VEGFR-1–Selective VEGF Homologue PlGF Is Arteriogenic Evidence for a Monocyte-Mediated Mechanism

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Abstract—Two signaling receptors for vascular endothelial growth factor (VEGF) in the vasculature are known with not yet well-understood roles in collateral vessel growth (arteriogenesis). In this study, we examined the involvement of the two VEGF receptors in arteriogenesis. Therefore, we used the VEGF homologue placenta growth factor (PIGF), which only binds to VEGFR-1 and VEGF-E, which only recognizes VEGFR-2. These peptides were locally infused over 7 days after ligation of the femoral artery in the rabbit. Evaluation of collateral growth by determining collateral conductance and angiographic scores demonstrated that the VEGFR-1–specific PlGF contributed significantly more to arteriogenesis than the VEGFR-2 specific VEGF-E. The combination of VEGF-E and PlGF did not exceed the effect of PlGF alone, indicating that cooperation of the two VEGF receptors in endothelial cell signaling is not required for arteriogenesis. In an in vitro model of angiogenesis, VEGF and VEGF-E were comparably active, whereas PlGF displayed no activity when given alone and did not further increase the effects of VEGF or VEGF-E. However, PlGF was as potent as VEGF when monocyte activation was assessed by monitoring integrin surface expression. In addition, accumulation of activated monocytes/macrophages in the periphery of collateral vessels in PIGF-treated animals was observed. Furthermore, in monocyte-depleted animals, the ability of PIGF to enhance collateral growth in the rabbit model and to rescue impaired arteriogenesis in PIGF gene–deficient mice was abrogated. Together, these data indicate that the arteriogenic activity observed with the VEGFR-1–specific PIGF is caused by its monocyte-activating properties. (Circ Res. 2003;92:378-385.)

Key Words: arteriogenesis ■ monocytes ■ endothelial cells ■ placenta growth factor ■ vascular endothelial growth factor receptors

In adult organisms, physiological blood vessel growth is restricted to the female reproductive system. In pathophysiological conditions such as tissue repair, blood vessel growth occurs either by the sprouting or intussusceptive growth of capillaries (also termed angiogenesis) or by the development of collateral arteries from preexisting anastomoses (also termed arteriogenesis). Whereas angiogenesis is predominately initiated by hypoxia, collateral artery growth is induced after occlusions in larger arterial vessels.1 In the latter case, blood flow is redistributed by the intravascular pressure difference along preexisting anastomoses. The thus generated increase in shear stress in these vessels is believed to activate the endothelium and to mediate the recruitment of circulating blood monocytes, which can support processes of vessel remodeling by the production of growth factors and by the release of metalloproteinases.2–4 As in the case of hypoxia-driven angiogenesis, collateral growth occurs as a pathophysiological compensatory process, which can be accelerated by the application of proteins or DNA. Various factors including the monocyte chemotactic protein (MCP)-1 and the angiogenesis factor VEGF (vascular endothelial growth factor) have been demonstrated to promote arteriogenesis.5,6

Because VEGF does not only recruit and activate endothelial cells but also monocytes,7 the mechanism by which VEGF can increase collateral growth could be mediated either by monocytes or endothelial cells. Previously, placenta growth factor (PIGF) was identified as a specific ligand of VEGFR-1, which is the exclusive VEGF receptor on monocytes.8–10 In endothelial cells, however, PIGF emerged to be much less or even not at all active.11 Furthermore, lack of the intracellular signaling domain of VEGFR-1 was not found to be associated with vascular defects during embryogenesis, leading to the hypothesis that the VEGFR-1 is dispensable and that VEGFR-2 is the predominant signaling receptor in endothelial cells for VEGF.12 The investigation of the roles of specific VEGF receptors has been helped by the recent
discovery of VEGF-E as a selective ligand for the VEGFR-2.\textsuperscript{13}

Previously, reduced collateral formation/arteriogenesis was demonstrated in PIGF gene-deficient mice, indicating that the VEGFR-1 is involved in this process.\textsuperscript{12} In the present study, we demonstrate the potent ability of PIGF to induce collateral growth in a rabbit model of hind limb ischemia by counting collateral vessels and measuring collateral flow perivascularly at the feeding artery. Surprisingly, the PIGF-mediated improvement in collateral numbers and perfusion was superior when compared with the VEGFR-2-specific ligand VEGF-E and displayed no additive activities when combined with VEGF-E. Depletion of monocytes could almost completely abolish the arteriogenic activity of PIGF, which is in support for the hypothesis that monocyte activation and recruitment is essentially involved in arteriogenesis.

