

VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B–PDGFR β signaling

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Summary

Combined stimulation with VEGF-A, FGF-2, or PDGF-BB has emerged as a potent strategy for therapeutic angiogenesis, although the mechanisms underlying the synergism of these factors are not well understood. In the present study, we investigated the mechanism of synergism between VEGF-A and FGF-2 by using Matrigel plug assay in vivo and embryonic stem cell (ESC)-derived VEGF receptor 2 (VEGFR2)-positive cells in vitro. Experiments in vitro revealed that, in addition to having direct mitogenic effects, these molecules enhance intercellular PDGF-B signaling in a cell-type specific manner: VEGF-A enhances endothelial *PDGF-B* expression, whereas FGF-2 enhances mural *PDGF receptor β (PDGFR β)* expression. Co-stimulation with VEGF-A and FGF-2 caused significant mural cell recruitment in vitro and formation of functional neovasculature in vivo, compared with single-agent stimulation. These effects were abrogated not only by anti-

PDGFR β neutralizing antibody, but also by exogenous PDGF-BB, which could overwhelm the endogenous PDGF-BB distribution. These findings indicated the importance of preservation of the periendothelial PDGF-BB gradient. Thus, we demonstrated that the directional enhancement of endogenous PDGF-B–PDGFR β signaling is indispensable for the synergistic effect of VEGF-A and FGF-2 on neoangiogenesis in adults. The findings provide insights into the mechanisms underlying the effects of co-stimulation by growth factors, which could lead to rational design of therapeutic angiogenic strategies.

Supplementary material available online at
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Key words: PDGF, FGF, VEGF, angiogenesis, mural cell, endothelial cell

Introduction

Therapeutic angiogenesis, a strategy in which angiogenic growth factors improve revascularization of ischemic tissues, has been extensively investigated (Ylä-Herttuala and Alitalo, 2003). The growth factors used for therapeutic angiogenesis in previous studies were principally vascular endothelial growth factor A (VEGF-A) and fibroblast growth factor 2 (FGF-2), both of which are well-investigated pro-angiogenic molecules. VEGF-A was originally identified as a specific mitogen for endothelial cells, and the importance of VEGF-A in vascular development in vivo has been established by studies investigating VEGF-A knockout mice (Carmeliet et al., 1996; Ferrara et al., 1996). FGF-2, by contrast, has been reported to act as a mitogen for both endothelial and mural cells (D'Amore and Smith, 1993; Bikfalvi et al., 1997). The roles of FGF signaling in vascular formation have recently been reported in mouse embryo and tissue explant models using dominant-negative FGF receptor 1 (FGFR1) (Lee et al., 2000; Rousseau et al., 2003), and in embryonic stem cells lacking FGFR1 (Magnusson et al., 2004), although they have not been clarified by studies using genetically engineered mice lacking FGF ligands (Miller et al., 2000) or FGF receptors

(FGFRs) (Arman et al., 1998; Deng et al., 1994; Yamaguchi et al., 1994).

Experimental studies on therapeutic angiogenesis in animals and humans using VEGF-A or FGF-2 had been carried out based on these findings. However, the results of three double-blind randomized controlled trials, one with recombinant VEGF-A and two with recombinant FGF-2, were less favorable than expected (Henry et al., 2003; Simons et al., 2002; Lederman et al., 2002). Stimulation with single angiogenic molecules thus appears to be insufficient to induce functional vessels in humans.

The combination of VEGF-A and FGF-2, however, has been reported to have potent synergistic effects on neovascular formation in experimental conditions both in vivo and in vitro (Pepper et al., 1992; Goto et al., 1993; Asahara et al., 1995). In addition to the combination of VEGF-A plus FGF-2, that of VEGF-A and platelet-derived growth factor BB (PDGF-BB) (Richardson et al., 2001), and that of FGF-2 and PDGF-BB (Cao et al., 2003) have also been recently reported to have a potent synergistic effect in inducing neovascularization in experimental animal models in vivo. However, the mechanisms underlying this synergism are not well understood.

In the present study, we examined the mechanism of angiogenic synergism between VEGF-A and FGF-2. Stimulation with VEGF-A alone directly promotes the proliferation of endothelial cells, while stimulation with FGF-2 alone directly promotes the proliferation of endothelial as well as mural cells. In addition to these direct effects, we demonstrate here that the co-stimulation with VEGF-A and FGF-2 induces efficient mural cell recruitment to nascent vessels through enhancement of endogenous PDGF-B–PDGFR β signaling in concert: VEGF-A enhances PDGF-BB secretion by the endothelium, and FGF-2 enhances PDGFR β expression in mural cells. Since endothelium-derived PDGF-BB protein has affinity to extracellular matrix (ECM) molecules, it is located in the vicinity of endothelial cells and may form a steep gradient in the periendothelial compartment (Abramsson et al., 2003). Mural cells with FGF-2-upregulated PDGFR β could detect the periendothelial PDGF-BB gradient induced by VEGF-A and be recruited to the endothelium. We also demonstrate that addition of exogenous PDGF-BB, which could overwhelm the periendothelial endogenous gradient of PDGF-BB, impairs the mural cell recruitment induced by co-stimulation with VEGF-A and FGF-2 in vitro and also functional neovascular formation in vivo. These findings identify the importance of preservation of the endogenous gradient of PDGF-BB in inducing successful neovascular formation, which should be considered in designing effective strategies in therapeutic angiogenesis.

Materials and Methods

In vivo Matrigel plug assay

Recombinant human VEGF-A (VEGF165, R&D systems, Inc.), FGF-2 (R&D systems), PDGF-BB (PeproTech, EC, Ltd), monoclonal antibody anti-PDGFR β (APB5, eBioscience) and non-specific IgG (eBioscience) were mixed by pipetting, in combination or separately, with regular Matrigel (BD Biosciences). FGF-2 was used with 0.1 mg/ml of heparin (Aventis Pharma, Japan) in all Matrigel plug assays in this study. The Matrigels (500 μ l each) were injected subcutaneously into the abdominal region of male ICR mice (CLEA Japan Inc.) after anesthesia with Avertin. Two plugs with different ligands were injected into each mouse to avoid differences between individuals. Each Matrigel plug was harvested on day 7 and divided into two blocks, one fixed overnight in 4% paraformaldehyde (PFA) and then paraffin embedded for Hematoxylin and Eosin (H & E) staining, and another directly frozen in dry-iced acetone for immunohistochemistry. For the in vivo permeability assay, FITC-conjugated dextran (M_r 2 \times 10⁶; Invitrogen Molecular Probes) was administered intravenously via lateral tail veins 10 minutes before harvesting the Matrigel plugs. Some samples were also fixed for electron microscopy in 2.5% glutaraldehyde. All experimental protocols were conducted in accordance with the policies of the Animal Ethics Committee at the University of Tokyo.

