs; it alters the pattern of EC gene expression, serves as an EC survival factor, and reverses EC senescence. However, the microvascular permeabilizing effects of VPF/VEGF become evident within seconds to minutes, whereas its other biological effects go into effect only over a matter of hours or days.

VPF/VEGF is thought to exert all of its effects by interacting with two receptor tyrosine kinases, Flk-1 (mouse; the human counterpart is KDR) and Flt-1, both members of the flk-like receptor tyrosine kinase family. With a few exceptions, these receptors are expressed exclusively on vascular endothelium, thereby accounting for the selective action of VPF/VEGF. Studies with cultured ECs have revealed that VPF/VEGF interaction with its receptors leads to a number of tyrosine phosphorylations, including those of both Flk-1/KDR and Flt-1, PLC-γ, and phosphatidylinositol 3'-kinase. Other signaling events include activation of protein kinase C and transient increases in intracellular calcium. Despite considerable recent progress, much remains to be learned about the signaling pathways that are initiated in vascular endothelium by VPF/VEGF. Whether Flt-1 and Flk-1 trigger different or similar pathways, the full extent of such pathways, and whether different receptors and/or pathways lead to the several different biological effects exerted by VPF/VEGF on vascular endothelium remain unclear.

To date, measurements of VPF/VEGF signaling have been performed exclusively on cultured ECs and, for the most part, on ECs derived from large blood vessels. Whereas this approach is convenient and allows precise control of the cellular microenvironment, it suffers from certain drawbacks. VPF/VEGF exerts its several biological effects primarily on microvascular endothelium. ECs derived from large blood vessels differ in many respects from microvascular endothelium; therefore, studies with cultured large vessel ECs may not accurately reflect the biological and biochemical responses of microvascular ECs. Moreover, whether isolated from large or small blood vessels, ECs lose certain differentiated properties in culture, and as isolated cells, they are not subject to regulatory mechanisms that may be imposed in vivo by neighboring cells. The signaling events associated with the dramatic early increases in microvascular permeability that occur in response to VPF/VEGF in vivo.

For these reasons, we set out to develop a system that would more closely mimic EC signaling events as they occur in vivo. We chose the mouse mesentery for this purpose because it offers a number of important advantages:
VPF/VEGF SIGNALING OF MOUSE MESENTERY ENDOTHELIUM

(a) Mesentery is rich in microvessels and contains relatively small numbers of a limited number of other cell types (mesothelial cells, fibroblasts, adipocytes, pericytes, and lymphocytes), none of which are known to express VPF/VEGF receptors.

(b) Within a few minutes after i.p. injection, VPF/VEGF profoundly increases the permeability of mesenteric microvessels (13). Moreover, when ascites tumor cells are transplanted to the peritoneal cavity, the mesentery exhibits the complete repertoire of biological responses that has been attributed to VPF/VEGF: microvascular hyperpermeability; fibrinogen extravasation; clotting of leaked fibrinogen to fibrin; replication of ECs; angiogenesis; and new stroma generation (12-14).

(c) Mesentery is abundant and easily obtained. It can be harvested rapidly from one or a small number of mice and can be cut into small pieces that can be tested individually for reactivity to agonists in short-term organ culture.

(d) Mesentery is extremely thin. Hence, VPF/VEGF and other agonists or antagonists rapidly penetrate mesentery to reach target microvessels.

(e) Mesentery permits the study of pathologically induced microvessels, e.g., tumor microvessels, if mesenteries are harvested from mice bearing ascites tumors.

(f) Experiments with mesentery can be carried out under physiological conditions. In contrast, ECs are typically cultured with growth factor-containing serum, and the response of cultured ECs to VPF/VEGF generally requires serum starvation.

Here we describe a system for measuring the response of mouse mesenteric blood vessel endothelium to VPF/VEGF in vivo and in vitro. We also compare the signaling responses of normal and tumor-generated mesenteric vessels with those reported for cultured ECs. Finally, using the mesentery system, we report new activation steps that follow exposure of microvessels to VPF/VEGF.