\section*{Materials and Methods}

\subsection*{Arteriogenic Peptides}

Monocyte chemoattractant protein-1 (MCP-1) was provided from Boehringer Ingelheim. Heparin-binding forms of VEGF(VEGF\textsubscript{165}) and PIGF(PIGF-2) were expressed in SF9-insect cells (Invitrogen, San Diego, Calif) using the baculovirus expression system, produced, and purified as described previously.\textsuperscript{9} To compare VEGF-E with these heparin-binding forms appropriately, a VEGF-E chimera containing the heparin-binding domain of VEGF was used, which enables this molecule to bind to the VEGF coreceptor neuropilin (M. Heil, R. Mittnacht-Krauss, K. Isbrücker, J. van den Heuvel, C. Dehio, W. Schaper, M. Clauss, H.A. Weich, unpublished data, 2003). This molecule was not changed in its binding properties to the VEGF coreceptor neuropilin (M. Heil, R. Mittnacht-Krauss, K. Isbrücker, J. van den Heuvel, C. Dehio, W. Schaper, M. Clauss, H.A. Weich, unpublished data, 2003). This molecule was not changed in its binding properties to the VEGF coreceptor neuropilin (M. Heil, R. Mittnacht-Krauss, K. Isbrücker, J. van den Heuvel, C. Dehio, W. Schaper, M. Clauss, H.A. Weich, unpublished data, 2003). This molecule was not changed in its binding properties to the VEGF coreceptor neuropilin (M. Heil, R. Mittnacht-Krauss, K. Isbrücker, J. van den Heuvel, C. Dehio, W. Schaper, M. Clauss, H.A. Weich, unpublished data, 2003). This molecule was not changed in its binding properties to the VEGF coreceptor neuropilin (M. Heil, R. Mittnacht-Krauss, K. Isbrücker, J. van den Heuvel, C. Dehio, W. Schaper, M. Clauss, H.A. Weich, unpublished data, 2003).

\subsection*{Animal Models}

The present study was performed with the permission of the State of Hessen, Regierungspräsidium Darmstadt, according to Section 8 of the German Law for the Protection of Animals. It conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animals were housed individually with free access to water and chow. No animals died during or after femoral artery ligation, and we did not observe any gangrene or gross impairment of hind limb function after femoral artery occlusion.

\subsection*{Ischemic Rabbit Hind Limb}

Thirty-five New Zealand White Rabbits (Elevage Scientifique, Chatillon, France; 2.8±0.4 kg body weight) were randomly assigned to one of the groups stated below (n=5 per group). For dissection and ligation of the femoral artery, the animals were anesthetized with an IM injection of ketamine hydrochloride (40 mg/kg) and xylazine (4 mg/kg). Animal groups received either 10 mg/mL albumin in isotonic buffer in the absence (controls) or presence of MCP-1, PIGF, vascular endothelial growth factor (VEGF, VEGF-E) or a combination of VEGF-E and PIGF locally via an osmotic minipump (Alzet Corp) for 1 week. Pumps were connected to the proximal stump of the ligated femoral artery by a catheter and then subcutaneously fixed in the flank. All animals received antibiotic (Enrofloxacine) and analgesic treatment (Buprenorphin).

\subsection*{Postmortem Angiography}

Hind limbs of euthanized rabbits were perfused with papaverine solution (4 mg/L) followed by a gelatin-bismuth-based contrast agent.\textsuperscript{14} Angiograms of each hindlimb were taken in a Balteau radiography apparatus (Machlett).\textsuperscript{3} Angiographically visible collateral arteries, spanning the arteriae profunda femoris and circumflexa femoris lateralis to the arteriae genualis descendens and caudalis femoris, according to the Longland classification,\textsuperscript{15} were counted (Figure 2A). According to the Poiseuille law, vessels with large diameters conduct more blood. To address the important role of vessel diameter, we used an angioscope by introducing weighting factors for vessels with larger sizes (Figure 2B). Therefore angiographically visible collaterals were weighted into groups according to distinct vessel diameters.

