Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2

RENHAI CAO[1], EBBA BRÅKENHIELM[1], ROBERT PAWLIUK[2], DAVID WARIARO[3], MARK J. POST[4], ERIC WAHLBERG[3], PHILIPPE LEBOULCH[2,5,6] & YIHAI CAO[1]

[1]*Laboratory of Angiogenesis Research, Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden*
[2]*Genetix Pharmaceuticals, Inc., Cambridge, Massachusetts, USA*
[3]*Department of Vascular Surgery, Karolinska Hospital, Stockholm, Sweden*
[4]*Department of Medicine, Dartmouth Medical School, Lebanon, New Hampshire, USA*
[5]*Harvard Medical School and Division of Genetics, Department of Medicine, Brigham & Women's Hospital, Boston, Massachusetts, USA*
[6]*INSERM EMI 0111, Hopital Saint-Louis, Paris, France*
Correspondence should be addressed to Y.C.; e-mail: yihai.cao@mtc.ki.se

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The establishment of functional and stable vascular networks is essential for angiogenic therapy. Here we report that a combination of two angiogenic factors, platelet-derived growth factor (PDGF)-BB and fibroblast growth factor (FGF)-2, synergistically induces vascular networks, which remain stable for more than a year even after depletion of angiogenic factors. In both rat and rabbit ischemic hind limb models, PDGF-BB and FGF-2 together markedly stimulated collateral arteriogenesis after ligation of the femoral artery, with a significant increase in vascularization and improvement in paw blood flow. A possible mechanism of angiogenic synergism between PDGF-BB and FGF-2 involves upregulation of the expression of PDGF receptor (PDGFR)-α and PDGFR-β by FGF-2 in newly formed blood vessels. Our data show that a specific combination of angiogenic factors establishes functional and stable vascular networks, and provides guidance for the ongoing clinical trials of angiogenic factors for the treatment of ischemic diseases.

The discovery of several potent angiogenic regulators has stimulated the development of therapeutic strategies to promote or inhibit angiogenesis[1–4]. As a result, several angiogenic molecules have been brought to clinical trials for the treatment of ischemic diseases, even without complete understanding of their mechanisms of action and long-term impact on blood vessels. Most of these approaches are based on the biological effect of a single angiogenic molecule[5–7]. The establishment of stable and functional blood vessel networks, however, is a complex process that requires several angiogenic factors to stimulate vessel sprouting and remodeling of the primitive vascular network[8–10]. Thus, a therapeutic strategy based on a single angiogenic factor may be insufficient to induce functional and stable vessels for treatment of ischemic disorders such as cardiac and limb ischemia. Indeed, clinical delivery of either vascular endothelial growth factor (VEGF) or FGF-2 alone has produced some controversial results in the treatment of cardiac ischemia[11,12]. In addition, studies of animals and human patients have shown that delivery of a single angiogenic agent can cause serious complications[8,11,13,14]. In animals, for example, unregulated VEGF expression in myocardium can lead to heart edema and formation of hemangioma, which may contribute to heart failure and even death[14]. These vascular complications are caused mainly by the instability and leakiness of the newly formed vascular networks. Thus, there is a great need to improve current angiogenic therapy by exploring combinatorial strategies for establishing stable blood vessels. FGF-2 and VEGF are potent angiogenic factors *in vivo* and PDGF-BB has a substantial role in the stabilization of newly formed blood vessels[10,15–17]. Little is known, however, about the impact of combinations of these factors on blood vessel growth and stability and therapeutic angiogenesis. In this report, we investigate whether FGF-2 or VEGF, when combined with PDGF-BB, can induce stable vascular networks. We specifically asked whether these combinations of angiogenic factors could improve collateral growth and functional recovery in ischemic tissues.

Angiogenic synergy between PDGF-BB and FGF-2

To study the angiogenic properties of combinations of PDGF-BB, FGF-2 and VEGF *in vivo*, we carried out two *in vivo* angiogenesis assays, the mouse corneal micropocket assay and the mouse Matrigel assay. All three dimeric isoforms of PDGF (PDGF-AA, PDGF-AB and PDGF-BB) induced angiogenesis in the mouse cornea. The angiogenic responses of corneas stimulated by PDGF-AB and PDGF-BB were robust with a large number of capillaries (Fig. 1*c* and *d*). The areas of neovascularization stimulated by equivalent amounts of each of these two factors were indistinguishable from each other (Fig. 1*j*). In contrast, vessel lengths, clock hours (the proportion of the circumference that is vascularized if the eye is viewed as a clock) and vascular areas stimulated by PDGF-AA were significantly ($P < 0.001$) less than those induced

