Angiogenesis stimulated by PDGF-CC, a novel member in the PDGF family, involves activation of PDGFR-αα and -αβ receptors

RENHAI CAO, EBBA BRÅKENHIELM, XURI LI,* KRISTIAN PIETRAS,† JOHAN WIDENFALK,‡ ARNE ÖSTMAN,‡ ULF ERIKSSON,* AND YIHAI CAO†

Microbiology and Tumor Biology Center, Karolinska Institute, S-171 77 Stockholm, Sweden; *Ludwig Institute for Cancer Research, Stockholm Branch, S-171 77 Stockholm, Sweden; †Ludwig Institute for Cancer Research, Uppsala Branch, S-751 24 Uppsala, Sweden; and ‡Institute of Neurobiology, Karolinska Institute, S-171 77 Stockholm, Sweden

ABSTRACT A newly discovered PDGF isoform, PDGF-CC, is expressed in actively angiogenic tissues such as placenta, some embryonic tissues, and tumors. We test the possibility that PDGF-CC promotes angiogenesis in vivo. The core domain (mature form) of human PDGF-CC is sufficiently potent to stimulate neovascularization in the mouse cornea. The corneal angiogenic response induced by PDGF-CC is robust although the area of neovascularization is smaller than those of FGF-2 and VEGF-stimulated angiogenesis. Similarly, PDGF-BB and PDGF-AB induce angiogenic responses virtually indistinguishable from PDGF-CC-stimulated vessels. In contrast, PDGF-AA displays only a weak angiogenic response in the mouse cornea. Although there was no significant difference in incorporation of mural cells to the newly formed blood vessels induced by PDGF-BB and -CC, the percentage of mural cell positive vessels induced by PDGF-AA was greater than those induced by FGF-2, PDGF-BB, and PDGF-CC. In the developing chick embryo, PDGF-CC induced branch sprouts from established blood vessels. In PDGF receptor-transfected endothelial cells, PDGF-CC activated the PDGF receptor alpha subunit (PDGFR-α). PDGF-CC, but not PDGF-AA, was able to activate PDGFR-β receptor in endothelial cells that coexpress both α and β forms of receptors. Thus, the PDGF-CC-mediated angiogenic response is most likely transduced by PDGFR-αα and -αβ receptors. These data demonstrate that the PDGF family is a complex and important group of proangiogenic factors.—Cao, R., Bråkenhielm, E., Li, X., Pietras, K., Widenfalk, J., Östman, A., Eriksson, U., Cao, Y. Angiogenesis stimulated by PDGF-CC, a novel member in the PDGF family, involves activation of PDGFR-αα and -αβ receptors. FASEB J. 16, 1575–1583 (2002)

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Platelet-derived growth factors (PDGFs) have important functions on many cell types including endothelial cells in vivo. For more than a decade, the PDGF family had been considered to be encoded by two genes (PDGF-A and PDGF-B), resulting in three dimeric isoforms of proteins: PDGF-AA, PDGF-BB, and PDGF-AB. The biologically actions of these growth factors are known to be mediated by three receptor dimeric structures (PDGFR-αα, PDGFR-ββ, and PDGFR-αβ) (1–3). Recent genetic studies in mice have suggested the possible existence of additional forms of PDGF. Mice lacking PDGFR-α exhibit a phenotype that is more severe than that caused by elimination of the PDGF-A gene (4–7). Deletion of the PDGF-A and PDGF-B genes does not reproduce the exact defects caused by PDGFR-α deficiency (2, 3). Thus, PDGFR-α may mediate more signals than just the effects of PDGF-AA, PDGF-BB, and PDGF-AB. A radical explanation for the more severe phenotype occurring in PDGFR-α-deficient mice is the presence of additional ligands for this receptor.

In agreement with the mouse genetic studies, sequence homology analysis revealed PDGF-CC as a novel member of the PDGF family (8). The same gene was identified almost simultaneously by an independent group and named spinal cord-derived growth factor (9). This novel factor contains a characteristic PDGF/vascular endothelial growth factor (VEGF) domain with eight invariant cysteine residues involved in inter- and intra-chain disulfide bonding (8). Such a structural pattern of conserved eight-cysteine residues is also present in the backbone structure of the VEGF family (10–14). More recently, another member of the PDGF family, PDGF-DD, has been identified based on its sequence homology to known PDGFs (15, 16). PDGF-DD selectively binds and activates PDGFR-ββ, but not PDGFR-αα (15, 16).