Immunohistochemistry of Matrigel plugs

Frozen Matrigel plugs were sectioned at 10 μ m thickness in a cryostat, and then briefly fixed with 4% PFA. The fixed sections were reacted with monoclonal antibodies, anti-murine PECAM1 (Mec13.3, BD Pharmingen 553370), CD34 (FITC-conjugated RAM34, BD Pharmingen 553733) or F4/80 (CI:A3-1, Serotec Inc. MCA497), or rabbit polyclonal antibodies against PDGFR β (Upstate Group, Inc. 06-498), NG2 (Chemicon International, Inc. AB5320) or collagen IV (Chemicon AB756P). Specimens were

subsequently stained with secondary antibodies conjugated with Alexa Fluor[®] 488, 594 or 647 anti-rat/rabbit IgG (Invitrogen Molecular Probes). Because anti-mouse secondary antibodies stain the whole Matrigel plug section, we used Cy3-conjugated murine monoclonal antibody for SMA (1A4, Sigma-Aldrich Corporate C6198) and murine monoclonal antibody for desmin (D33, Dako Cytomation M0760) prestained with the Zenon labeling kit (Invitrogen Molecular Probes).

Quantification in Matrigel plug assay

We quantified the formation of vessels and vessel-like structures in Matrigel plugs by measuring lengths in H & E-stained samples in 0.3 mm² microscopic fields. First, we classified vasculatures into three categories as follows: (1) vessels with red blood cells (RBCs), (2) vasculatures without RBCs, and (3) lined cells without cavities. When PDGF-BB or the anti-PDGFR β neutralizing antibody was mixed with VEGF-A and FGF-2, an additional category was included, i.e. (4) vessels with extravascular RBCs, which indicate hemorrhage. We drew lines manually along each category of vasculature using Adobe PhotoShop (Adobe systems, Inc). Then we calculated the total lengths of the lines in each category using ImageJ software (National Institute of Health). Microsoft Excel (Microsoft Corporation) software was used for statistical analysis. The data are average of nine samples from three independent Matrigel plugs.

Cell culture and sorting

Maintenance, differentiation, culture and magnetic-bead cell sorting of MGZ5 ES cells (gift from H. Niwa) using an anti-VEGFR2 monoclonal antibody (phycoerythrin-conjugated Avas12a1; eBioscience), were performed as described previously (Yamashita et al., 2000). We plated 2.5 \times 10⁴ VEGFR2-positive (VEGFR2⁺) cells per well on 8-well CultureSlides (BD Falcon) for immunostaining or 2.0 \times 10⁵ cells per well on 1-well CultureSlides for total RNA preparation. CultureSlides were coated with 30 μ g/ml of collagen IV (Nitta Gelatin) prior to use. The cells were incubated in serum-free conditions for 1.5 days as described previously (Hirashima et al., 2003), in the presence of various ligands. FGF-2 was used with 1 μ g/ml of heparin in all in vitro experiments in the present study. For staining of acetylated low-density lipoprotein (AcLDL) in endothelium, we used Alexa Fluor[®] 594-conjugated AcLDL (Invitrogen Molecular Probes) following the manufacturer's protocol.

Quantitative RT-PCR analysis

Total RNAs from ESC-derived VEGFR2⁺ cells treated with various ligands were extracted using the RNeasy Mini Kit (QIAGEN). RNAs were reverse transcribed by random hexamer priming using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR analysis was performed using the GeneAmp 5700 system (Applied Bioscience) and SYBR[®] Green (Applied Bioscience). All expression data were normalized to *GAPDH*. Primer sequences were: *PDGF-B* (NM_011057) 5'-agcagagcctgctgtaacg-3', 5'-ggctctttcgcaaatc-3'; *PDGFR β* (NM_008809) 5'-tgccagttccacctgaa-3', 5'-agtgtgctcagctctgctt-3'; *PECAM1/CD31* (NM_008816) 5'-ccaaa-cagaaacccgtggagat-3', 5'-gtaatggctgttgctccaca-3'; *SMA α /Acta2* (NM_007392) 5'-agcgtgagattgccgtgacat-3', 5'-gcgttcgttccaatgtga-3'; and *GAPDH* (NM_008084) 5'-tgcagtgcaagtggagatt-3', 5'-tgccgtgaattgccgt-3'.

Immunocytochemistry

Cells were fixed in 1:1 acetone-methanol solution and incubated with antibodies against PECAM1 (Mec13.3), SMA (1A4, Sigma-Aldrich

Corporate A2547) and PDGFR β (APB5, eBioscience). The cells were then incubated with secondary antibodies and Sytox Green nuclear counterstain (Invitrogen Molecular Probes). For staining of PDGF-B, we used a rabbit polyclonal antibody (Abcam ab15499) at 1:100 dilution with specimens fixed in 10% formalin.

Quantification of endothelial-mural cell communication in vitro

Distances between the edge of each SMA⁺ mural cell and that of its nearest PECAM1⁺ endothelial sheet were measured using Adobe Photoshop. We analyzed the data statistically using Microsoft Excel. Experiments were performed in triplicate, and the data for statistical analyses were taken from six fields of microscopic views from three independent sets of experiments.

Video microscopy

A long-running video of the live cells on the CultureSlides (BD Falcon) was made using a Leica DM IRB microscope equipped with a hardware-controlled motor stage. The video images were analyzed using ImageJ software (National Institutes of Health, USA).

Results

Co-stimulation with VEGF-A and FGF-2 induces formation of mature blood vessels in vivo

We used the Matrigel plug assay in vivo to investigate synergism by co-stimulation with VEGF-A and FGF-2 (referred to as the V/F treatment; Fig. 1A). We examined the effect of stimulation with VEGF-A alone (V, Fig. 1B) or FGF-2 alone (F, Fig. 1C), and compared them with the V/F treatment. Newly formed vessels and vessel-like structures in Matrigel plugs were quantified by measuring their lengths. We classified them into the following three categories: (1) red blood cell (RBC)-containing vessels (red bars in Fig. 1D; scattergram shown in Fig. 1E), (2) cavities surrounded by cells but without RBCs (light blue bars in Fig. 1D), and (3) cells arrayed in line but without cavities (blue bars in Fig. 1D). The function of the neovasculature was also investigated by a permeability assay using intravenously administrated FITC-conjugated dextran (M_r 2 \times 10⁶).

The V/F treatment was most successful in inducing functional blood vessels, which contained RBCs and dextran