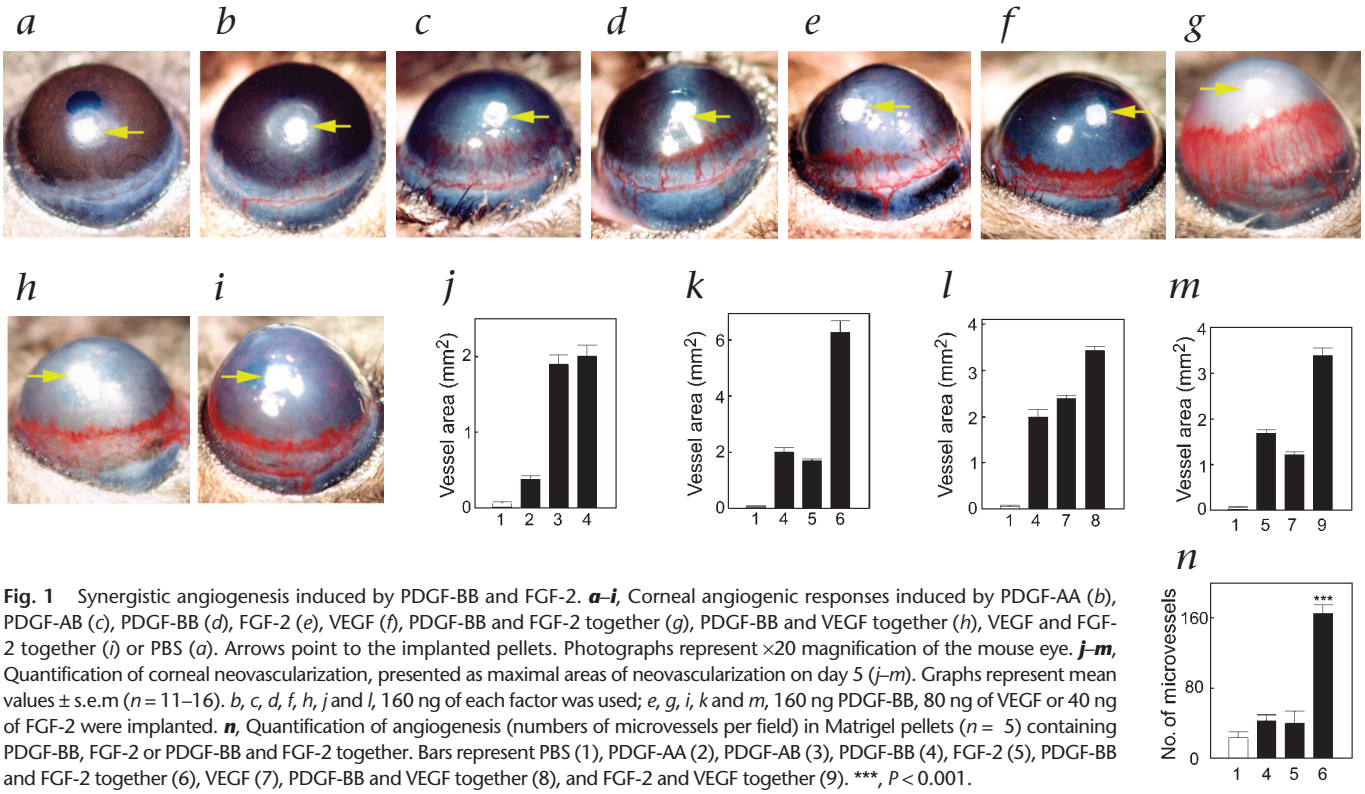

Fig. 1 Synergistic angiogenesis induced by PDGF-BB and FGF-2. ***a–i***, Corneal angiogenic responses induced by PDGF-AA (*b*), PDGF-AB (*c*), PDGF-BB (*d*), FGF-2 (*e*), VEGF (*f*), PDGF-BB and FGF-2 together (*g*), PDGF-BB and VEGF together (*h*), VEGF and FGF-2 together (*i*) or PBS (*a*). Arrows point to the implanted pellets. Photographs represent ×20 magnification of the mouse eye. ***j–m***, Quantification of corneal neovascularization, presented as maximal areas of neovascularization on day 5 (*j–m*). Graphs represent mean values ± s.e.m ($n = 11–16$). *b, c, d, f, h, j* and *l*, 160 ng of each factor was used; *e, g, i, k* and *m*, 160 ng PDGF-BB, 80 ng of VEGF or 40 ng of FGF-2 were implanted. ***n***, Quantification of angiogenesis (numbers of microvessels per field) in Matrigel pellets ($n = 5$) containing PDGF-BB, FGF-2 or PDGF-BB and FGF-2 together. Bars represent PBS (1), PDGF-AA (2), PDGF-AB (3), PDGF-BB (4), FGF-2 (5), PDGF-BB and FGF-2 together (6), VEGF (7), PDGF-BB and VEGF together (8), and FGF-2 and VEGF together (9). ***, $P < 0.001$.

by PDGF-AB or PDGF-BB (Fig. 1*b* and *j*). Pellets without growth factors did not induce corneal neovascularization (Fig. 1*a* and *j*).

Because PDGF-BB showed a robust angiogenic response in this rigorous angiogenic model, we focused on this molecule in our subsequent studies. PDGF-BB is expressed with other angiogenic factors, including FGF-2 and VEGF, in angiogenic tissues such as placenta and embryonic tissue[18,19], which suggests that it might modulate the angiogenic activity of these factors. To test this possibility, PDGF-BB and either FGF-2 or VEGF were co-implanted into mouse corneas. As expected in this model, FGF-2 or VEGF alone induced intense corneal neovascularization (Fig. 1*e* and *f*). A marked synergistic effect on neovascularization was observed in corneas co-implanted with PDGF-BB and FGF-2 (Fig. 1*g* and *k*). The measured area of neovascularization induced by PDGF-BB and FGF-2 together was significantly greater than the sum of the effects obtained with either of the two factors alone ($P < 0.001$). The newly formed blood vessels were well organized, with distinct vascular tree-like structures and branch formations. In contrast to PDGF-BB– or FGF-2–induced vascular networks, VEGF-stimulated capillary networks appeared as disorganized primitive vascular plexuses with sinusoidal blobs at the leading edges (Fig. 1*f*). These primitive vascular networks probably represented the vascular permeability feature of premature and leaky vessels induced by VEGF. Combination of PDGF-BB with VEGF did not result in synergistic angiogenesis, other than a slightly additive angiogenic effect (Fig. 1*h* and *l*). PDGF-BB did not improve the quality of the VEGF-induced vascular networks, which appeared to be leaky and tortuous. In fact, these premature vessels seemed to be even more leaky than the vessels induced by VEGF alone, with capillaries that were probably erupted at the leading edges (Fig. 1*h*). As a control, a combination of VEGF and FGF-2 produced an additive effect in this system (Fig. 1*i* and *m*).

In an *in vivo* Matrigel model, PDGF-BB or FGF-2 released from heparin-Sepharose beads stimulated an approximately two-fold increase in the number of microvessels observed as compared with the heparin-Sepharose control sample (Fig. 1*n*; Supplementary Fig. 1 online). Notably, the number of microvessels induced by PDGF-BB and FGF-2 in combination was more than eight-fold greater than in controls (Fig. 1*n*). Again, neovascularization stimulated by PDGF-BB and FGF-2 together was significantly greater than the sum of the effects obtained with either factor used alone ($P < 0.001$). These data show that a combination of PDGF-BB and FGF-2 has a synergistic efficacy in induction of neovascularization in two *in vivo* model systems.

Establishment of stable vessels by PDGF-BB and FGF-2

Previous work has suggested that PDGF-BB is centrally involved in vascular network maturation and remodeling by recruiting mural cells (pericytes and smooth muscle cells) onto the nascent endothelium[20]. To study the vascular remodeling function of growth factors alone or in various combinations, mouse corneal vascular networks were followed for more than 210 d. Although PDGF-BB alone induced a relatively robust angiogenic response in corneas between days 5 and 12, these vessels were unstable and had completely vanished by day 24 (Fig. 2*a*). FGF-2–induced blood vessels remained relatively stable for 40 d after implantation (Fig. 2*b*), but almost all regressed by day 70. Overwhelming corneal neovascularization in corneas implanted with PDGF-BB and FGF-2 between days 7 and 10 resulted in the loss of all implanted pellets. Notably, corneal neovascularization continued to reach a maximal level around day 12 despite the loss of the PDGF-BB and FGF-2 implants; these vascular networks remained stable for over 210 d (Fig. 2*c*). At the time this report was prepared, the vessels had remained stable for more than one year

without further regression. In addition to vascular stability, the vascular networks induced by PDGF-BB and FGF-2 together were remodeled into well-defined vascular tree-like structures (Fig. 2*c*). Similar to FGF-2-stimulated vessels, VEGF-stimulated vessels were completely regressed within 70 d (Fig. 2*d*) although the initial nascent vascular network with sinusoidal blob-like structures underwent marked remodeling into well-defined networks by day 24 (Fig. 2*d*). A combination of PDGF-BB and VEGF slightly improved the remodeling and stability of blood vessels compared with either factor alone (Fig. 2*e*). After a prolonged period (~70 d), however, only a barely detectable number of vessels remained.

In the corneas implanted with both FGF-2 and VEGF, early vascular plexuses underwent extensive remodeling into well-defined vascular networks (Fig. 2*f*). In the presence of implanted growth factors, these well-organized vessels remained for a relatively long period (~100 d) but disappeared by day 175. Quantification analysis showed that the angiogenic response and the number of stable blood vessels stimulated by PDGF-BB