Despite the structural similarities of PDGF-CC and the VEGF family, PDGF-CC does not interact with the three VEGF high-affinity receptors (VEGFR-1, VEGFR-2, and VEGFR-3) (8). Instead, the core domain of PDGF-CC
specifically interacts with the PDGFR-α and PDGFR-αβ heterodimeric receptors but not with PDGFR-ββ homodimers (8, 17). Similar to PDGF-AA, the core domain of PDGF-CC induces tyrosine phosphorylation of the PDGFR-α receptor and cell proliferation of fibroblasts (8, 18). It should be emphasized that the full-length PDGF-CC does not activate the PDG receptors (8, 17). Similarly, proteolysis is also required for PDGF-DD to interact with PDGFR-ββ (15, 16). Thus, proteolytic processing is required to release the active form of PDGF-CC and PDGF-DD. In vitro, plasmin can cleave the full-length PDGF-CC to generate the active core domain. This feature seems to be unique to PDGF-CC and PDGF-DD because both PDGF-AA and PDGF-BB can bind and activate their receptors without extracellular proteolytic cleavage (8, 15, 16).

In adult human tissues, high levels of PDGF-CC expression were detected in heart, kidney, liver, pancreas, ovary, and placenta (8). In contrast, PDGF-AA, another PDGFR-α receptor ligand, is expressed at lower levels in these tissues with the exception of heart and pancreas (8). The distribution patterns of PDGF-CC and PDGF-AA in various tissues suggest that these ligands may have different although overlapping target cells. In the developing embryo, PDGF-CC is predominately expressed in the spinal cord, epidermis, and epidermal openings such as mouth, nostrils, ears, and eyelids (8, 9). Similar to other PDGFs, PDGF-CC is also widely expressed in muscle tissues including the myoblasts of smooth and skeletal muscles (19). This expression pattern is in close proximity to the sites of PDGFR-α and -β receptor expression (8). These findings suggest that PDGF-CC could activate PDGFR-αβ in vivo. The biological functions of PDGF-CC in vivo remain poorly characterized. A recent study reports that PDGF-CC is an oncogenic protein that transforms murine fibroblast cell line and induces tumor formation (18). PDGF-CC has been found to stimulate vascular smooth muscle cell growth in vitro (20).

The high levels of expression of PDGF-CC in angiogenic tissues such as the placenta, ovary, and embryonic tissues suggest that this factor may induce new blood vessel growth in vivo. To test this possibility, we determined the angiogenic activity of PDGF-CC in two angiogenesis models, the chick chorioallantoic membrane (CAM) and the mouse corneal model. Other isoforms of the PDGF family have been reported to stimulate angiogenesis in the CAM assay (21, 22). Here we show that PDGF-CC is able to induce new blood vessel formation in the CAM model and the mouse corneal model. This factor activates PDGFR-α and -β receptors in endothelial cells that coexpress both types of receptors. In this work, we describe our findings of the angiogenic property of PDGF-CC in comparison with other members of the PDGF family and other known angiogenic factors.

**MATERIALS AND METHODS**

Reagents, cells, and animals

Recombinant human PDGF-CC core domain protein was expressed and purified to homogeneity as described previously (8). Two micrograms of PDGF-CC was analyzed on a 4–12% gradient BisTris NuPAGE (Novex) polyacrylamide gel followed by staining with Coomassie blue. Under nonreducing conditions, the core domain of PDGF-CC appeared as dimers with the molecular mass of 31 kDa (Fig. 1A, lane 2). The dimeric forms of PDGF-CC were converted to monomers (~22 kDa) under a reducing condition in the presence of DTT (Fig. 1A, lane 3). The dimeric forms of PDGF-CC under a nonreducing condition moved faster than those linear molecules under a reducing condition. Thus, the molecular weight of dimeric PDGF-CC under a nonreducing condition did not represent the double molecular weights of monomeric PDGF-CC under a reducing condition. PDGF-AA, PDGF-BB, PDGF-AB, and recombinant human VEGF_165 were kindly provided by Dr. Monica Tsang (R&D Systems Inc., Minneapolis, MN). Recombinant human FGF-2 was obtained from Pharmacia & UpJohn (Milan, Italy). Male 5- to 6-wk-old C57BL/6 mice were acclimated and caged in groups of six or less. Animals were anesthetized by injection of a mixture of dormicum and hypnorm (1:1) before all procedures and killed with a lethal dose of CO₂. All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Board.
Chick embryo chorioallantoic membrane assay