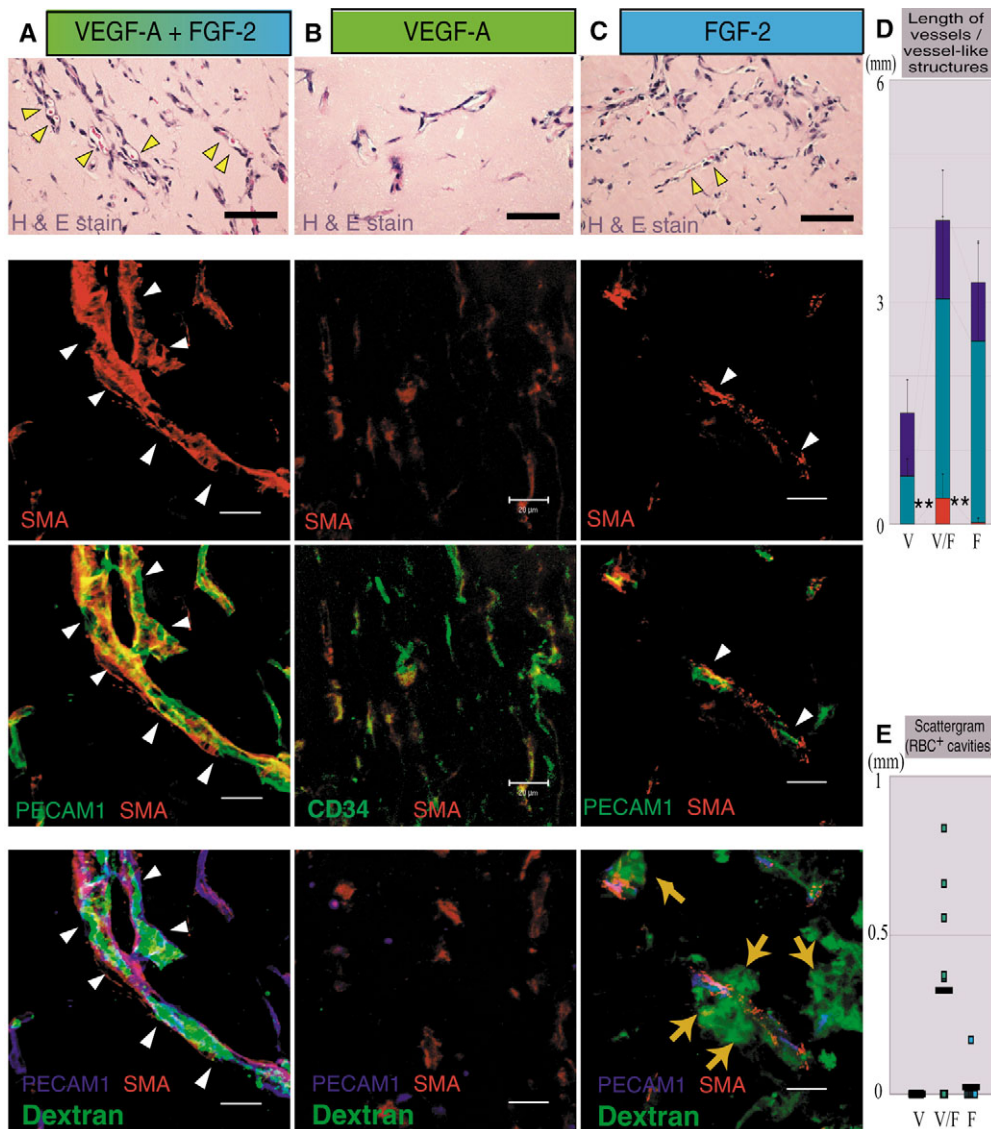


Fig. 1. Comparison of effects of VEGF-A/FGF-2 co-stimulation with those of VEGF-A or FGF-2 stimulation alone in a Matrigel plug assay in vivo. (A–C) Matrigel plugs mixed with 1 μ g/ml FGF-2 and 200 ng/ml VEGF-A (A), 1 μ g/ml VEGF-A alone (B), or 1 μ g/ml FGF-2 alone (C) were subcutaneously injected into the abdomen of mice. After 7 days, gels were extracted and examined after Hematoxylin and Eosin (H & E) staining (A–C upper rows; Scale bars: 60 μ m), and by immunohistochemistry with anti-PECAM1 or CD34, SMA, or by intravenously administrated FITC-conjugated dextran, as indicated in the figure (lower rows; scale bars: 20 μ m). (D,E) Quantification of the formation of new vessels in Matrigel plugs by their lengths is shown. Vessels and vessel-like structures were classified into three categories as follows: mature blood vessels containing red blood cells (RBCs) (red bars), cavities surrounded by cells but without RBCs (light blue), and cells arraying in line but without cavities (blue). * P <0.05, ** P <0.01 by Student's t -test. Corresponding figures for NG2 and desmin staining, and F4/80 staining under the VEGF-A-stimulated condition are shown in Figs S1 and S2, respectively.

in the vessel lumen with no leakage (Fig. 1A,D,E). Significantly more RBC-containing vessels were formed with the V/F treatment (** $P < 0.01$) than with stimulation with VEGF-A or FGF-2 alone. Immunohistochemical analysis revealed that PECAM1⁺ endothelial cells in the V/F-induced blood vessels were surrounded by and attached tightly by SMA⁺ mural cells. Injected dextran was observed strictly within the vessel lumen (Fig. 1A, white arrowheads). PECAM1⁺ cells in newly formed blood vessels were also positive for an endothelial marker, CD34 (data not shown) (Simmons et al., 1992). We investigated perivascular SMA⁺ mural cells with other molecules reported to be markers of less mature pericytes, NG2 and desmin (Gerhardt and Betsholtz, 2003) (Fig. S1 in supplementary material). The SMA⁺ mural cells in Matrigel plugs were all positive for NG2, whereas only main cellular pericytic process, which firmly attached to endothelium, was positive for desmin as reported.

In VEGF-A-treated samples, neither RBC- nor dextran-containing vessels were observed (Fig. 1B,D, and Fig. S2 in supplementary material); only some linear cavities surrounded by cells were found. From electron microscopic examination cells did not appear to form continuous layers (data not shown). Although cells were all negative for PECAM1, major parts of them were either positive for CD34 or SMA. Moreover, some of these cells were also positive for F4/80, a murine macrophage marker. The findings suggest that there could be inflammatory hematopoietic cells in the Matrigel plug under VEGF-A stimulation (Fig. S2 in supplementary material). SMA⁺ cells in gels under VEGF-A stimulation were positive for NG2 but negative for desmin (Fig. S1 in supplementary material). These findings suggest that stimulation with VEGF-A alone was not sufficient for inducing mature blood vessel formation in the present experimental conditions.

FGF-2 stimulation induced significantly less RBC-containing blood vessels than did V/F treatment (yellow arrowheads in Fig. 1C and the red bar in Fig. 1D). A permeability assay revealed that the vessels treated with FGF-2 alone are leaky, and an immunohistochemical study showed less extensive SMA⁺ mural coverage of endothelium than in V/F treatment (Fig. 1C). SMA⁺ cells treated with FGF-2 were NG2⁺ and desmin⁻ as were the VEGF-A-treated cells. The total

length of RBC-containing blood vessels induced by VEGF-A alone and FGF-2 alone was significantly less than that induced by V/F treatment (red bars in Fig. 1D). Thus the V/F stimulation had a synergistic effect on mature neovascular formation.

Since mural coverage of endothelial tubes is considered important for stabilization of newly formed blood vessels (Carmeliet, 2004), we focused on this cell-cell interaction in the following experiments.

In vitro model of mural cell recruitment induced by V/F treatment

To model the mural-endothelial communication induced by V/F treatment in vitro, we used embryonic stem cell (ESC)-derived VEGFR2⁺ cells cultured under serum-free condition on collagen IV-coated dishes (Yamashita et al., 2000; Watabe et al., 2003). PECAM1⁺ endothelial sheets, which were also positive for uptake of acetylated low-density lipoprotein (data not shown), appeared exclusively in the presence of VEGF-A, whereas SMA⁺ mural cells appeared in the presence of PDGF-BB as described previously (Yamashita et al., 2000) (Fig. 2B,D).

Under the V/F treatment both endothelial and mural lineages appeared (Fig. 2A,E). In addition, mural cells were recruited to the endothelial sheets (Fig. 2A, and corresponding long-running video-microscopic records available on-line: see Movie 1 in supplementary material), reflecting the findings in the in vivo Matrigel plug assay under V/F treatment. Video-microscopic observation for more than 12 hours revealed that, although the mural cells exhibited high motility, they still move toward the endothelium.