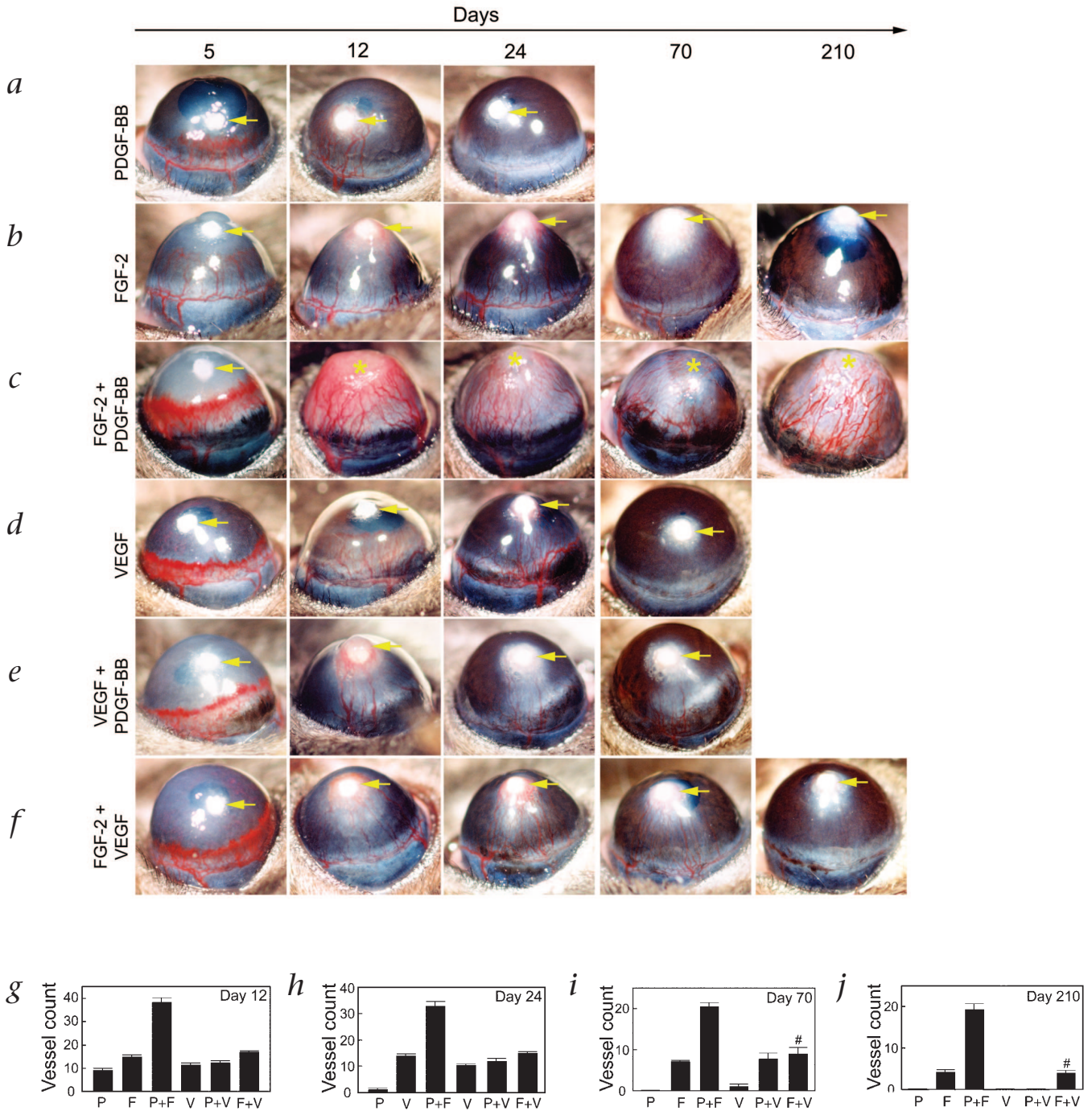

Fig. 2 Blood vessel stability. ***a–f***, Corneal neovascularization was examined at the indicated time points. Arrows indicate the implanted pellets; asterisks indicate positions of pellets in those corneas that lost implanted pellets. ***g–j***, Bars represent vessel counts of corneas implanted with PDGF-BB (P), FGF-2 (F), PDGF-BB and FGF-2 together (P+F), VEGF (V), PDGF-BB and VEGF together (P+V) and FGF-2 and VEGF together (F+V). Values are presented as mean determinants ± s.e.m.(*g–j*). #, day 54 or day 175 (*i* and *j*); (n = 4–8).

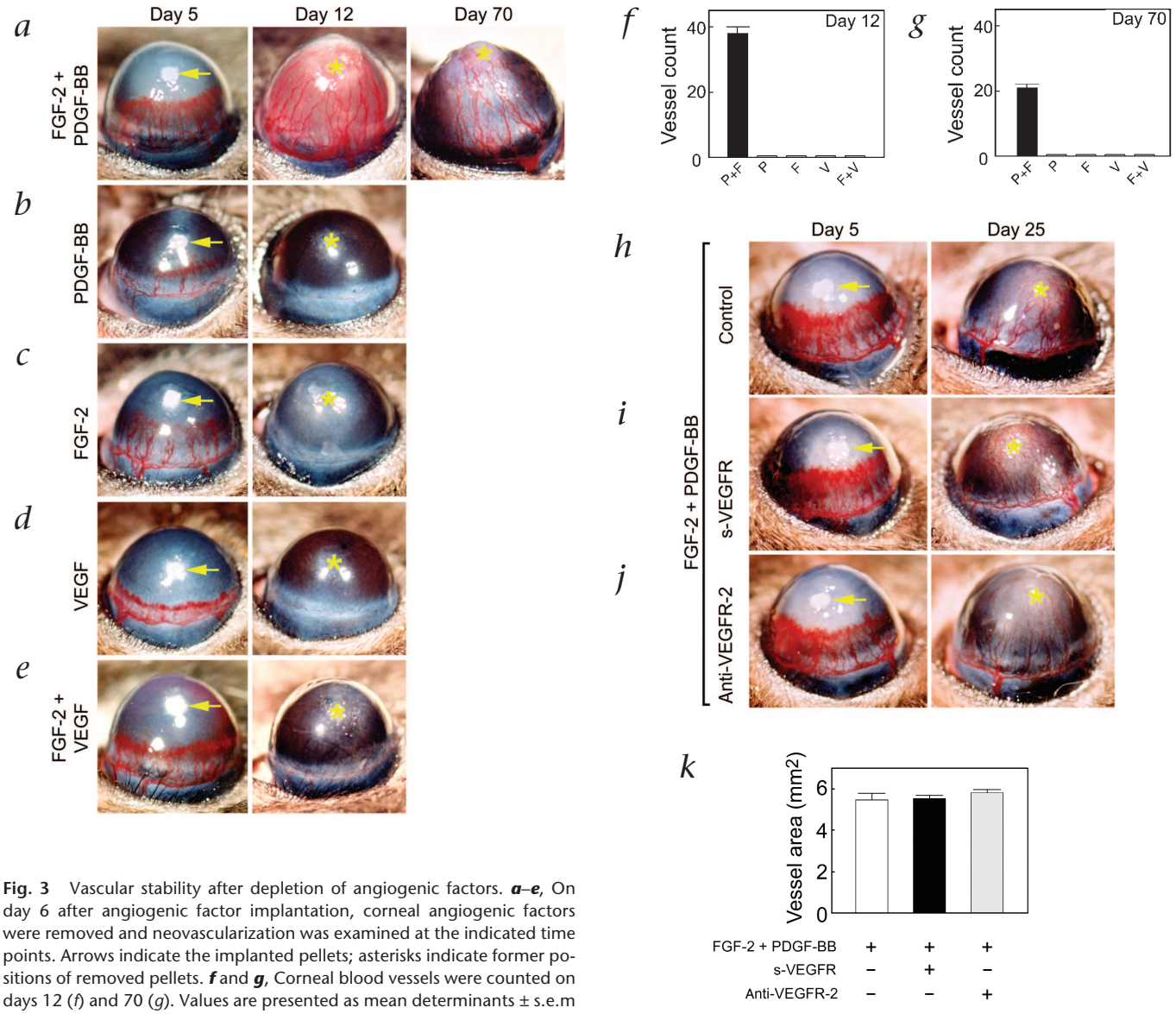

Fig. 3 Vascular stability after depletion of angiogenic factors. ***a–e***, On day 6 after angiogenic factor implantation, corneal angiogenic factors were removed and neovascularization was examined at the indicated time points. Arrows indicate the implanted pellets; asterisks indicate former positions of removed pellets. ***f*** and ***g***, Corneal blood vessels were counted on days 12 (*f*) and 70 (*g*). Values are presented as mean determinants ± s.e.m ($n = 4–8$). Bars represent vessel counts per cornea implanted with PDGF-BB and FGF-2 together (F+P), PDGF-BB (P), FGF-2 (F), VEGF (V) and FGF-2 and VEGF together (F+V). ***h–k***, PDGF-BB and FGF-2 were co-implanted in the absence (*h*) or presence of a soluble VEGFR-2 receptor (s-VEGFR; *i*) or a VEGFR-2 neutralizing antibody (anti-VEGFR-2; *j*). Neovascularization was examined on days 5 and 25 and areas of vascularization were quantified (day 5; *k*); ($n = 8–9$).

and FGF-2 together were far greater than those induced by either FGF-2, PDGF-BB or VEGF alone, or by combinations of PDGF-BB and VEGF or FGF-2 and VEGF (Fig. 2*g–j*). We should emphasize that vascular stability was determined in the absence of PDGF-BB and FGF-2 implants and in the presence of implants containing other factors either alone or in combinations. Thus, only a specific combination of angiogenic factors could stabilize the nascent vascular network.