The CAM assay was performed according to published methods (23, 24). Briefly, 3-day-old fertilized white Leghorn eggs (OVA Production, Sorgen, Sweden) were cracked and chick embryos with intact yolks were carefully placed in 20 × 100 mm plastic Petri dishes. After 6 days of incubation in 4% CO₂ at 37°C, a disk of methylcellulose containing 2.5 μg of PDGF-CC dried on a nylon mesh (3×3 mm) was implanted on the CAM of individual embryos. The nylon mesh disks were made by desiccation of 20 μL of 0.45% methylcellulose in H₂O. After 4–5 days of incubation, embryos and CAMs were examined for the formation of new blood vessels in the field of the implanted disks by a stereoscope. Disks of methylcellulose containing 2.5 μg of BSA were used as negative controls. Five to nine embryos/sample were used for each experiment.

Mouse corneal micropocket assay

The mouse corneal assay was performed according to described procedures (23, 25). Corneal micropockets were created with a modified von Graef cataract knife in male 5- to 6-wk-old C57BL/6 mice. A micropellet (0.35×0.35 mm) of sucrose aluminum sulfate (Bukh Meditec, Copenhagen, Denmark) coated with hydrogel polymer type NCC (IFN Sciences, New Brunswick, NJ) containing various amounts of PDGF-AA, PDGFB-AB, PDGF-BB, PDGF-CC, FGF-2, or VEGF was implanted into each corneal pocket. Control animals were implanted with pellets containing no growth factor only phosphate-buffered saline (PBS). The pellet was positioned 1.2–1.4 mm from the corneal limbus. After implantation, erythromycin/ophthalmic ointment was applied to each eye. Eyes were examined by a slit-lamp biomicroscope on day 5 after pellet implantation. Vessel lengths and clock hours of circumferential neovascularization were measured.

Immunohistochemistry

The growth factor-implanted mouse eyes were enucleated at day 5 after implantation, immediately frozen on dry ice, and stored at −80°C before use. Frozen sections of 10 μm were cut using a cryomicrotome. Sections were air-dried for 10 min, fixed with acetone, and blocked with 30% non-immune goat serum. Endogenous biotin was blocked using an avidin-biotin reagent (Vector laboratories, Burlingame, CA). A mixture of primary antibodies consisting of a rat anti-mouse CD51 antibody (1:100, PharMingen, San Diego, CA) and a mouse anti-human desmin (1:50 NOVO Castra, Newcastle on Tyne, UK) antibody was added and incubated for 1 h at room temperature. After intensive washing, secondary antibodies of a rabbit anti-rat-IgG labeled with FITC (Dako A/S, Glostrup, Denmark) and a biotinylated goat anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL) were added to the tissue sections and incubated for 30 min. After rigorous rinsing, streptavidin-conjugated Cy3 (1:2500, Jackson ImmunoResearch, West Grove, PA) was added to samples and incubated for 30 min. After washing in PBS, slides were mounted in 90% glycerol and examined under a fluorescence microscope (Nikon) at 20× magnification. Images were collected with a digital camera system and further analyzed with Adobe Photoshop 6.0 software program.

Immunodetection of activation of PDGF-α and -β receptors

Porcine aortic endothelial cells expressing PDGFR-α and PDGFR-β (26) were grown in 60 mm Petri dishes and starved in serum-free medium containing 0.1 mg/mL BSA. Cells were stimulated with PDGF-AA, PDGF-BB, or PDGF-CC at a concentration of 100 ng/mL or without growth factors for 60 min on ice. After washing with serum-free medium, cells were lysed by a lysis Tris-HCl buffer containing 0.5% Triton X-100, 0.5% sodium deoxocholate, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 5 mM EDTA. Equal amounts of cell lysates of each sample were immunoprecipitated with a rabbit anti-PDGFR-α polyclonal antiserum (27) or a mixture of two monoclonal antibodies against PDGFR-β (28). Immunocomplexes were washed three times with the lysis buffer, followed by 20 mM Tris-HCl, pH 7.5, and separated onto a 7% SDS-PAGE. Western blotting for phosphorylaserine was subsequently performed using the P-I-99 mouse monoclonal antibody (Santa Cruz, Santa Cruz, CA).