Upon treatment with FGF-2 alone, various lineages, including cells negative for PECAM1 and SMA, were observed (Fig. 2C). Notably, apparent endothelial-mural cell interaction was not observed in cells treated with FGF-2, although both endothelial and mural lineages were observed.

VEGF-A enhances expression of *PDGF-B* whereas FGF-2 enhances that of *PDGFR β*

To examine the mechanism of mural-endothelial interaction during V/F treatment, we determined the levels of expression

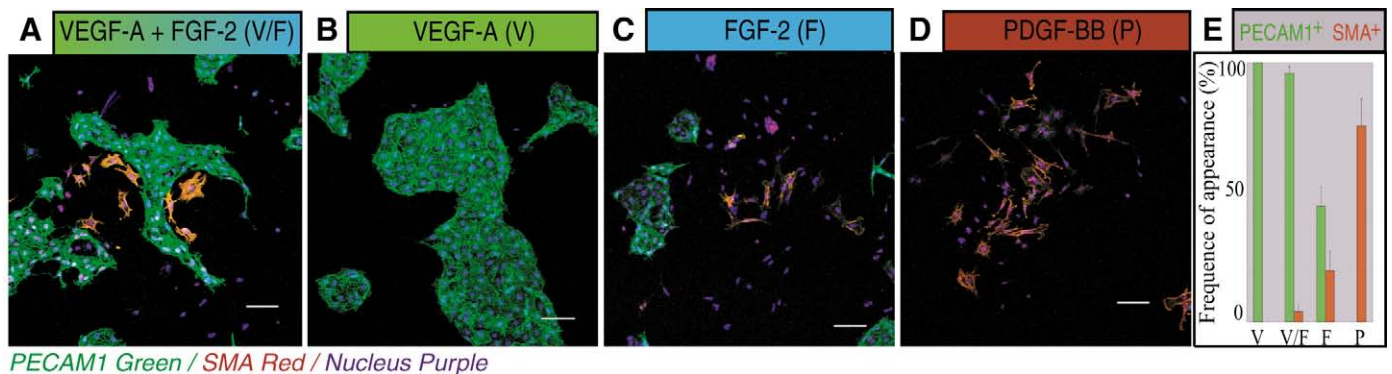


Fig. 2. Effects of growth factors on ESC-derived VEGFR2⁺ cells. (A–D) ESC-derived VEGFR2⁺ cells were treated with growth factors for 1.5 days, followed by immunostaining with anti-PECAM1 (green) and anti-SMA (red). Cells were treated with (A) a combination of VEGF-A (30 ng/ml) and FGF-2 (10 ng/ml; represented by a gradation of green and light blue in this and subsequent figures), (B) VEGF-A (green), (C) FGF-2 (light blue), or (D) PDGF-BB (20 ng/ml; red). Scale bars: 100 μ m. (E) Rates (%) of appearance of PECAM1⁺ cells (green bars) and SMA⁺ cells (red bars). Error bars represent the standard deviation (s.d.). Corresponding video microscopy recordings taken for a half-day prior to fixation can be seen in Movies 1–4 in supplementary material.

of *PDGF-B* and *PDGFRβ*, because these two molecules constitute one of the key signaling pathways in endothelial-mural communication, promoting mural cell migration to endothelium (Lindahl et al., 1997). *PECAM1* and *SMA*, which are marker genes for endothelium and mural cells, respectively, were assessed as well (Fig. 3A). All data were normalized to *GAPDH*.

High expression of *PECAM1* was observed in VEGF-A-treated and V/F-treated cells, reflecting the formation of endothelial sheets. *SMA* was mainly expressed in PDGF-BB-treated cells. These results are consistent with those obtained by immunohistochemical staining (Fig. 2). Expression of *PDGF-B* was high in VEGF-A-treated as well as in V/F-treated cells (colored bars), suggesting that expression of *PDGF-B* was induced by VEGF signaling. Immunostaining of V/F-treated cells (Fig. 3B) confirmed high expression of PDGF-BB in endothelial sheets compared to that in mural cells (red arrow).

Expression of *PDGFRβ* (which mediates PDGF-BB

signaling) was induced in PDGF-BB-treated cells, a finding attributable to the expression of *PDGFRβ* in mural cells. In FGF-2-treated cells, levels of expression of *PECAM1* and *SMA* correlated well with the results of immunostaining. Notably, expression of *PDGFRβ* was enhanced while that of *SMA* remained low in FGF-2-treated cells (blue bars, Fig. 3A; PDGF-BB-treated cells are represented by red bars, for comparison). These findings suggest the possibility that FGF-2 enhances *PDGFRβ* expression not only in mural cells but also in *PECAM1/SMA* double-negative cells. The level of expression of *PDGFRβ* was also higher in V/F-treated cells than in VEGF-A-treated cells. We then immunohistochemically determined the presence of *PDGFRβ*-expressing cells (Fig. 3C). With VEGF-A treatment, no cells expressed *PDGFRβ*. With V/F treatment, *PDGFRβ* was detected only in *SMA*⁺ cells (yellow arrows). With FGF-2 treatment, *PDGFRβ* was detected not only in *SMA*⁺ cells, but also in *PECAM1/SMA* double-negative cells (arrowheads). We