Transient exposure to PDGF-BB and FGF-2 leads to stable blood vessels

The differential effects of various angiogenic factors, either alone or in combinations, on vascular stability could be due to differences in bioavailability of these factors after they are released from the slow-release polymer. To exclude this possibility, we deliberately removed the implanted growth factor pellets in the corneas 6 d after implantation. As expected, all nascent vessels induced by PDGF-BB, FGF-2 or VEGF completely regressed within one week after removal of growth factors (Fig. 3*b–f*). In contrast, vessels induced by PDGF-BB and FGF-2 together remained without regression for 70 d (Fig. 3*a* and *g*). At the time this report was prepared, these well-established and defined vascular trees had remained for more than one year without further regression. In contrast, blood vessels induced by a combination of FGF-2 and VEGF, the two most potent angiogenic factors, regressed within 7 d after depletion of angiogenic factors (Fig. 3*e–g*). These data indicate that PDGF-BB, FGF-2, VEGF and the combination of FGF-2 and VEGF act as survival factors for their own premature vessels. The long-term stability of vascular networks independent from the resource of growth factors might be determined at an early phase of neovascularization by transient exposure to a specific combination of angiogenic factors such as FGF-2 and PDGF-BB.

Vessel stability is independent of VEGF

The synergistic angiogenesis and long-lasting vessels induced by PDGF-BB and FGF-2 together could involve VEGF-mediated signaling pathways activated indirectly by these two factors. To explore this possibility, VEGF antagonists, including soluble and neutralizing VEGFR-2 antibodies, were applied to corneas co-implanted with PDGF-BB and FGF-2. These reagents can specifically block VEGF-induced angiogenesis[15]. Neither the soluble nor the neutralizing antibody blocked PDGF-BB– and FGF-2–induced angiogenic synergy or vascular stability as measured at different time points (Fig. 3*h–k*). These data, together with those from the above experiments, seem to indicate that VEGF does not have an important role in the angiogenic synergy and vascular stability induced by PDGF-BB and FGF-2 together.

Correlation of mural cell association with corneal vessel stability

Mural cells have an essential role in blood vessel maturity[21,22]. To assess the maturity of corneal vessels induced by various growth factors, we examined corneal sections for the presence of CD31, an endothelial cell marker, and desmin, a marker specifically expressed in pericytes and smooth muscle cells. In general, desmin-positive corneal microvessels were less prevalent than CD31-positive vessels (Fig. 4*a–c*). A closely located but non-overlapping pattern was detected with both CD31- and desmin-positive signals when images of the same sections were combined digitally (Fig. 4*a–c*). The desmin signals were located at the outer edges of CD31 staining, indicating the attachment of mural cells to the newly formed endothelium. A maturation index (percentage of vessels coated with periendothelial cells) of about 15–20% was found on day 5 in corneal vessels induced by FGF-2 and PDGF-BB alone or together; no significant difference was recorded among different groups (Fig. 4*d*). On day 12, the maturation index in the PDGF-BB–induced vessels (~60%) was significantly ($P < 0.001$) higher than in the FGF-2–induced vessels (~30%; Fig. 4*e*). A significant ($P < 0.01$) increase in smooth muscle cell coating (~45%) was also found in the corneas co-implanted with PDGF-BB and FGF-2. At day 25, more than 70% of corneal vessels induced by both FGF-2 and PDGF-BB were desmin positive, whereas fewer than 40% of FGF-induced vessels were associated with mural cells (Fig. 4*f*). By this time, all PDGF-BB–induced vessels had regressed. In addition, it seemed that corneal vessels induced by both PDGF-BB and FGF-2 were non-leaky, as detected by extravasation of FITC-labeled dextran. Whereas VEGF-induced vessels were permeable to FITC-dextran, resulting in a completely diffused pattern,

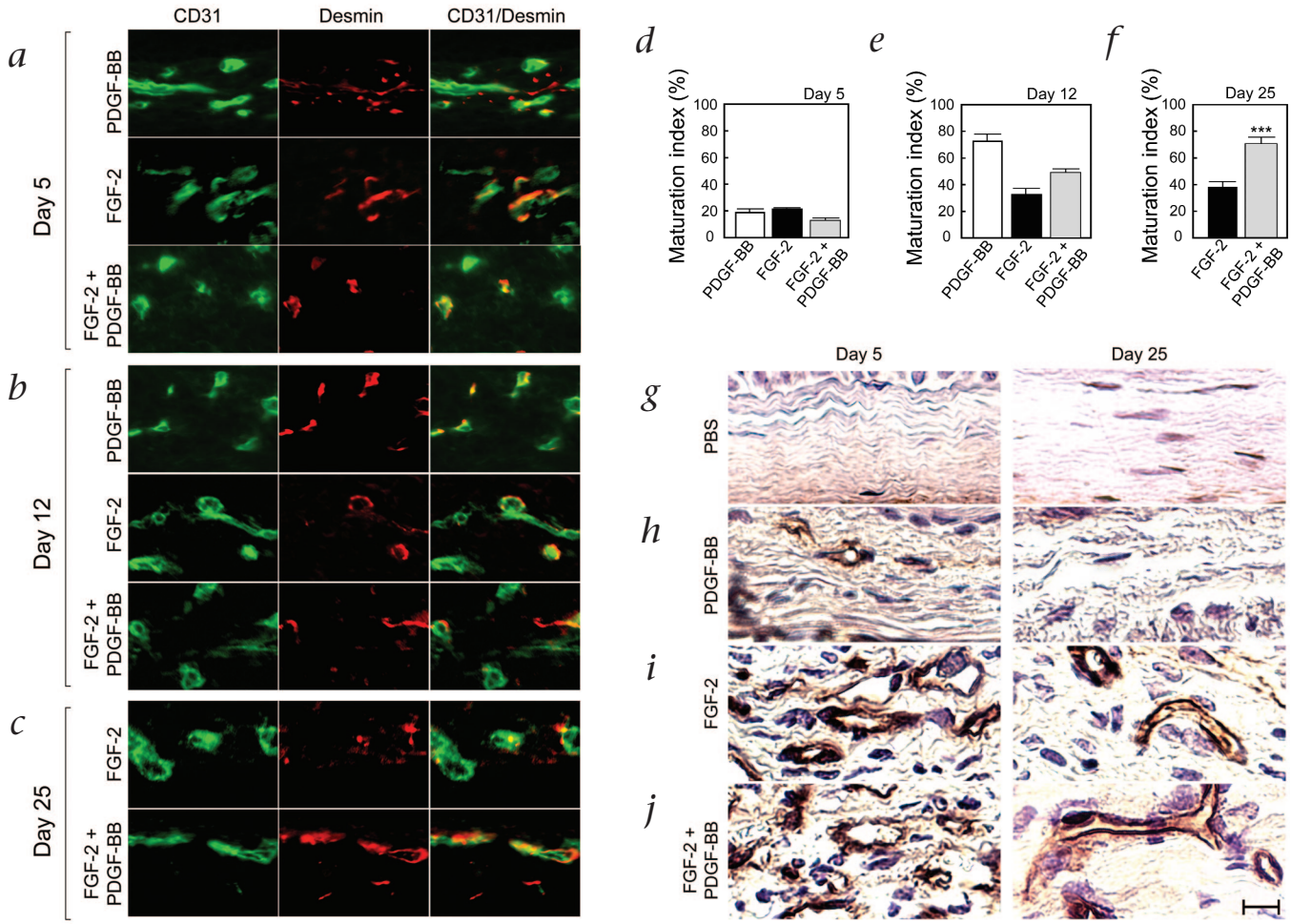

Fig. 4 Histological analysis of corneal neovascularization. ***a–c***, Corneas implanted with FGF-2, PDGF-BB or PDGF-BB and FGF-2 together were analyzed on days 5, 12 and 25 by immunohistochemical double labeling for CD31 and desmin. Green, CD31-positive signals; red, desmin-positive signals; yellow, double-labeling signals. Magnification, ×20. ***d–f***, Vessel maturation index as percentages of mural-positive vessels at different time points. Data is presented as mean determinants (± s.e.m.; $n = 4–12$). ***, $P < 0.001$. ***g–j***, Non-fluorescent anti-CD31 peroxidase staining followed by hematoxylin counter-staining was performed on treated corneas at the indicated time points. Scale bar, 10 μm.