\subsection*{In Vivo Calculation of Collateral Conductance}

Seven days after ligation of the femoral artery, animals were again anesthetized and maintained as described.\textsuperscript{16} Systemic pressure was measured in the right carotid artery, peripheral pressures in both saphenous arteries, each connected by catheters to P32DC pressure transducers (Statham, Spectramed). Synchronously, hindlimb blood flow was measured at increasing adenosine concentrations (30 to 600 μg/kg per min) infused through a thin catheter positioned cranial to the aortic bifurcation using an animal research flowmeter and perivascular ultrasonic flow probes (2.5 mm; Transonic), which were placed around both external iliac arteries.\textsuperscript{17,18} All data were recorded on a computer using an ADI-transducer and commercially available software (MacLab, Macintosh). The maximal collateral conductance (CC) was calculated from the systemic (SP) and peripheral pressures (PP) and external iliac blood flow (Q\textsubscript{e}) at maximal vasodilatation using the following equation:

\begin{equation}
CC(\text{mL/min per 100 mm Hg}) = \frac{Q_{\text{e}}(\text{mL/min})}{SP-PP(\text{mm Hg})} \times 100
\end{equation}

\subsection*{In Vivo Localization of Macrophages}

X-gal staining was used to localize and quantify activated macrophages according to previous reports showing that activated rabbit tissue macrophages posses specific endogenous galactosidase activity.\textsuperscript{19,20} Three days after femoral artery ligation and infusion of PIGF or albumine, rabbits were fixed by perfusion; the vastus intermedius muscle was excised and cryopreserved. Sections (10 to 12-μm thick) were air dried, washed with PBS, and incubated at 37°C for 24 to 48 hours with X-gal solution (0.1% X-gal, 5 mmol/L potassium ferri-cyanide, 5 mmol/L potassium ferrocyanide, 1 mmol/L magnesium chloride, 0.002% NP-40, 0.01% sodium deoxycholate and PBS; pH 7.0). In some cases, nuclei were counterstained with hematoxylin. Total number of positive macrophages per section was counted using an optical microscope (Leitz Aristoplan). Photomicrographs of each section were then obtained and computerized, and the total area of each section was measured by planimetry using the NIH Image 1.62 software for Macintosh. Finally, numerical density of activated macrophages per mm\textsuperscript{2} was calculated.

\subsection*{PIGF\textsuperscript{−/−} Mice}

Anesthetized PIGF\textsuperscript{−/−} mice were unilaterally ligated and collateral growth was quantified as previously described.\textsuperscript{12} Either 5 μg PIGF or solvent (0.1% albumin) (each n=5) were infused for 7 days via microsomatic pumps as described above. Blood flow measurements in paws of anesthetized mice were performed at different time points using a laser Doppler perfusion imager (MLDI 5063, Moor Instruments Ltd). The ligated-to-nonligated side perfusion ratios were calculated for each mouse after subtracting previously determined background flux values.\textsuperscript{21}

\subsection*{Monocyte Depletion}

Bisphosphonates were encapsulated in liposomes and injected IV 24 hours prior to femoral ligation in rabbits in order to deplete monocytes as described previously.\textsuperscript{22} Loss of CD14-positive cells was tested immediately before ligation by FACS analysis (data not shown). In PIGF\textsuperscript{−/−} mice, monocytes were depleted by a single injection of 150 mg/kg 5-fluorouracil (5-FU) as described previously.\textsuperscript{23}
In Vitro Models

Sprout Formation Assay
Angiogenesis in vitro was tested as described previously. Briefly, human lung–derived microvascular endothelial cells (HLMECs) were grown to confluence on cytodex-3 microcarrier beads and placed into a fibrinogen gel containing VEGF, VEGF-E, PlGF, or solvent. Polymerization was started by adding 0.65 U/mL thrombin. After 18 hours, gel was fixed in 1% PFA/PBS and number of sprouts per 50 beads was counted under the microscope. Experiments were performed in triplicates.

Monocyte Stimulation and Flow Cytometric Analysis
Whole blood was drawn from healthy donors and anticoagulated using citrate. Blood aliquots were incubated with VEGF, PlGF, or VEGF-E for 2 hours at 200 ng/mL. Thereafter, either 50 µL of these samples were incubated with one of the following combinations of two monoclonal antibodies: phycocerythrin (PE)-conjugated monoclonal antibody (mab) against CD14 (clone MY4, Beckman-Coulter) served for monocyte identification. Fluorescein-isothiocyanate (FITC)-conjugated mab against each examined integrin subunit (α<sub>i</sub>, α<sub>IIb</sub>, or β<sub>3</sub>, integrin monomers; all from DIANOVA) were used to quantify integrin expression on monocyte surface. After incubation, samples were lysed in ammoniumchloride buffer, washed, and fixed with 2% paraformaldehyde. Flow cytometry was performed on a FACS Calibur (BD). CD14-positive monocytes were identified by their PE-fluorescence and gated for analysis. FITC-fluorescence corresponding to the expression of either one of the analyzed integrins was measured and examined statistically using Cellquest (BD).