In situ hybridization

In situ hybridization was carried out according to a standard method using radioabeled oligonucleotide probes and high stringency conditions. Two probes complementary to PDGF-α (nucleotides 423–470 and 3083–3130) and two probes complementary to PDGF-β (946–996 and 2610–2657) were used (29, 30). All probes were used separately and did not match any known sequence in Genbank except those of the intended genes. A control 50-mer random probe not complementary to any sequence deposited in the Genbank was also used. After 3’-end labeling with [35S]dATP (NEN Dupont) by terminal deoxynucleotidyl transferase (Amer sham, Arlington Heights, IL), probes were purified (QIAquick™ Nucleotide Removal Kit Protocol, QIAGEN, Chats worth, CA). Slides were incubated overnight (42°C) with 0.1 mL hybridization mixture containing 50% formamide, 4× SSC (0.15 M NaCl, 0.015 M sodium citrate pH 7.0), 1× Denhardt’s solution, 1% sarcosyl, 0.02 M Na3PO4, pH 7.0, 10% dextran sulfate, 0.06 M DTT, 0.1 mg/mL sheared salmon sperm DNA, and hot probe. Slides were then rinsed four times (45 min) in 1× SSC at 60°C and allowed to adjust to room temperature during a fifth rinse in 1× SSC. Further rinsing was carried out in distilled water and increasing concentrations of ethanol. Air-dried slides were then dipped in emulsion (Kodak NTB2, diluted 1:1 with water). After 5 wk of exposure, slides were developed, counterstained with cresyl violet, and mounted (Entellan™, Merck, Rahway, NJ). The control probe was hybridized and processed together with the other probes and gave rise to no specific pattern of hybridization signals in the mouse tissue. Specific labeling was confirmed by similar expression patterns revealed by two probes (complementary to different parts of the mRNA) for each PDGFR-α and PDGFR-β. Detection of positive autoradiographic signals was based on serial observations of adjacent sections from each tissue specimen; accumulation of silver grains in the emulsion above specific cells and tissues was identified by the staining procedures. Only cells over which silver grain accumulation was clearly above surrounding background levels and could be confirmed by both dark-field and high magnification bright-field were regarded as positive.

RESULTS

Stimulation of neovascularization in the chick embryo

The CAM assay is one of the most widely used in vivo angiogenesis assays and detects angiogenic activity of compounds during embryonic development (31). The early embryos in this angiogenesis assay lack immune response, thus allowing the study of angiogenesis without the interference of the host's immune response.
reactions and inflammatory influences on growing vessels. To study whether PDGF-CC could induce angiogenesis in vivo, the core domain of PDGF-CC protein was implanted onto the chick chorioallantoic membrane in the developing embryo. PDGF-CC at the dose of 2.5 μg/disk was able to stimulate microvessel growth in each implanted chick embryo (Fig. 1C). A significant increase in neovascularization with high vessel density was observed in the surrounding areas of the PDGF-CC implants. PDGF-CC induced the formation of new branches and vessel sprouts (arrows in Fig. 1C) from the existing vessels that grew toward the implanted disks. These vessel sprouts appeared as ‘red dots’ budding from blood vessels adjacent to the implanted factors. During prolonged incubation, these sprouts and branches would be further intensified. However, it was difficult to follow the development of these vessels due to the short life span of the embryos. In contrast, disks without growth factors did not stimulate neovascularization in chick embryos (Fig. 1B).

**Stimulation of neovascularization by PDGF-CC in mouse corneas**

The corneal angiogenesis model is one of the most rigorous mammalian angiogenesis models and requires a putative compound to be sufficiently potent in order to induce neovascularization in the avascular corneal tissue. Potent angiogenic factors including FGF-2 and VEGF have profound effects in this system (23–25, 31–33). Micropellets of aluminum sulfate coated with the slow-release polymer hydron containing PDGF-CC, FGF-2, or VEGF were surgically implanted into the corneas of C57BL/6 mice. Stimulation of new blood vessel growth was examined on day 5 after implantation. The angiogenic response of corneas stimulated by 160 ng of PDGF-CC was robust with a high number of capillaries (Fig. 2B). The newly formed as well as the limbal vessels were markedly dilated in the PDGF-CC-implanted corneas (Fig. 2B). The length of capillary vessels (~0.8 mm) in corneas implanted with PDGF-CC was similar to that found in VEGF-induced vessels (Fig. 2B, D, E). The overall angiogenic response induced by PDGF-CC (Fig. 2B) appeared similar to that induced by FGF-2 (Fig. 2C), albeit less potent than FGF-2. PDGF-CC- and FGF-2-induced microvessels were well organized and separated (Fig. 2B, C). In contrast, the VEGF-induced blood vessels seemed to be leaky, hemorrhagic, and likely to rupture (Fig. 2D). At the front edge, VEGF-induced capillaries were fused into disorganized and sinusoidal structures (Fig. 2D). Thus, angiogenic responses induced by PDGF-CC and VEGF were markedly different whereas PDGF-CC- and FGF-2-induced vessels were similar.