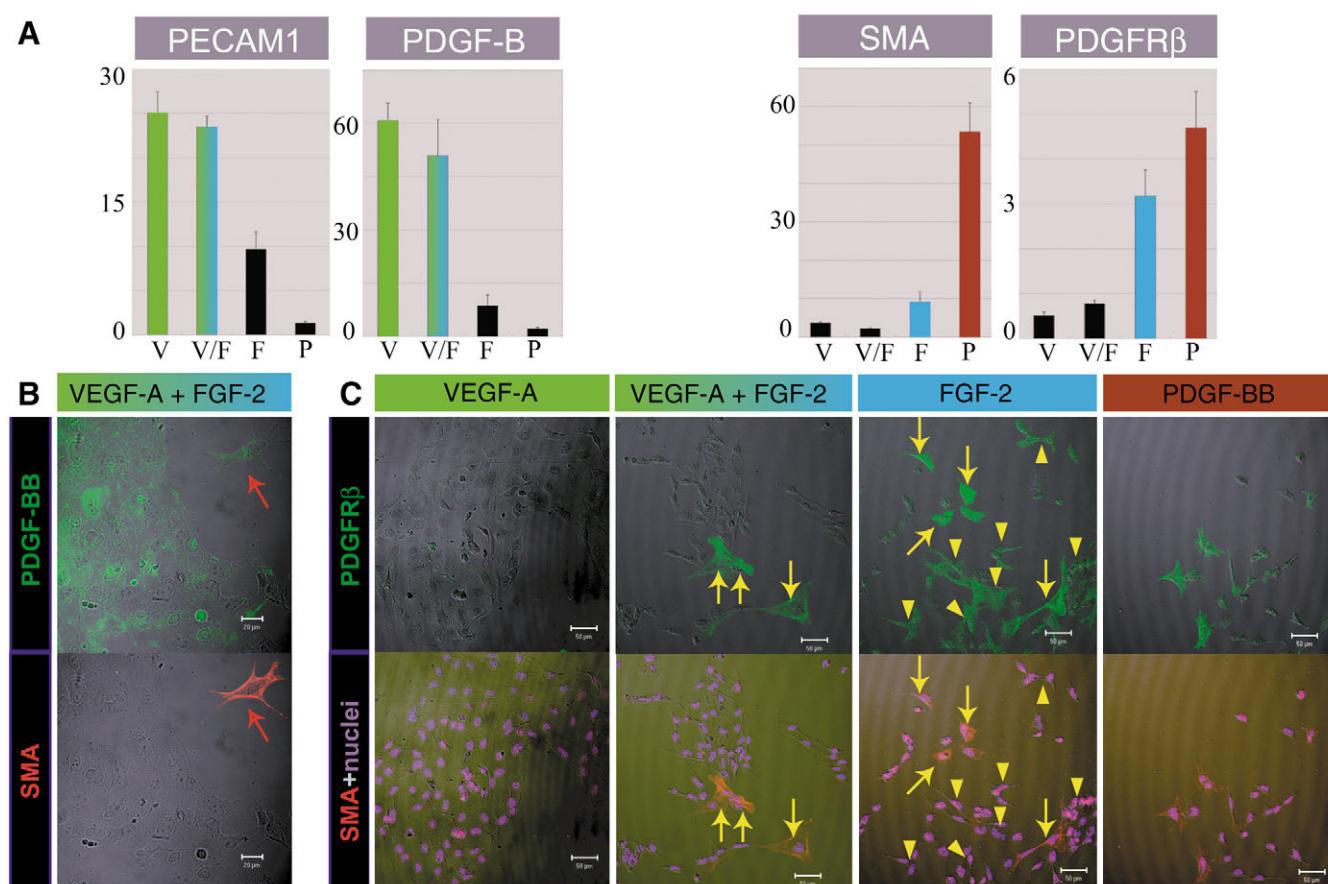


Fig. 3. Expression of markers and *PDGF-B/PDGFRβ* in ESC-derived VEGFR2⁺ cells treated with various growth factors. (A) Expression of *PECAM1*, *SMA*, *PDGF-B* and *PDGFRβ* was determined by quantitative RT-PCR; VEGFR2⁺ cells treated with VEGF-A (V), VEGF-A and FGF-2 (V/F), FGF-2 (F) or PDGF-BB (P) for 1.5 days. Vertical axes show relative expression. All expression data were normalized to *GAPDH*. Results were classified into two groups according to the pattern of expression. Expression of *SMA* and *PDGFRβ* in FGF-2-treated (F; blue bars) and PDGF-BB-treated (P; red bars) cells, and that of *PECAM1* and *PDGF-B* in VEGF-treated cells (V and V/F; green and blue/green bars, respectively). FGF-2- and PDGF-BB-treated cells (labelled F and P, respectively) in the two images on the left, and VEGF-A- and VEGF-A-FGF-2-treated cells (labelled V and V/F, respectively) in the two images on the right are shown as black bars because expression levels of corresponding genes were not markedly induced. (B,C) Expression of *PDGF-B* (B) and *PDGFRβ* (C) was immunohistochemically determined in cells treated for 1.5 days. Distribution of *SMA* is shown for comparison in the lower panels. While *PDGF-B* is expressed more in endothelium than in mural cells in the V/F treatment (red arrow), *PDGFRβ* was present in *SMA*⁺ cells (yellow arrows) as well as in *PECAM1/SMA*⁻ cells (yellow arrowheads), but not in *PECAM1*⁺ cells. Bright-field images have been merged in all pictures to demonstrate the presence of endothelial sheets. Scale bars: 20 μm (B) and 50 μm (C).

thus concluded that FGF-2 enhances expression of PDGFR β in cells of non-endothelial lineage, including the double-negative cells.

These findings explain well the mural cell recruitment observed with V/F treatment: VEGF-A makes the endothelial sheet more 'attractive' to mural cells by enhancing PDGF-BB secretion, while FGF-2 makes mural cells more 'sensitive' by enhancing mural PDGFR β expression. Endothelium-derived PDGF-BB may be located in the vicinity of endothelial sheets by binding to the ECM and form a gradient of PDGF-BB, resulting in effective recruitment of mural cells. VEGF-A and FGF-2 thus appear to synergistically enhance endogenous PDGF-B–PDGFR β signaling to promote migration of mural cells and their attachment to endothelial cells.

Co-stimulation by VEGF-A and FGF-2 enhances endothelial-mural interaction through PDGF-B–PDGFR β signaling

We added exogenous PDGF-BB (V/F/P treatment; Fig. 4A) or APB5, a neutralizing antibody against PDGFR β (Sano et al., 2001) (V/F/Ab treatment; Fig. 4C) to V/F-treated cells (Fig. 4B) in order to examine the possibility that the enhancement of endogenous PDGF-B–PDGFR β signaling is a key mechanism of V/F co-stimulation. Excessive exogenous PDGF-BB may distribute homogeneously and could disrupt mural cell migration by overwhelming the endogenous ECM-bound PDGF-BB gradient around the endothelial sheets.

Endothelial sheets and mural cells were significantly more distant from each other in both the V/F/P and V/F/Ab treatments than in the control V/F treatment (Fig. 4D). A total of 82% of mural cells were within 10 μ m (one-tenth of the scale bars in Fig. 4A–C) of the endothelial sheets in the V/F treatment, whereas only 20% of the mural cells were within 10 μ m in V/F/P and V/F/Ab treatments. Non-specific immunoglobulin had no effect when added to the V/F treatment (data not shown). With regard to cell populations, more SMA $^{+}$ cells were induced by V/F/P (Fig. 4E), possibly because of mitogenic function of exogenous PDGF-BB. Both PECAM1 $^{+}$

cells and SMA $^{+}$ cells appeared in all treatments tested, including the V/F/Ab treatment.

We could thus speculate that the mural cell recruitment induced by V/F treatment is mediated through enhancement of an endogenous gradient of periendothelial PDGF-BB, as well as mural PDGFR β expression.

VEGF-A and FGF-2 synergism through enhanced endogenous PDGF-B–PDGFR β signaling in vivo

We further examined the same set of conditions in vivo in the Matrigel plug assay (Fig. 5). VEGF-A and FGF-2 (Fig. 5B) were mixed with PDGF-BB (V/F/P treatment; Fig. 5A) or APB5 (V/F/Ab treatment; Fig. 5C). Lengths of vessels and vessel-like structures in Matrigel plugs were quantified as in Fig. 1, but an additional category was included, i.e. vessels with extravascular RBCs, which indicate hemorrhage. Significantly more vessels with extracellular RBCs were observed in the V/F/P or V/F/Ab treatments than in the V/F treatment (orange arrowheads in Fig. 5A,C and orange bars in Fig. 5D; * P <0.05, ** P <0.01). Significantly fewer RBC-containing vessels were formed in these conditions (Fig. 5D,E). The immunohistochemical study revealed defective attachment of SMA $^{+}$ cells to PECAM1 $^{+}$ cells in the V/F/P and V/F/Ab treatments (Fig. 5A,C, white arrowheads). Some SMA $^{+}$ cells did not attach to PECAM1 $^{+}$ cells under these conditions (yellow arrows), and leakage of dextran was observed (arrows). All perivascular SMA $^{+}$ cells were NG2-positive, but desmin-negative, in V/F/P and V/F/Ab treatments (Fig. S3 in supplementary material). Electron microscopic study revealed that the cells in the V/F/P or V/F/Ab treatments failed to attach to each other and formed neither continuous layers nor tight junctions in such vessel-like structures. Non-specific IgG had no effect on V/F-induced blood vessel formation (data not shown). These findings suggest the importance of preservation of the endogenous periendothelial gradient of PDGF-BB, in ligand-stimulated neovascularization in vivo as well.