Statistics
Results are presented as mean±SEM. Comparison between two mean values was performed using the unpaired Student’s t test, between 3 or more groups using a one-way ANOVA followed by a Student’s t test with a post hoc Bonferroni correction. P<0.05 was considered to be statistically significant.

Results
Arteriogenic Properties of Placenta Growth Factor
Based on a previous study showing that PlGF gene–deficient mice are severely impaired in their ability to promote arteriogenesis in response to femoral ligation, we tested whether PlGF infusion can rescue this phenotype in these animals. PlGF was applied by micropumps into PlGF<sup>−/−</sup> mice and perfusion was assessed by laser Doppler measurements in the paw. With this regimen, a slight improvement of blood flow after 3 days and significantly increased blood perfusion after 7 days could be observed (Figure 1).

Because rabbits more easily comply with the requirements to perform detailed hemodynamic measurements, we performed further studies with these animals. After femoral ligation and local infusion of proteins for 7 days, collateral vessel formation was assessed by angiography and collateral conductance was calculated.

Post-mortem angiograms after 1 week of femoral artery ligation and local albumin treatment (control) showed characteristic corkscrew-like collateral vessels (Figure 2A). In the VEGF-treated group (3.0 µg/kg), an increased number of visible collaterals per hind limb was observed (14.3±0.4 versus 11.6±0.9; P<0.01). However, most of these collaterals only reached diameters less than 200 µm. In contrast, when PlGF was applied at the same doses, the number of collaterals (16.7±0.4; P<0.001) and their average diameter (300±50 µm) were significantly increased (Figure 2 and Table).

To address the important role of vessel size for blood perfusion, we introduced a diameter-weighted angiographic score analysis of collaterals (Table). One week after femoral artery ligation, all VEGF animals showed only a moderately increased angioscore per limb (91.7±5.1 versus 66.7±7.1; P<0.01). However, the PlGF-treated group revealed an improvement of about 50% (105.2±13.9; P<0.001) that was almost close to the effect (139.4±12; P<0.001) observed with MCP-1 (0.5 µg/kg). Further hemodynamic in vivo measurements showed that compared with controls, VEGF improved collateral conductance values about 30% (133±5.1 versus 101.3±9.9 mL/min per 100 mm Hg; P<0.01), whereas application of PlGF and of MCP-1 increased flow about 65% (165±5.1 mL/min per 100 mm Hg; P<0.001) and 100% (220±5.1 mL/min per 100 mm Hg; P<0.001), respectively (Figure 3). Together these findings indicate that stimulation of the VEGFR-1 can increase both number and size of angiographically visible collateral vessels and improve blood flow.
Combination of VEGF-E and PlGF Does Not Further Improve PlGF Accelerated Arteriogenesis

Having shown that PlGF and VEGF can both accelerate processes of collateral formation, we next assessed whether specific activation of VEGFR-2 can promote arteriogenesis. Therefore, we used the VEGFR-2-selective ligand VEGF-E, which was further modified to bind to heparin and thus could be compared with the heparin-binding isoform of PlGF and VEGF used in this study. Infusion of VEGF-E (1.5 μg/kg) increased the number and size of collaterals visible by angiography (13 ± 1.4 versus 11.6 ± 0.9 collaterals per leg; *P = NS) although not to the same extent as was observed with PlGF. When VEGF-E (1.5 μg/kg) was combined with PlGF (1.5 μg/kg) at the same concentration as in the single agent studies, no further improvements of collateral numbers and collateral angioscore were observed compared with the application of PlGF alone (14.3 ± 1.1 versus 16 ± 0.6 collaterals per leg; Table). When collateral conductance was assessed, VEGF-E had a mild effect compared with the control group (124 ± 8.6 mL/min per 100 mm Hg; *P < 0.05). Again, the combination of the same concentration of PlGF and VEGF-E failed to even reach conductance values of PlGF alone (129 ± 21.5 versus 141 ± 9.2 mL/min per 100 mm Hg; Figure 3). However, when the applied PlGF concentration was doubled (3.0 μg/kg), a significant improvement in collateral growth (16.7 ± 0.4 collaterals per leg and 165 ± 5.1 mL/min per 100 mm Hg) versus PlGF (1.5 μg/kg) or VEGF-E (1.5 μg/kg) could be demonstrated (Figure 3 and Table; **P < 0.01). These data indicate that VEGFR-1 and VEGFR-2 mediated processes do not cooperate.