MATERIALS AND METHODS

Antibodies. IgG fractions of polyclonal antisera raised against NH2-terminal peptides of KDR (33 amino acids, Ala2-Thr32) and Flt-1 (30 amino acids, Ser21-Gly25) were kind gifts of Dr. Donald Senger (Beth Israel Deaconess Medical Center, Boston, MA). These antibodies recognize both human and murine isoforms of KDR/Flk-1 and Flt-1, respectively. A monoclonal antibody to phosphotyrosine (4G10, both free and agarose-conjugated) and a polyclonal murine isoforms of KDR/Flk-1 and Flt-1, respectively. A monoclonal antibody to phosphotyrosine (4G10, both free and agarose-conjugated) and a polyclonal

In addition to SDS-PAGE and immunoblotting, with antibody against phospho-specific Elk-1 (Ser187). For JNK assays, c-Jun fusion protein [GST-NH2-terminal c-Jun (1-89)] bound to glutathione-Sepharose beads was used to bind JNK present in tissue lysates, and kinase assays were performed as described above, using an antibody against phospho-specific c-Jun (Ser63) for immunoblotting.

In situ Detection of VPF/VEGF-induced MAPK Phosphorylation and Nuclear Translocation in Cultured ECs. Semiconfluent cultures of early passage (5-6) HUVECs (Clonetic Corporation, San Diego, CA) and human dermal microvascular ECs (the kind gift of Dr. Michael Detmar) were serum-starved (0.5% serum in EMB medium overnight) and treated with VPF/VEGF (100 ng/ml) for different intervals at 27°C. After washing, cells were fixed in 4% paraformaldehyde in PBS at pH 7.4, and stained with antibody against phospho-specific Elk-1 (Ser187). For JNK assays, c-Jun fusion protein (GST-NH2-terminal c-Jun (1-89)] bound to glutathione-Sepharose beads was used to bind JNK present in tissue lysates, and kinase assays were performed as described above, using an antibody against phospho-specific c-Jun (Ser63) for immunoblotting.

RESULTS AND DISCUSSION

Distribution of VPF/VEGF Receptors in Mouse Mesentery. VPF/VEGF initiates signaling by interacting with two protein receptor tyrosine kinases, Flk-1 and Flt-1; with a few exceptions, these receptors are expressed exclusively on vascular ECs (8, 22, 25-27). To determine whether this distribution was also true in mesentery, we stained mouse mesenteries with antibodies specific for each receptor. Both Flk-1 and Flt-1 were expressed exclusively on the mesenteric vasculature; ECs lining small vessels stained strongly, whereas those lining large muscular arteries and veins did not stain (Fig. 1). Therefore, we concluded that signaling events measured in mesentery after stimulation with VPF/VEGF could be safely attributed to events taking place in the vascular endothelium of the microvasculature.

Tyrosine Phosphorylation of Flk-1 and PLC-γ Induced in Mouse Mesentery by VPF/VEGF and by MOT Ascites Tumor
Cells. Both Flk-1 and Flt-1 as well as other proteins undergo phosphorylation when cultured ECs are exposed to VPF/VEGF (28–30).

To determine whether similar phosphorylations occurred in mesentery, VPF/VEGF was injected i.p., and mesenteries were harvested at different intervals. Mesenteric extracts were then prepared as described in "Materials and Methods" and analyzed by immunoblotting with antiphosphotyrosine antibodies.

In agreement with results obtained with cultured ECs, mesenteries stimulated in vivo by i.p. injection of VPF/VEGF exhibited increased tyrosine phosphorylation of a number of different proteins (Fig. 2, Lanes 2–4). The approximate *M*ₙ of the major proteins that underwent phosphorylation in response to VPF/VEGF are 240,000, 220,000, 145,000, 120,000, 84,000, 70,000, 58,000, 45,000, 36,000, and 26,000, in addition to some smaller proteins. A similar pattern of phosphorylations was observed when portions of mesentery that had been harvested from untreated mice were incubated with VPF/VEGF for 3 min in vitro (Fig. 2, Lane 5).