Combined stimulation by PDGF-BB with VEGF-A (V/P treatment) or FGF-2 (F/P treatment) was also compared with the

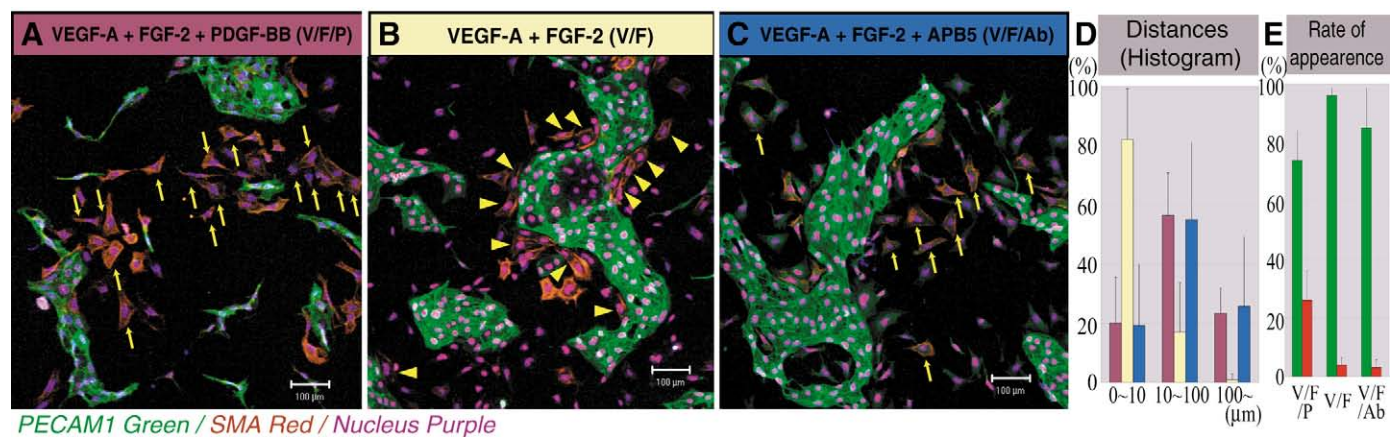


Fig. 4. Detachment of mural cells from endothelial sheets by exogenous PDGF-BB or anti-PDGFR β neutralizing antibody (APB5). (A–C) PDGF-BB (20 ng/ml; A) or anti-PDGFR β neutralizing antibody (APB5, 50 μ g/ml; C) was added to cells cultured with VEGF-A and FGF-2 (B). Green: expression of PECAM1; red: SMA; purple: nuclear counterstain. Arrows in A and C indicate detached mural cells; arrowheads in B indicate attached mural cells. Scale bars: 100 μ m. (D) Bar chart of distances between the nearest edges of SMA $^{+}$ mural and PECAM1 $^{+}$ endothelial sheets. Frequency is shown as the percentage of SMA $^{+}$ cell present in each distance class. (E) Comparison of the rates of appearance of PECAM1 $^{+}$ and SMA $^{+}$ cells.

V/F treatment in our experimental system (Fig. 6). Significantly fewer mature vessels were formed in V/P or F/P treatments than in the V/F treatment. Although SMA⁺ cells were observed near the PECAM1⁺ cells (especially in the F/P treatment), their attachments were defective. Leakage of dextran was also observed with F/P treatment. SMA⁺ cells were NG2-positive and desmin-negative as in other non-V/F treatments (Fig. S4 in supplementary material). Addition of exogenous PDGF-BB thus appeared to disrupt the perivascular gradient of PDGF-BB in vivo, leading to formation of less mature vessels.

Discussion

Therapeutic angiogenesis, a strategy to induce neovascularization using angiogenic molecules, has been expected to be useful as an alternative method of treatment in

patients with ischemic cardiovascular diseases. However, three large-scale clinical trials using recombinant VEGF-A or FGF-2 alone yielded results less significant than anticipated (Henry et al., 2003; Simons et al., 2002; Lederman et al., 2002). By contrast, combined stimulation with two angiogenic molecules from among VEGF-A, FGF-2 and PDGF-BB has been reported to be potent in inducing neovascular formation at least in experimental conditions. However, the mechanisms of synergistic effect are still not well enough understood, to be able to design optimal combinations of such agents. In the present study, we addressed the mechanism of the synergism of VEGF-A and FGF-2. We found that these factors play unique roles in synergistic enhancement of endogenous PDGF-B-PDGFR β signaling, to promote mature blood vessel formation, in addition to their known mitogenic effects (summarized schematically in Fig. 7).