VEGF and VEGF-E, but not PlGF, Stimulate Angiogenesis In Vitro

To cross-characterize the VEGF receptor-selective ligands, we compared them in a model of sprouting angiogenesis in vitro by using human lung microcapillary endothelial cells grown on microbeads and embedded into a fibrin gel. In this assay, VEGF and the VEGFR-2-selective ligand VEGF-E induced sprout formation to comparable extents, whereas the VEGFR-1-selective ligand PlGF had no effect even at higher concentrations (Figure 4). To address a potential synergy between the two VEGF receptors, various concentrations of PlGF were combined with VEGF-E. Again, no significant increase in sprout formation was observed, indicating that VEGFR-1 activation is not required for VEGFR-2 signal transduction leading to sprout formation (Figure 4).

PlGF and VEGF Equally Stimulate Integrin Expression in Human Peripheral Monocytes, Whereas VEGF-E Is Inactive

We next compared the ability of these receptor-specific ligands to activate monocytes, which only express the VEGFR-1. This receptor was shown to be activated by PlGF and VEGF but not by VEGF-E. Whole blood samples were stimulated for 2 hours either with VEGF, PlGF, or VEGF-E
and integrin expression on monocytes was assessed by flow cytometry (Figure 5). Both VEGF and PlGF induced expression of CD11a and CD18 on monocytes within the same range, indicating that the preparations of PlGF and VEGF were of comparable activity. However, when monocytes were stimulated with the VEGFR-2–specific ligand VEGF-E no significant increase in monocyte integrin expression was observed, which confirms previous reports.25 In conclusion, the higher activity of PlGF versus VEGF in vivo cannot be explained by higher bioactivity of PlGF because in vitro experiments with monocytes revealed equal activities of the VEGF protein used in this study.

**PIGF-Induced Collateral Growth Is Associated With Increased Macrophage Accumulation**

In order to provide further evidence for the hypothesis that the arteriogenic activity of PlGF is the consequence of an increased recruitment and activation of macrophages in the vicinity of growing collateral arteries, we localized and quantified activated macrophages according to their ability to express endogenous galactosidase activity.19,20 As shown in Figure 6, increased numbers of activated mononuclear phagocytes can be observed around grown collateral vessel. Quantification of activated macrophages in the vastus intermedius muscle (Figure 6) revealed a 3-fold increase in the PlGF-treated compared with the control group (62±6.7 versus 16.5±4.9 macrophages/cm²; \(P<0.001\)).

**Monocyte Depletion Abolishes PIGF-Induced Collateral Growth**

In order to provide further functional evidence for the hypothesis that monocytes mediate collateral growth in response to PIGF, we depleted monocytes from rabbits by using a recently developed protocol.22 This treatment caused almost total loss (more than 90%) of CD14-positive mononuclear cells as assessed by FACS analysis (data not shown). When femoral arteries were ligated 24 hours after the infusion of the depleting agent, the arteriogenic effect of PIGF was totally abolished as assessed by angiograms and perfusion studies (Figures 7A through 7C). Furthermore, in mononuclear cell–depleted mice by 5'-fluorouracil treatment,23 PIGF-administration via minipump failed to rescue collateral vessel formation in PIGF−/− mice (Figure 7D).
extravasated activated cells of the monocyte/macrophage lineage in PI GF-treated animals versus controls. This observation correlates with the finding that monocyte numbers are reduced in growing collaterals of the hind limb in PI GF gene–deficient animals.\textsuperscript{12} In addition, this finding is in line with the reported strong involvement of the monocyte chemokine MCP-1 and recent findings showing that addition of isolated monocytes can rescue impaired arteriogenesis after chemical depletion of mononuclear cells in rabbits.\textsuperscript{23} In conclusion, VEGF and PI GF by stimulating monocytes start processes of monocyte adherence to sites of increased shear stress, which occurs within preexisting collateral or small anastomotic vessels in close proximity to the occlusion. Activated monocytes invade the vessel wall and then by release of proteases and further growth factors start or direct remodeling processes.