Like most tumors that have been studied, MOT cells secrete large
amounts of VPF/VEGF (MOT ascites fluid contains VPF/VEGF in concentrations in excess of 40 nm; Ref. 13). Therefore, we prepared extracts of mesentery harvested from MOT ascites tumor-bearing mice and tested them for protein tyrosine phosphorylations. We found that these extracts exhibited the same phosphorylation pattern that we observed in mesenteries exposed to VPF/VEGF in vivo or in vitro (Fig. 2, Lanes 6–8). In contrast, phosphorosynosine immunoblots performed on MOT cell lysates revealed a very different pattern of protein phosphorylations (Fig. 2, Lane 9). These results provide strong evidence that mesenteric ECs in ascites tumor-bearing mice undergo phosphorylations that are similar to those induced by VPF/VEGF alone.

Among other proteins, Flk-1 and PLC-γ have been shown to be phosphorylated when BAECs are cultured with VPF/VEGF (30). To determine whether these proteins were also phosphorylated in mesenteric vessels, we collected mesenteries from normal mice that had received VPF/VEGF i.p. and also from mice bearing MOT ascites tumors. Immunoblots demonstrated that both proteins were phosphorylated in response to injected VPF/VEGF as well as in supernatants derived from mice bearing MOT ascites tumors (Fig. 3, a and b).

**Association of Flk-1 with PLC-γ after VPF/VEGF Stimulation.**

Guo et al. (30) reported that PLC-γ was phosphorylated after interaction of BAECs with VPF/VEGF and also became associated with several other proteins including tyrosine-phosphorylated Nck, two GAP-associated proteins, and an unknown 200-kDa tyrosine-phosphorylated protein. Because Flk-1 has a molecular mass of ~200 kDa, we considered the possibility that Guo’s unknown protein might be Flk-1. To test this possibility, mesentry extracts prepared from mice injected i.p. with VPF/VEGF were immunoprecipitated with antibodies against Flk-1 and then immunoblotted with antibodies against PLC-γ. Fig. 3c demonstrates that PLC-γ formed a complex with Flk-1, and that this complex increased significantly in response to VPF/VEGF. Similar complexes were demonstrated in mesenteric extracts derived from MOT ascites tumor-bearing mice. We also performed the reciprocal experiment, immunoprecipitating extracts with antibodies to PLC-γ, followed by immunoblotting with antibodies to Flk-1 (Fig. 3d). Similar results were obtained. Taken together, these experiments show that upon VPF/VEGF stimulation, the receptor tyrosine kinase Flk-1 comes to be associated with PLC-γ in mesenteric ECs. Extrapolating our results to BAECs, it seems likely that Flk-1 is the unidentified 200-kDa protein that Guo et al. (30) described as part of a PLC-γ multimer complex after VPF/VEGF stimulation.

**Phosphorylation of PYK2 and FAK Induced by VPF/VEGF.**

It has been shown previously that VPF/VEGF induces increased intracellular calcium in cultured HUVECs (31). Lev et al. (32) have demonstrated that the protein tyrosine kinase PYK2, also known as related adhesion focal tyrosine kinase (33), is rapidly phosphorylated on tyrosine residues in response to stimuli that increase intracellular calcium. To determine whether PYK2 in mesenteric endothelium was activated by VPF/VEGF, extracts from mice injected i.p. with VPF/VEGF were immunoprecipitated with antibodies against phosphotyrosine and then immunoblotted with antibody against PYK2. Fig. 4a shows a specific protein band corresponding to PYK2, demonstrating increased tyrosine phosphorylation after VPF/VEGF treatment. Mesenteries taken from MOT ascites tumor-bearing mice also demonstrated increased PYK2 phosphorylation (Fig. 4). By analogy with work in other systems (31), this result suggests that the increased intracellular calcium induced by VPF/VEGF is associated with PYK2 activation.

We also found that FAK (34), the founding member of the FAK family that also includes PKY2, underwent phosphorylation in mesentery after i.p. injection of VPF/VEGF (Fig. 4b). Therefore, both members of the FAK family of protein kinases were activated by VPF/VEGF. Activation of FAK would be expected to lead to alterations in the organization of the cellular cytoskeleton; such cytoskeletal rearrangements could account for EC contraction events that have been associated with VPF/VEGF-induced increases in microvascular permeability (35).