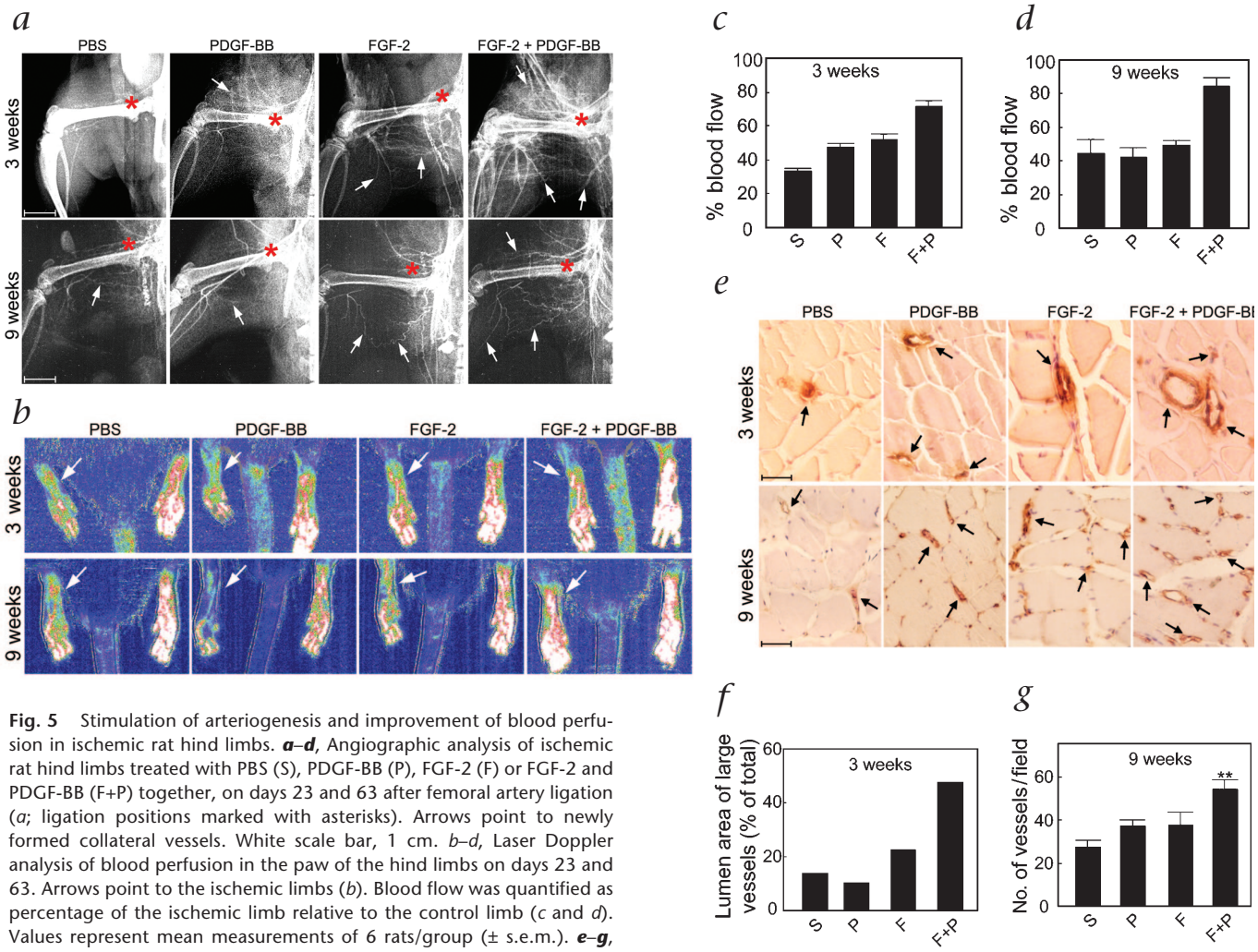

Fig. 5 Stimulation of arteriogenesis and improvement of blood perfusion in ischemic rat hind limbs. ***a*–*d***, Angiographic analysis of ischemic rat hind limbs treated with PBS (S), PDGF-BB (P), FGF-2 (F) or FGF-2 and PDGF-BB (F+P) together, on days 23 and 63 after femoral artery ligation (*a*; ligation positions marked with asterisks). Arrows point to newly formed collateral vessels. White scale bar, 1 cm. *b*–*d*, Laser Doppler analysis of blood perfusion in the paw of the hind limbs on days 23 and 63. Arrows point to the ischemic limbs (*b*). Blood flow was quantified as percentage of the ischemic limb relative to the control limb (*c* and *d*). Values represent mean measurements of 6 rats/group (± s.e.m.). ***e*–*g***, Anti-SMA staining of histological sections of ischemic hind-limb muscle tissue treated with PBS, PDGF-BB, FGF-2 or FGF-2 and PDGF-BB together, on days 23 (*e* and *f*) and 63 (*e* and *g*). Arrows point to newly formed arterial vessels (*e*). *f* and *g*, Quantification of large vessel lumen areas (>700 μm^2) as mean percentage of total vessel lumen areas on day 23 (*f*) and total numbers of arterial vessels on day 63 (*g*). Black scale bar, 50 μm. **, $P < 0.01$; $n = 5$–6.

PDGF-BB– and FGF-2–induced vessels resisted leakage, so the majority of FITC-dextrans were limited to the lumenal areas of the vessels (see Supplementary Fig. 1 online).

We used non-fluorescent immunohistological analysis to assess whether co-implantation with PDGF-BB and FGF-2 could elicit tissue reactions other than neovascularization. CD31 and hematoxylin staining of corneas at days 5 and 25 did not detect significant numbers of non-vascular cells (Fig. 4*g*–*j*). H&E staining showed that corneal neovascularization induced by FGF-2 and PDGF-BB together did not differ significantly from that induced by FGF-2 or PDGF-BB alone (see Supplementary Fig. 1 online). The only difference was that corneas stimulated with both PDGF-BB and FGF-2 contained a higher density of microvessels than those stimulated with either growth factor alone. Similarly, H&E staining of *in vivo* Matrigel implants did not detect additional infiltration of cell types other than a high density of newly formed vessels (see Supplementary Fig. 1 online). These data indicate that microvessel stability induced by both PDGF-BB and FGF-2 correlated with the increase of mural cell association to newly formed vessels and did not result in obvious tissue reactions other than neovascularization.

Stimulation of rat collateral growth and blood perfusion

In a rat hind-limb ischemic model, angiographic analysis at day 23 after ligation of the femoral artery revealed an increased number of collateral vessels in the FGF-2–treated group (Fig. 5*a*). Delivery of PDGF-BB to ischemic muscles was only moderately effective in stimulating collateral growth. These data were consistent with the corneal model in which FGF-2 displayed more potent angiogenic activity than PDGF-BB (Fig. 2). FGF-2 and PDGF-BB together potently stimulated collateral growth and resulted in a high density of collateral vessels, which were distributed in a broad area of ischemic muscles near the lesion site and extended to the distal regions of the ischemic limb (Fig. 5*a*, arrows). As a negative control, PBS did not significantly induce collateral growth (Fig. 5*a*).