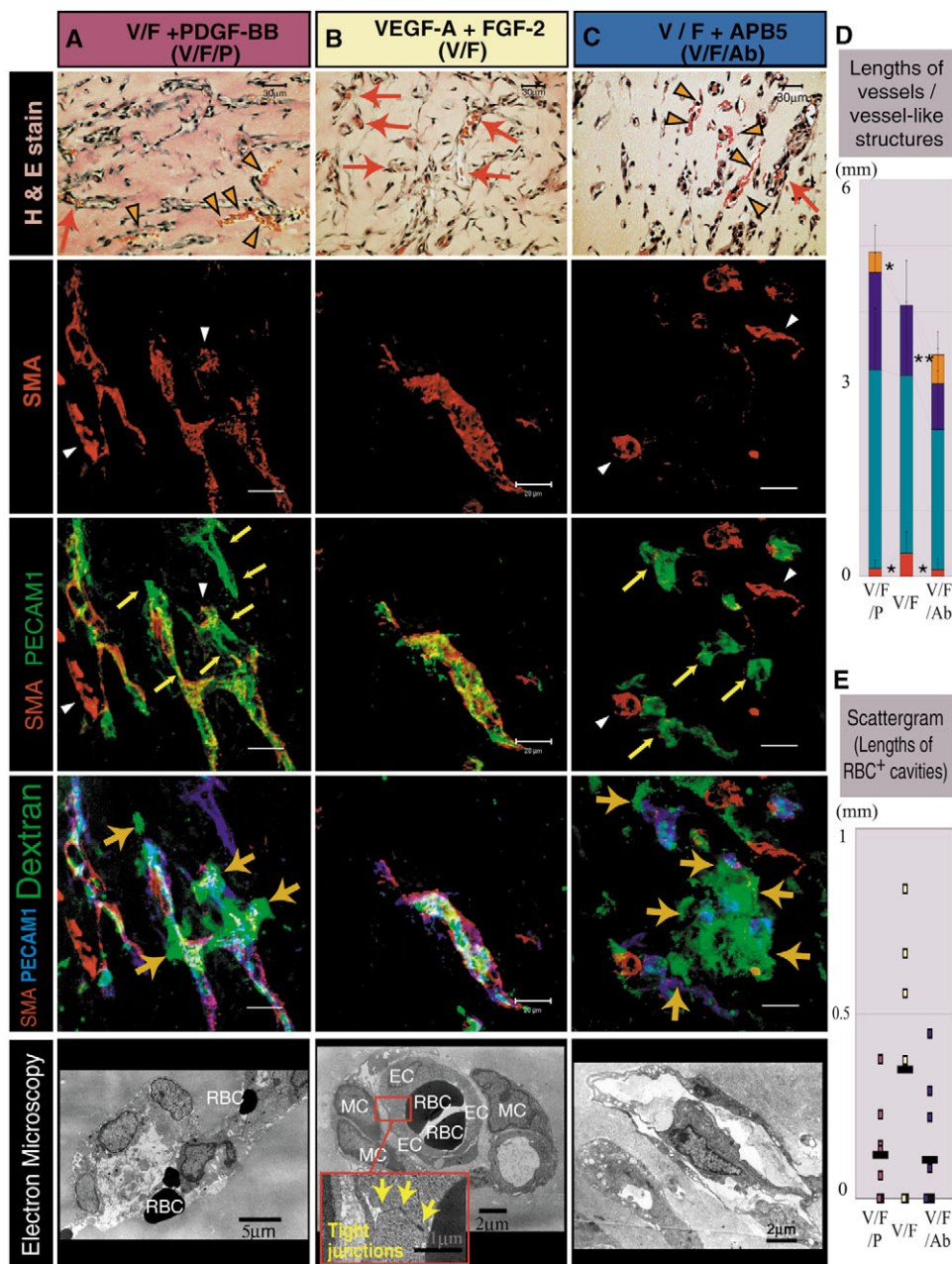
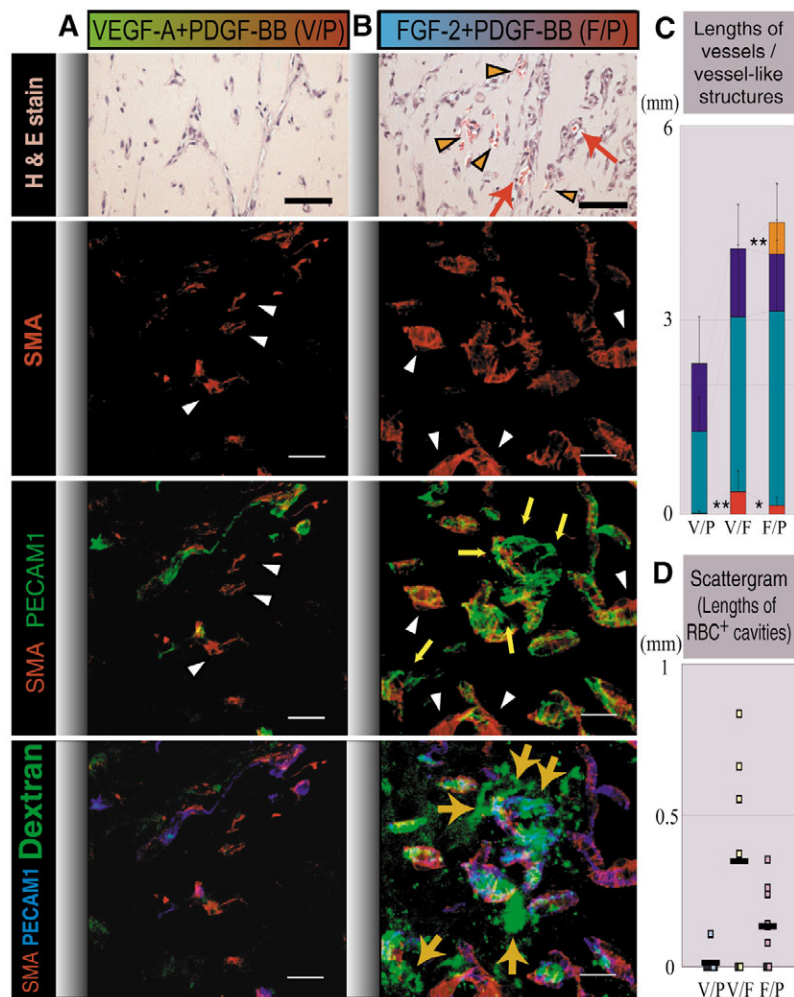


Fig. 5. Effects of exogenous PDGF-BB and anti-PDGFR β neutralizing antibody (APB5) in the Matrigel plug assay in vivo. (A-C) PDGF-BB (1 μ g/ml; A) or the anti-PDGFR β neutralizing antibody (APB5, 100 μ g/ml; C) was mixed with VEGF-A (200 ng/ml) and FGF-2 (1 μ g/ml) in the Matrigel plug assay. (B) V/F treatment only. (Top row) H & E staining. Scale bars: 30 μ m. Red arrows show mature blood vessels containing RBCs; orange arrowheads indicate vessels with extravascular RBCs. (Second and third rows) Immunohistochemistry. SMA staining (red) is shown in the second row, and merged images with PECAM1 staining (green) are shown in the third row. White arrowheads indicate detached SMA⁺ cells, and arrows indicate PECAM1⁺ cells not covered by SMA⁺ cells. (Fourth row) Permeability assay using intravenously administered dextran (green), merged with SMA (red) and PECAM1 (blue) images. Arrows indicate leakage of dextran. Scale bars: 20 μ m. (Fifth row) Electron micrographs. Scales are indicated in each photograph. MC, mural cells; EC, endothelial cells; RBC, red blood cells; yellow arrows, tight junctions formed between endothelial cells. (D,E) Induction of new vessels and vessel-like structures in Matrigel plugs was quantified as in Fig. 1 but with a new category (orange bars) representing vessels with extravascular RBCs, which indicate hemorrhage. * P <0.05, ** P <0.01 by Student's t -test. Corresponding figures for NG2 and desmin staining are shown in Fig. S3 in supplementary material.

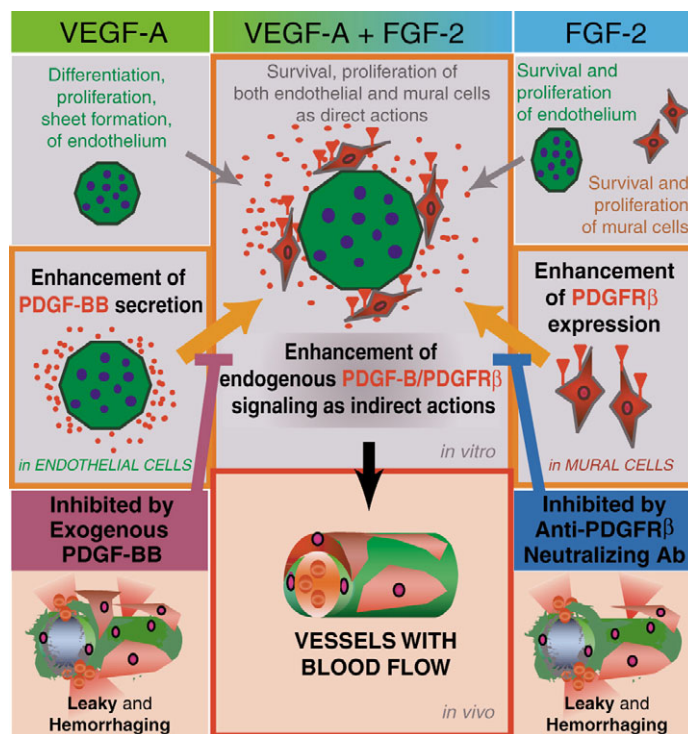
Fig. 6. Matrigel plug assay with other types of combined stimulation. (A,B) Combined stimulation with VEGF-A (200 ng/ml) plus PDGF-BB (1 μ g/ml) (A, V/P treatment) and FGF-2 (1 μ g/ml) plus PDGF-BB (1 μ g/ml) (B, F/P treatment). Red arrows and orange arrowheads in H and E stained section (first row) show mature blood vessels and vessels with extravascular RBCs, respectively. White arrowheads and yellow arrows in immunohistochemically stained sections (second and third row) indicate detached SMA⁺ cells and PECAM1⁺ cells not covered by SMA⁺ cells, respectively. Arrows in the permeability-assay section (fourth row) indicate leakage of dextran. Scale bars: 60 μ m in H and E stained sections; 20 μ m in immunohistochemically stained sections. (C,D) Newly formed vessels and vessel-like structures were quantified using the same method as in Fig. 5. * P <0.05, ** P <0.01. Corresponding figures for NG2 and desmin staining are shown in Fig. S4 in supplementary material.