**Activation and Translocation of MAPK after VPF/VEGF.**

To further dissect the signaling pathways induced in mesenteric ECs by VPF/VEGF, we assessed MAPK phosphorylation after i.p. injection of VPF/VEGF. As shown in Fig. 5a, VPF/VEGF induced increased amounts of tyrosine-phosphorylated MAPK; similar increases were found in mesenteries harvested from animals bearing MOT ascites tumors. Control mesenteries that were not exposed to exogenous VPF/VEGF also showed small amounts of phosphorylated enzyme, possibly reflecting a low level of endogenous VPF/VEGF activity present in blood or synthesized locally or, alternatively, activation by some other mechanism.

We also assayed mesenteric MAPK activity using the GST-Elk-1 fusion protein as a substrate. Tissue lysates were immunoprecipitated with an antibody (PY204) specific for tyrosine-phosphorylated MAPK, and the resulting immunocomplexes were incubated with GST-Elk-1 fusion protein in the presence of ATP. Fig. 5b shows that VPF/VEGF increased Elk-1 phosphorylation ~2-fold. Mesentery extract supernatants prepared from MOT ascites tumor-bearing mice showed a similar increase in phosphorylated Elk-1.

**MAPK has been shown to translocate to the cell nucleus after activation (36). Therefore, we sought to detect translocated MAPK immunohistochemically, applying a phospho-specific antibody to sections of VPF/VEGF-treated mesentery.** Because of high tissue background, we were unable to demonstrate significant differences of activated MAPK translocation to the nucleus after VPF/VEGF treatment. However, after exposure to VPF/VEGF, we were able to demonstrate increased amounts of nuclear phosphorylated MAPK in serum-starved cultures of both HUVECs (Fig. 6) and human dermal microvascular ECs (data not shown). Together, these data suggest that...
Fig. 4. Activation of PYK2 and FAK in mouse mesenteries after i.p. injection of either 500 ng of VPF/VEGF (harvested at 10 min) or 10^6 MOT cells (harvested at 13 days). Mesentery lysates were immunoprecipitated with antiphosphotyrosine antibody (PTyr) and immunoblotted with antibodies to PYK2 or FAK (stained bands in a and b, upper left panels, illustrate phosphorylated PYK2 and FAK, respectively). IgG bands (lower left panels in a and b) show that loading of immunocomplex extracts was equivalent in C, VPF, and MOT lanes. Right panels of a and b illustrate immunoblots prepared with 30 μg of the same extracts and stained with anti-PYK2 and anti-FAK antibodies, respectively, to provide a measure of the amounts of protein present in the extracts. These experiments were performed twice with identical results.

Fig. 5. VPF/VEGF and MOT cell activation of MAPK and JNK. Mesenteries were harvested from control (C), VPF/VEGF-injected, and MOT ascites tumor-bearing mice (13 days), as in previous figures. a, immunoblots of mesentery extracts with phospho-specific MAPK antibody (upper panel) and with MAPK antibody (lower panel). Phosphorylation of Elk-1 fusion protein was visualized by immunoblotting with phospho-Elk-1 (Ser^381) antibody. c, JNK assays were performed as described in "Materials and Methods." Phosphorylation of c-Jun was measured by immunoblotting with phospho-c-Jun (Ser^63) antibody. In the lower panel, Ponceau S staining reveals that comparable amounts of GST fusion proteins were bound to the beads. Similar results were obtained in three independent experiments.

**Activation of JNK and Phosphorylation of c-Jun after VPF/VEGF.** The proto-oncogene c-Jun is known to be regulated by protein phosphorylation (37, 38). Cellular stress, tumor necrosis factor α, and UV radiation all activate JNKs (also known as stress-activated protein kinases; Refs. 37–40) to phosphorylate Ser^63 and Ser^73 in the c-Jun transactivation domain. Because JNK has 40–50% sequence homology with MAPKs, we considered the possibility that JNK might also be activated as the result of VPF/VEGF signaling. To test this possibility, mesentery extracts were incubated with GST-NH₂-terminal c-Jun (1–89) fusion protein bound to glutathione-Sepharose
Fig. 6. Immunocytochemical staining of cultured HUVECs for phospho-MAPK at time 0 (A), at 10 min (B–E), and at 30 min (F) after addition of 100 ng/ml VPF/VEGF. A, B, D, and F were taken with bright-field optics, whereas C and E, representing the same fields as B and D, respectively, were taken with phase-contrast optics to allow positive identification of nuclear and cytoplasmic borders. Note the greatly increased intensity of overall staining in B–F as compared with control A. Also, this staining is predominantly nuclear as demonstrated by phase-contrast microscopy. Staining was less intense at 30 min than at 10 min although still largely nuclear. Bar, 20 μm.