An independent study was carried out for nine weeks to assess whether these arterial vessels could remain for a relatively long period. By the end of week 9, several newly formed collateral vessels were detectable in the PBS control group (Fig. 5*a*). Such a compensatory effect of ischemia-induced collateral growth was usually seen in our model 6–9 weeks after ligation. Delivery of PDGF-BB alone did not elicit a significant increase in numbers of

collaterals. In contrast, after treatment with FGF-2 alone, the detectable number of collaterals was markedly higher than after induction by PDGF-BB alone or in controls (Fig. 5*a*, arrows in nine-week photographs). As expected, the high numbers of collaterals induced by PDGF-BB and FGF-2 together at the early time point were maintained for more than 60 d without regression (Fig. 5*a*). The overall analysis showed that the number of collaterals induced by PDGF-BB and FGF-2 together was substantially higher than that induced by FGF-2 alone, although the angiographic results were difficult to quantify.

Paw blood perfusion in ischemic hind limbs treated with PDGF-BB and FGF-2 together, measured by Doppler analysis on day 23, was markedly improved to an average of about 75% of the blood flow in non-ischemic control limbs, whereas blood flow in the PBS-treated control limbs was 35% of non-ischemic control (Fig. 5*b* and *c*). Delivery of FGF-2 or PDGF-BB alone at this time point produced only moderate improvement in blood flow (40–50%). At day 63, paw blood flow in the ischemic limbs treated with PDGF-BB and FGF-2 together continued to increase (to nearly 90%), whereas there were no apparent differences in blood perfusion in the groups treated with PDGF-BB, FGF-2 or PBS alone (Fig. 5*b* and *d*).

Because angiographic analysis can detect only relatively large collateral vessels, we carried out immunohistochemical analyses

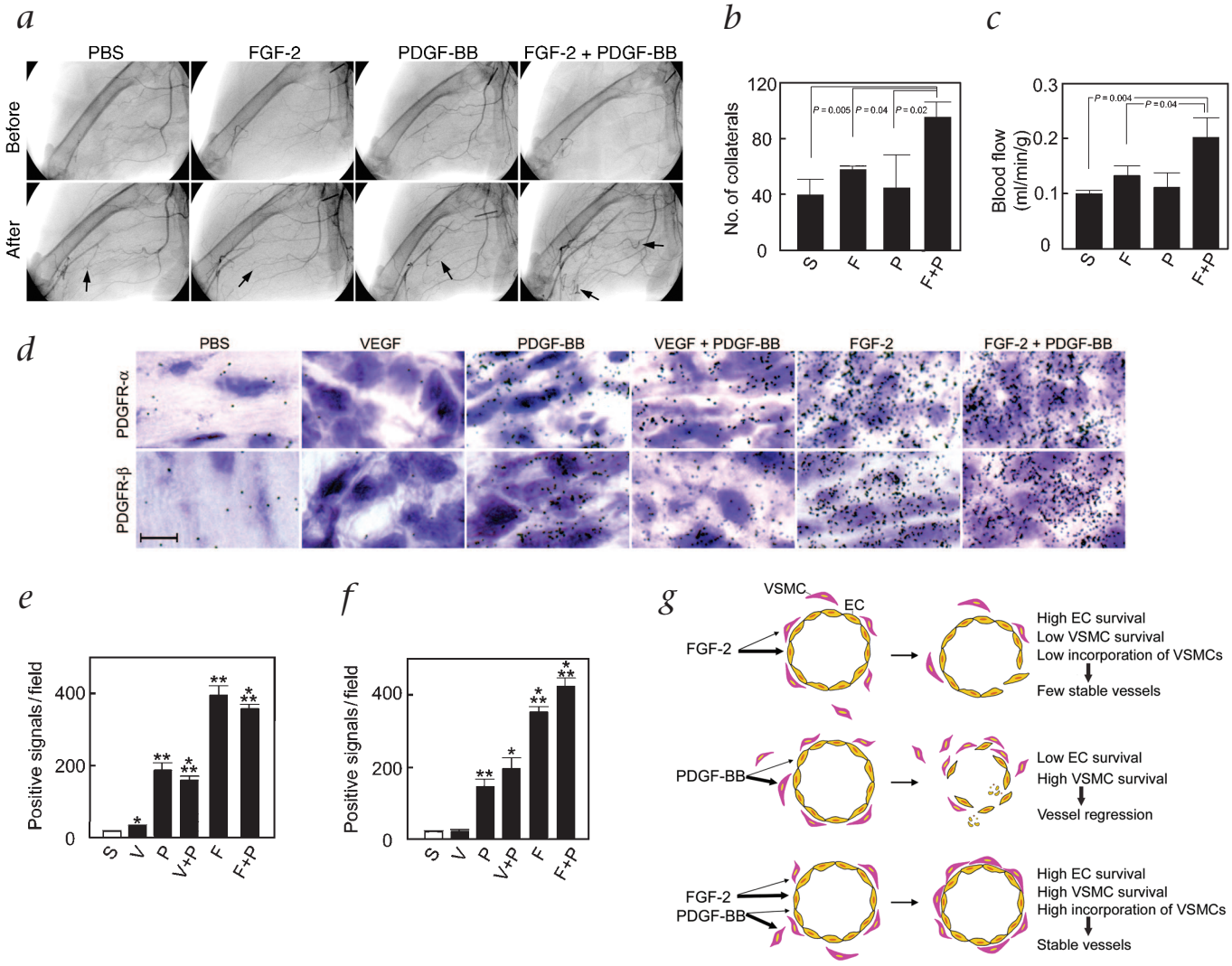

Fig. 6 Treatment of ischemic rabbit hind limbs and *in situ* detection of PDGFR-α and PDGFR-βon newly formed blood vessels. ***a–c***, Angiographic analysis of ischemic rabbit limbs was done on day 0 (before) and day 7 (after). *a*, Arrows point to the newly formed arterial vessels. *b*, Numbers of collaterals were quantified (n = 5–7) *c*, Blood flow was analyzed using a microsphere method (n = 5–7) Various growth factor–implanted corneal tissue sections were hybridized with the oligonucleotide probes for mouse PDGFR-α and PDGFR-β. ***d–f***, Bright-field images. Scale bar, 10 μm. Positive signals of PDGFR-α (*e*) and PDGFR-β (*f*) were quantified (n = 5–12) Graphs represent mean values (± s.e.m.) per field (1,884 μm^2). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *b*, *c*, *e* and *f*, Bars represent samples treated with PBS (S), VEGF (V), PDGF-BB (P), VEGF and PDGF-BB together (V+P), FGF-2 (F) and FGF-2 and PDGF-BB together (F+P). ***g***, Schematic representation of the role of FGF-2 and PDGF-BB in blood vessel remodeling and stability. EC, endothelial cells; VSMC, vascular smooth muscle cells. FGF-2 is a potent angiogenic factor that preferentially acts on endothelial cells *in vivo*. FGF-2 is also a key survival factor for endothelial cells. However, FGF-2 is unable to induce survival signals for vascular smooth muscle cells (VSMC). Thus, FGF-2–induced vessels are unstable after withdrawal of the growth factor. PDGF-BB is a potent mitogenic and chemotactic factor for VSMC but a poor survival factor for endothelial cells. As a result, PDGF-BB–induced vascular networks regress. FGF-2 and PDGF-BB combined produce not only a synergistic effect on angiogenesis, but also confer a potent survival effect on both endothelial and VSMC. Thus, these vessels remain stable.

of muscle tissue using an antibody against smooth muscle actin (SMA). Consistent with the angiographic analysis, dual angiogenic factor delivery after three weeks resulted in a marked increase in the number of relatively large arterial vessels (Fig. 5*e*). The lumenal areas of these large arterial vessels (>700 μm^2) occupied nearly 50% of the total lumen area in histological sections of the dual angiogenic factor–treated group (Fig. 5*f*). In contrast, FGF-2– or PDGF-BB–stimulated collaterals consisted mainly of smaller arterial vessels with an average lumenal area of <400 μm^2 in the majority of vessels (Fig. 5*e*). No obvious tissue reactions were found. Quantification analysis revealed that large arterial vessels (>700 μm^2) in the FGF-2–treated and PDGF-BB–treated groups occupied only about 24% and 10% of total lumen areas, respectively (Fig. 5*f*). Delivery of PBS control induced a similarly weak collateral response at this time point.