Although the direct effects of PI GF on mononuclear phagocytes are well documented,\textsuperscript{9,10,26,27} VEGFR-1–mediated signaling events in the endothelium cannot be totally excluded to be involved in arteriogenesis. One possible mechanism could be the release of the arteriogenic chemokine MCP-1,\textsuperscript{5} which reportedly is induced by VEGF in endothelial cells in vitro.\textsuperscript{28} However, the signaling receptor for VEGF has not been identified for this activity. Furthermore, the proarteriogenic activities of VEGF and its homologues could also be explained by the ability of VEGF to mobilize hematopoietic stem cells. These could give rise to monocyte precursor or endothelial progenitor cells (EPCs) and thereby may induce collateral growth.\textsuperscript{29–31} A functional role of the VEGFR-1 in recruiting not only EPCs but also other myeloic cells is shown by systemic treatment with PI GF.\textsuperscript{32} Mobilization of monocyte precursor cells would be in line with our present data showing that monocytes are essential for PI GF-induced collateral growth and our previous finding that CD11b (Mac-1)–purified blood monocytes can rescue impaired collateral growth–dependent blood flow restoration in monocyte-depleted mice.\textsuperscript{23} However, our local infusion of VEGF homologues does not lead to significant measurable systemic values as determined by use of a commercially available ELISA for VEGF and PI GF (data for not shown), which makes mobilization of bone marrow–derived cells less likely. Furthermore, we hypothesize that by local infusion via the minipump the heparin-binding VEGF homologues accumulate at the heparin-proteoglycans of the endothelium and thus build up a gradient for peripheral blood monocytes.

In addition to monocytes, VEGFR-1 has also been reported in nonendothelial vascular cells, namely in coronary smooth muscle cells.\textsuperscript{33} A mechanism involving smooth muscle cells (SMCs) in PI GF- and VEGF-induced arteriogenesis would be plausible because smooth muscle cell proliferation is both observed in and required for growth of arterial vessels.\textsuperscript{2} Vascular smooth muscle cells in vitro display both VEGF receptors and they migrate in response to VEGF-E and VEGF, but in response to PI GF only at higher concentrations.\textsuperscript{33} However, in vivo so far only the VEGFR-1 could be detected.\textsuperscript{34} In this context, migration of VEGFR-1–expressing SMCs can explain the increase in vessel diameter, which is more significant in response to treatment with the VEGFR-
1–specific ligand PI GF in comparison to the VEGFR–2–specific ligand VEGF–E.

Increased angiographic collateral scores in the PIGF–treated animals did also match with flow studies. The rabbit hind limb model used preferentially in this study is more sensitive than the equivalent mouse model in assessing the gain of SMC layers, because after femoral artery ligation, rabbit collaterals can increase in diameter from about 50 μm directly after ligation up to 400 μm. In contrast, in mice these diameters increase only from about 30 μm to about 80 μm and they develop one to two layers of SMCs after femoral ligation.35

By measuring flow in the external iliac arteries and determining the arterial pressure gradient across the occlusion site specifically, the collateral arterial conductance has been calculated for this study. Thus, the major increases of conductance by VEGF–E cannot be explained by effects on promoting angiogenesis in the peripheral ischemic vascular bed. The different mechanisms leading to arteriogenesis and angiogenesis are in line with our finding that VEGFR–1 and VEGFR–2 contribute differently to these processes. Whereas in arteriogenesis PIGF displays the major activity, in angiogenesis VEGF–E appears to be more important. Our observation that in an in vitro model of sprouting angiogenesis both VEGF and VEGF–E but not PIGF are potent stimulators of sprout formation is in line with previous reports showing that angiogenesis is dependent on VEGFR–2 signaling.12,25 However, lack of cooperation between the two VEGF receptors is not restricted to arteriogenesis but also observed in the sprout formation assay. In these experiments, addition of various concentrations of PIGF could not enhance VEGF or VEGF–E–induced sprout formation.

In conclusion, this study demonstrates a strong involvement of VEGFR–1 and VEGFR–1–expressing monocytes in arteriogenesis. This is supported by the major effect of the VEGFR–1–selective ligand PIGF compared with the minor effect of the VEGFR–2–specific ligand VEGF–E to stimulate collateral artery growth and the requirement of monocytes for PIGF to be arteriogenic. Furthermore, cooperation of the two VEGF receptors, either on cellular or on signaling levels, does not appear to be required for their affects on collateral growth stimulation in vivo and sprouting angiogenesis into fibrin in vitro.

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