During development, mural cells are considered to be required for establishment of the integrity of new vessels (Sims, 1986). Mural cell deficiency induces aberrant microvascular formation (Betsholtz, 2004). One of the key signaling pathways in mural cell recruitment has been reported to be the PDGF-B–PDGFR β pathway, as demonstrated in embryonic blood vessel formation of the brain capillaries and the kidney glomerular capillary tuft (Lindahl et al., 1997) and in postnatal renal and retinal function (Lindblom et al., 2003; Uemura et al., 2002). Recently, the PDGF-B–PDGFR β pathway was also shown to support postnatal tumor vessel formation (Abramsson et al., 2003). We here demonstrate that the PDGF-B signaling pathway is also indispensable for adult neovascular formation induced by exogenous ligands, VEGF-A and FGF-2.

One of the major roles of FGF-2 in the co-stimulation system appears to be enhancement of expression of *PDGFR β* in non-endothelial cells, in addition to stimulating the proliferation of both mural and endothelial cells. Enhancement of expression of *PDGFR α* and *PDGFR β* by FGF-2 was recently reported in newly formed vessels in the rabbit ischemic hind limb model, as determined by in situ hybridization (Cao et al., 2003), although the types of cells expressing PDGFRs were not specified. Another report

Fig. 7. A scheme of synergistic effects of VEGF-A and FGF-2 in neovasculture formation through enhancement of endogenous PDGF-B–PDGFR β signaling. VEGF-A induces secretion of PDGF-BB from endothelial cells in addition to its mitogenic effects on endothelium. FGF-2 enhances PDGFR β expression in mural cells, in addition to its mitogenic effects on both endothelial and mural cells. Combined stimulation with these two angiogenic molecules thus synergistically enhances intercellular communication through PDGF-B–PDGFR β signaling and causes mural cell recruitment and formation of mature vessels. These effects are blocked by either addition of exogenous PDGF-BB, which could overwhelm the local distribution of endogenous PDGF-BB, or inhibition of PDGFR β by specific antibodies, leading to formation of leaky and hemorrhaging vessels.



noted that expression of *PDGFR α* , but not of *PDGFR β* , was enhanced in vascular smooth muscle cells by FGF-2 (Schöllmann et al., 1992). We found that FGF-2-induced enhancement of expression of *PDGFR β* , but not of *PDGFR α* , is functionally important during blood vessel formation, as revealed by the effects of neutralizing antibody against *PDGFR β* in the present study. In Matrigels treated with FGF-2, however, PECAM1⁺ cells and SMA⁺ cells appeared to communicate less with each other than when treated with V/EGF-A plus FGF-2. This decrease in intercellular communication might be explained by the low level of expression of *PDGF-B* with administration of FGF-2 alone, in contrast to the high level of expression of *PDGF-B* in the endothelium in the V/F treatment, as suggested by in vitro experiments (Fig. 3).

Induction of expression of *PDGF-B* by VEGF-A contributes to effective enhancement of mural cell recruitment. However, VEGF-A itself failed to induce mature vessels in Matrigel plugs (Fig. 1B) but it did induce sheets of PECAM1⁺ cells in the in vitro ESC-derived system on collagen IV-coated dishes (Fig. 2B). At present, the reasons for this discrepancy in findings are unclear. One possible explanation is that formation of endothelial sheets requires layers of collagen IV (Hangai et al., 2002; Carmeliet, 2004). Consistent with this possibility, VEGF-treated gels were negative for collagen IV staining, whereas V/F-treated gels were positive for collagen IV around mature vessels with surrounding mural cells (our unpublished observation).

Therapeutic angiogenic strategies using exogenous PDGF-BB as a component have emerged recently, in addition to the combination of VEGF-A and FGF-2. However, the endothelial-mural interaction did not occur efficiently when Matrigel plugs or ESC-derived VEGFR2⁺ cells were treated with a combination of three growth factors, VEGF-A, FGF-2 and PDGF-BB, although both endothelial and mural cells were observed in these systems (Fig. 4A, Fig. 5A). V/F/P treatment induced mural cells more effectively than the treatment without PDGF-BB, probably due to the mitogenic effect of exogenous PDGF-BB. By contrast, exogenous PDGF-BB appeared to have an inhibitory effect on local mural cell migration, possibly by disruption of the local gradient of PDGF-BB derived from endothelial cells by excessive exogenous PDGF-BB. It remains unclear, however, to what extent mural cell migration and neoangiogenesis could be affected by differences of ECM molecules bound by PDGF-BB molecules, or differences in local concentration of exogenous PDGF-BB.

The effect of exogenous PDGF-BB on disruption of neoangiogenesis was further confirmed in the Matrigel plug assays. In a recent study using a variety of combinations of growth factors, including VEGF-A and FGF-2 (V/F treatment), FGF-2 and PDGF-BB (F/P treatment), were found to yield the best results in inducing stable vessels in an in vivo corneal assay (Cao et al., 2003). The combination of VEGF-A and PDGF-BB (V/P treatment) was also reported to be effective in inducing new vessels in a polymer gel system for stepwise and gradual release of VEGF-A followed by PDGF-BB. By contrast, in our Matrigel plug assay, V/F treatment induced more mature vessels than did the V/P or F/P treatments (Fig. 6). We thus concluded that exogenous PDGF-BB, which may distribute homogeneously and overwhelm the endogenous distribution of the molecule, could in fact impair blood vessel

stabilization by mural cells. Preservation of the endothelial cell-derived local PDGF-BB gradient thus appears important for appropriate migration of mural cells towards endothelial cells.

Use of the combination of VEGF-A and FGF-2 thus has the outstanding advantage in therapeutic angiogenesis that the ligand and the receptor are properly induced in a cell-type-specific manner: PDGF-BB is induced in endothelial cells by VEGF-A, and *PDGFR β* is induced in mural cells by FGF-2, resulting in enhancement of the cell-derived endogenous gradient of PDGF-BB and appropriate targeting of migrating mural cells. Understanding of the endogenous gradients of signaling molecules, as shown in this study, will enable rational design of effective strategies for induction of mature and functional blood vessels in vivo.

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