beads; such beads preferentially capture activated JNK. After washing the beads, kinase activity was measured as described in “Materials and Methods.” Fig. 5c shows an immunoblot prepared with antibody against phospho-specific c-Jun (Ser63). VPF/VEGF treatment induced an ~2-fold increase in JNK activity in mesentery extracts, and mesentery extracts from MOT ascites tumor-bearing mice showed an approximately equivalent increase. Thus, VPF/VEGF activates both the MAPK and JNK pathways in ECs; also, ascites tumor cells, presumably because they secrete VPF/VEGF, activate these same pathways. These results indicate that VPF/VEGF, unlike some other growth factors, activates both MAPK and JNK pathways (41).

Conclusions. We have described a system for measuring activation events induced by VPF/VEGF in mouse mesentery blood vessels. This approach offers many advantages, including the ability to study the responses of ECs in intact microvessels under physiological conditions as well as the ECs of pathological blood vessels such as those induced by tumors and, potentially, by other angiogenic stimuli. Therefore, this system can be regarded as a benchmark against which studies on cultured ECs can be compared for relevance to the response of vascular endothelium that occurs in vivo in both physiological and pathological settings. As with cultured ECs, mesenteric microvascular ECs responded to VPF/VEGF by phosphorylating a number of different proteins including both VPF/VEGF receptors (Flk-1 and Flt-1), PLC-γ, and MAPK (28–30). These results therefore validate earlier studies performed on cultured ECs. They also demonstrate that similar events occur in the microvasculature of tumor vessels, presumably because these cells secrete large amounts of VPF/VEGF.

In addition, we have used the mesenteric system to discover several new activation events that occur in mesenteric ECs after VPF/VEGF stimulation. These include the formation of a physical association between Flk-1 and PLC-γ, phosphorylation of both PYK2 and FAK, and activation of JNK with resulting phosphorylation of c-Jun.

Integrin-ligand interaction has previously been linked to activation of the MAPK pathway through FAK activation (34); therefore, it is not surprising to find that VPF/VEGF, which also activates MAPK, should activate FAK as well. That VPF/VEGF should also lead to increased tyrosine phosphorylation of PYK2 is not unexpected, in that PYK2 is a member of the FAK family, and others have shown that PYK2 is rapidly tyrosine phosphorylated in response to other stimuli that increase intracellular calcium concentrations (32). The MAPK activation demonstrated here may, in turn, be mediated through PYK2 and Ca2+-dependent PKC pathways. Lev et al. (32) have presented clear evidence that Ca2+-activated PYK2 stimulates the MAPK cascade. We also demonstrated activation and translocation of MAPK to the cell nucleus in two types of cultured ECs. VPF/VEGF activation of JNK further complicates the picture, in that JNK activation has previously been associated with cell stress (42). Taken together, these results have further elucidated the cell signaling pathways that follow exposure of ECs to VPF/VEGF.

A challenge for the future is to determine how the divergent signaling pathways initiated by VPF/VEGF interconnect to regulate EC permeability, proliferation, motility, and altered gene expression. The mesenteric system that we have described should be useful for this purpose. It affords the great advantage of allowing the study of metabolic events in a time frame that corresponds to the activities of VPF/VEGF as they occur in vivo. For example, VPF/VEGF causes increased microvascular permeability within a matter of a few minutes, whereas EC division, altered gene expression, and angiogenesis do not occur for some hours or even days. Here we have measured metabolic events taking place in mesenteric endothelium within a few minutes of stimulation with VPF/VEGF, therefore events that correlate temporally with increased microvascular permeability. We cannot say that the biochemical events that occur in this time frame are necessarily responsible for or are even associated with increased vessel per-
meability: they could instead be precursors to later events relating to EC division, altered gene expression, and so forth. However, biochemical events that do not proceed within this time frame cannot be associated with increased microvascular permeability.

REFERENCES


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