After the nine-week treatment, collaterals induced with PDGF-BB and FGF-2 together underwent substantial remodeling. Early large-lumenal collaterals were replaced by a large number of small and medium arterial vessels, although a few large-lumenal vessels still remained (Fig. 5*e* and *g*, arrows). Although the mechanism of this remodeling process is unclear, it is possible that these early larger 'mother' vessels later divide into smaller 'daughter' vessels. These well-organized arterial vessels were mainly distributed around muscle tissues co-implanted with PDGF-BB and FGF-2 and extended to distal sites. No obvious collagenous or elastic tissues were detected. FGF-2 alone was able to maintain a smaller number of arterial vessels. Similarly, increased numbers of collaterals were found after nine weeks in PDGF-BB– and in PBS-treated tissues as compared with those found at earlier time points. An independent study using various doses and ratios of FGF-2 and PDGF-BB suggested that the dose of FGF-2 used in our rat hind-limb model studies was critical, with the optimal ratio between PDGF-BB and FGF-2 being 4:1 (data not shown). These data suggest that dual angiogenic factor delivery induces the growth of functional collaterals.

Stimulation of rabbit collateral growth and blood flow

We used a similar ischemic hind-limb model with rabbits to further assess the stimulatory effect of PDGF-BB and FGF-2 on ischemic collateral growth. On day 7 after treatment, angiographic analysis showed that significant numbers of collaterals were readily detectable in ischemic hind limbs treated with PDGF-BB and FGF-2 together (Fig. 6*a*). These collaterals represented newly formed arteries, as they were not detectable before treatment. Quantification analysis showed that the increased numbers of collaterals induced by PDGF-BB and FGF-2 together were significantly ($P < 0.05$) greater than those found in other groups (Fig. 6*b*). FGF-2 or PDGF-BB alone did not significantly induce collateral growth above the control in the rabbit ischemic limbs (Fig. 6*a* and *b*). Microsphere analysis revealed that blood flow was significantly ($P < 0.05$) improved in the ischemic muscle tissue of rabbits treated with PDGF-BB and FGF-2 together, as compared with those treated with other factors or control (Fig. 6*c*). These results provide independent support for our rat model findings that PDGF-BB and FGF-2 in combination stimulate functional collateral growth in ischemic limbs.

Upregulation of PDGF receptors by FGF-2

To investigate the molecular mechanism of the observed synergistic effects of FGF-2 and PDGF-BB on angiogenesis and vascular stability, we examined the PDGF receptors, PDGFR-α and PDGFR-β, in newly formed mouse corneal blood vessels. Expression of *Pdgfra* and *Pdgfrb* mRNA was determined by quantitative *in situ* hybridization using probes specific for these receptors[23]. Extremely high *Pdgfra* and *Pdgfrb* expression was detected in FGF-2–induced blood vessels (Fig. 6*d*). Both PDGFR-α– and PDGFR-β–positive signals were distributed in these newly formed blood vessels, as confirmed by co-localization of endothelial staining for CD31 (data not shown). The combination of PDGF-BB and FGF-2 did not seem to significantly elevate the expression levels of these two receptors in newly formed blood vessels. In contrast, both PDGFR-α and PDGFR-β were expressed at only moderate levels in the PDGF-BB–induced microvessels (Fig. 6*d*). Upregulation of PDGF receptors seemed to be specific for FGF-2, as PDGFR-α and PDGFR-β receptor expression in VEGF-induced new blood vessels was nearly undetectable and barely distinguishable from background (Fig. 6*d*). Stimulation with a combination of PDGF-BB and VEGF did not result in greater expression of the two PDGF receptors as compared with induction by PDGF-BB alone (Fig. 6*d*). Expression of PDGFR-α and PDGFR-β, as measured by quantification analysis, was more than ten-fold higher on FGF-2–induced vessels than on VEGF-induced vessels (Fig. 6*e* and *f*). We should emphasize that these experiments were evaluated using two independent sets of probes specific for each receptor; nearly identical results were obtained using each set of receptor probes. These findings offer an explanation of why FGF-2, but not VEGF, synergistically induces angiogenesis and vascular stability with PDGF-BB. The molecular mechanism of the angiogenic synergy may, at least in part, involve upregulation by FGF-2 of the expression of PDGF receptors, which transduce angiogenic and arteriogenic signals triggered by PDGF-BB.

Discussion

Our work shows that single angiogenic factors, including FGF-2, VEGF and PDGF-BB, were unable to establish stable vascular networks. In contrast, a combination of PDGF-BB and FGF-2, but not PDGF-BB and VEGF or VEGF and FGF-2, synergistically induces angiogenesis and long-lasting functional vessels. Induction of robust angiogenic responses and establishment of long-lasting, functional arterial vessels are both important in angiogenic therapy for ischemic heart and limb diseases. Current angiogenic therapies in human trials are based on single-agent approaches. Although some of these trials have produced encouraging short-term results, they lack long-term beneficial effects. A recent FGF-2–based random, double-blind, controlled trial of FGF-2 in the treatment of coronary artery disease showed trends toward short-term, but not long-term, symptomatic improvement[24]. Consistent with this early clinical data, our present work shows that FGF-2, VEGF and PDGF-BB are indeed able to stimulate angiogenesis in the short term, but none of these factors alone is able to maintain these newly formed vessels. These results suggest that separate mechanisms control blood vessel growth and maintenance of nascent vasculature.

During embryonic development, the growing vascular system is exposed to numerous angiogenic factors and vascular modulators that coordinately regulate vascular growth and remodeling. Likewise, growth and stabilization of functional vessels in adult tissues may require joint effects of multiple factors that target different cell populations in the vasculature. How can we identify the combinations that synergistically stimulate and stabilize blood vessels? The mouse corneal model allows us to assess multifactorial impact on angiogenesis and vascular stability. The

finding that PDGF-BB and FGF-2 together, but not PDGF-BB and VEGF or FGF-2 and VEGF, can synergistically stimulate angiogenesis and vascular stability indicates that only a specific combination of growth factors can accomplish this task. We also found that blood vessels continue to grow and remodel after the depletion of PDGF-BB and FGF-2. Vessels induced by PDGF-BB and FGF-2 together undergo extensive remodeling and remain permanently in the cornea. These results indicate that the decision between maintenance and regression of newly formed vessels is made when the vessels are born, depending upon exposure to a set of angiogenic factors. With regard to the treatment of ischemic diseases, our data implies that a short exposure of the ischemic tissue to PDGF-BB and FGF-2 would be sufficient to establish stable and functional vessels. Although this observation requires further validation, it may change our current therapeutic strategy from sustained release of angiogenic factors to 'one-shot' delivery of PDGF-BB and FGF-2. In our rat ischemic hind-limb model, short-term delivery of PDGF-BB and FGF-2 led to establishment of stable arterial vessels.

The mechanisms underlying the angiogenic synergy and vascular stability induced by PDGF-BB and FGF-2 may be complex. Here we provide important observations as to how these two factors produce a synergistic impact on blood vessels. The fact that only FGF-2, but not other tested factors, upregulates both PDGFR-α and PDGFR-β indicates that activation of PDGF receptor–transduced signaling pathways in newly formed blood vessels is crucial for blood vessel stability. During simultaneous exposure of the vasculature to these two factors, FGF-2 may have a critical role in triggering an initial robust angiogenic response. Because FGF-2–induced vessels express PDGF receptors at extremely high levels, PDGF-BB, an active ligand for both the α and β receptors, subsequently displays potent angiogenic activity as well. Thus, PDGF-BB and FGF-2 can synergistically induce angiogenesis. In the absence of FGF-2, PDGF-BB only moderately upregulates its own receptors and thus lacks the ability to elicit a robust angiogenic response. As maximal angiogenic responses induced by PDGF-BB and FGF-2 take only a few days (7–12 d.), the next task is to remodel these massive vascular networks. In the absence of PDGF-BB and FGF-2, how do these vessels become stable? One possible explanation is that PDGF receptors expressed at high levels can form aggregates on the cell surface and be autophosphorylated in the absence of ligands. The initial receptor aggregation process, however, may be triggered by PDGF-BB and subsequently become ligand independent. Such a mechanism of PDGF receptor activation in the absence of ligands has been previously reported[25]. Thus, constant activation of PDGF receptors on both endothelial and mural cells may lead to remodeling and stability of these newly formed vessels in the absence of exogenous PDGF-BB and FGF-2.

We have provided several independent lines of evidence suggesting that VEGF may not be a crucial part of PDGF-BB– and FGF-2–induced angiogenic synergy and vascular stability. (i) A combination of VEGF and PDGF-BB did not synergistically stimulate angiogenesis or induce vascular stability. (ii) VEGF antagonists, including a VEGFR-2–neutralizing antibody and a soluble VEGFR-2 receptor, were unable to block PDGF-BB– and FGF-2–induced angiogenic synergy and vascular stability. (iii) Combinations of VEGF and FGF-2 or PDGF-BB and VEGF were unable to stabilize newly formed blood vessels for longer periods. (iv) VEGF alone was unable to induce stable blood vessels; the VEGF-induced vessels were leaky, whereas the vessels induced by PDGF-BB and FGF-2 together were non-leaky. (v) VEGF, PDGF-BB or both did not substantially upregulate PDGF receptor expression. We cannot, however, completely exclude the possibility that VEGF may have a role in vascular stability by activating VEGFR-1. Our study clearly shows that PDGF-BB is not a stabilizer for the VEGF-induced vessels. The molecular basis for this differential effect is that, unlike FGF-2, VEGF is unable to induce PDGF receptor expression. What, then, would be the vascular stabilization partner for VEGF? Our unpublished data suggest that the angiopoietin–Tie-2 system may have a role in stabilization of VEGF-induced vessels. Although the underlying molecular mechanisms are not fully understood, our findings may provide an important therapeutic guideline that combinations of various angiogenic factors can induce functionally stable vessels and that sustained exposure of angiogenic factors may not necessarily be required.

Methods

Mouse corneal micropocket assay. The mouse corneal assay was performed as previously described[26]. Micropellets containing 160 ng of VEGF (R&D Systems, Minneapolis, Minnesota), 160 ng of PDGF-BB, PDGF-AA or PDGF-AB (R&D Systems), or 80 ng of FGF-2 (Pharmacia & Upjohn, Milan, Italy) were implanted into mouse corneal micropockets. Half-amounts of FGF-2 were used in PDGF-BB or VEGF combinatorial experiments. Where indicated, a PDGF-BB–FGF-2 pellet containing 1 μg of neutralizing or soluble antibody against mouse VEGFR-2 was implanted. Vessel lengths, clock hours of circumferential neovascularization and vascularization areas were measured at various time points. For quantification of vessel numbers, see Supplementary Methods online. All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Board.

Matrigel assay. We used a previously published Matrigel pellet model[27]. Briefly, heparin-Sepharose beads were mixed with PDGF-BB, FGF-2 or both (1 μg each). The growth factor–loaded beads were mixed with regular Matrigel (Becton Dickinson, Bedford, Massachusetts) and subcutaneously injected into mice. After 2 weeks, animals were killed and Matrigel pellets were collected and fixed in paraformaldehyde and embedded in paraffin. Thin sections (5 μm) were stained with H&E. Ten high-power fields were used for quantification of blood vessels (see Supplementary Methods online).

Rat ischemic hind-limb model. The rat ischemic hind-limb model is a modification of a 2-stage procedure previously described[28]. Ischemia was created in the left hind limb; the right leg served as control. All left-side branches of the aorta distal to the renal arteries and of the iliac artery were ligated. After 5 d, the femoral artery was ligated. Animals were randomly divided into groups (n = 5–6 per group) and treated with FGF-2, PDGF-BB, FGF-2 and PDGF-BB combined, or PBS. On the same day as the second operation, growth factors (800 ng FGF-2, 1,600 ng PDGF-BB or 800 ng FGF-2 and 1,600 ng PDGF-BB together) in slow-release polymers were implanted into intramuscular pockets near the ligation site. After the operation, soluble growth factors (1.5 μg FGF-2, 3 μg PDGF-BB or 1.5 μg FGF-2 and 3 μg PDGF-BB together) were injected into 3 sites close to the femoral ligation; the treatment continued every other day for 12 d (see Supplementary Methods online).

Rabbit hind limb ischemic model. New Zealand White rabbits were anesthetized and the right femoral artery and its branches were ligated. Animals were randomized to receive vehicle, FGF-2, PDGF-BB or FGF-2 and PDGF-BB together (n = 8–9 per group). An Alzet mini-pump (Alza Corp., Palo Alto, California) loaded with test agent or vehicle (10 μg/kg) was placed proximal to the occlusion. Limb blood perfusion was determined before and immediately after ligation by injection of microspheres (see Supplementary Methods online). After 7 d of treatment, blood flow microsphere analysis was repeated. The rabbit hind-limb ischemic model was approved by the Institutional Animal Care and Use Committee of Dartmouth Medical School.

Laser Doppler imaging and angiography. A laser Doppler imager (Moor Instruments, Axminister, England) was used to assess rat limb blood perfusion[29]. At days 23, 42 and 63 after the second operation, blood flow in the ischemic and control hind paws of each animal were calculated as the average of the percentages of perfusion in the ischemic paw and the control paw. Collateral growth was analyzed by angiography in the rat hind limbs after treatment, and in the rabbit hind limbs before and after treatment (see Supplementary Methods online for details and quantification of rabbit angiograms).

Immunohistochemistry. The growth factor–implanted mouse eyes were enucleated on indicated days and fixed in paraformaldehyde or immediately frozen on dry ice and stored at –80 °C. Frozen sections (10 μm) were incubated with a mixture of rat antibody against mouse CD31 and mouse antibody against human desmin. Immuno-positive signals were developed using a FITC-conjugated secondary antibody and Cy3-conjugated streptavidin, and examined under a fluorescent microscope (Nikon, Melville, New York) at ×20 magnification. For the peroxidase staining, thin paraffin sections of corneas were stained with a biotinylated rat antibody against mouse CD31. Rat muscle tissues from the ischemic and healthy hind limbs were dissected at various time points and fixed in paraformaldehyde. Thin paraffin sections were incubated with a mouse antibody against SMA, followed a secondary mouse antibody labeled with horseradish peroxidase. The reaction was developed by diaminobenzidine substrate and sections were counterstained with hematoxylin. See Supplementary Methods online for quantification of blood vessel numbers, lumen areas and detailed methods.

***In situ* hybridization.** We used 2 probes complementary to PDGFR-α (nucleotides 423–470 and 3,083–3,130) and 2 probes complementary to PDGFR-β (nucleotides 946–996 and 2,610–2,657)[23,30]. All probes were used separately and did not match any known sequence in GenBank except those of the intended genes. Corneal histological sections were hybridized with the ^{33}P-labeled probes. Slides were rinsed, dehydrated and emulsion dipped. After 5 weeks of exposure, slides were developed and counterstained with cresyl violet. Specific labeling was confirmed by similar expression patterns revealed by 2 probes each (complementary to different parts of the mRNA) for PDGFR-α and PDGFR-β. See Supplementary Methods online for details and quantification of autoradiographic signals.

Note: Supplementary information is available on the Nature Medicine *website.*

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Competing interests statement

The authors declare that they have no competing financial interests.

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