**Comparison of angiogenic responses induced by the members of the PDGF family**

The finding of angiogenic activity of PDGF-CC promoted us to investigate the angiogenic responses induced by other isoforms of the PDGF family in mouse corneas. The angiogenic features of the PDGF family in mammals have not been previously studied although several reports have demonstrated that PDGF-BB induced angiogenesis in the CAM (21, 22). In the present study, we were interested in a comparison of the angiogenic features of each PDGF isoform in the same angiogenesis model setting. As shown in Fig. 3, besides PDGF-CC, all other dimeric isoforms of PDGFs (including PDGF-AA, PDGF-AB, and PDGF-BB) were able to induce angiogenesis in the mouse cornea. Homodimers of PDGF-BB (Fig. 3C) and PDGF-CC (Fig. 3D) and the heterodimeric PDGF-AB (Fig. 3B) induced a similar angiogenic pattern in the mouse cornea. The measured vessel lengths (Fig. 3E), clock hours (Fig. 3F), and areas of neovascularization (Fig. 3G) stimulated by the same amount of these three isoforms were nearly indistinguishable. In contrast, the vessel lengths (Fig. 3E) vessel clock hours (Fig. 3F), and vascular areas (Fig. 3G) stimulated by PDGF-AA were significantly less than those induced by PDGF-AB, PDGF-BB, or PDGF-CC. Blood vessels stimulated by all four isoforms of the PDGFs were dilated (Fig. 3A–D).

**Figure 2. Comparison of corneal neovascularization induced by PDGF-CC, FGF-2, and VEGF.** Micropellets containing 160 ng PDGF-CC (B), 80 ng FGF-2 (C), or 160 ng VEGF (D) were implanted into mouse corneal micropockets. An example of a PBS pellet-implanted cornea (A). Corneal neovascularization was measured and photographed on day 5 after growth factor implantation. Arrows point to the implanted pellets. Photographs represent 20x amplification of the mouse eye. Quantification of corneal neovascularization is presented as maximal vessel lengths (E), clock hours of circumferential neovascularization (F), and areas of neovascularization (G). Graphs represent mean values (±SE) of 11–16 eyes (6–8 mice) in each group.
Association of mural cells to the newly formed blood vessels

Immunohistological studies using an anti-CD31 antibody revealed that microvessel densities induced by FGF-2, PDGF-AB, PDGF-BB, and PDGF-CC were virtually identical (Figure 4A, G, J, M, P). In contrast, vessel density (Fig. 4D, P) stimulated by PDGF-AA was significantly less than that induced by FGF-2, PDGF-AB, PDGF-BB, or PDGF-CC (Fig. 4P, P<0.001). Associations of mural cells (pericytes and smooth muscle cells) with newly formed microvessels have been correlated with vessel maturation (34). To investigate this association, we stained corneal tissue sections containing microvessels induced by FGF-2 and various forms of PDGF for the presence of desmin. This marker is specifically expressed in pericytes and smooth muscle cells associated with endothelial cells in blood vessels (35). In general, the number of corneal vessels positively stained by desmin (Fig. 4B, E, H, K, N) were fewer compared with CD31 positive vessels. Quantification of desmin positive signals revealed no significant difference in FGF-2, PDGF-AB, PDGF-BB, or PDGF-CC-induced vessels. An overlapping pattern was detected with CD31 and desmin positive signals when images in the same sections were combined using a digital program (yellow in Fig. 4C, F, I, L, O). Although PDGF-AA displayed the weakest angiogenic response in the PDGF family (Fig. 4P), the percentage of desmin positive vessels induced by PDGF-AA was significantly higher than that induced by FGF-2, PDGF-BB, or -CC (Fig. 4R). These data suggest that PDGF-CC, like other members in the PDGF family, may play a critical role in blood vessel growth and maturation by recruiting mural cells to the newly formed vasculature.

Expression of PDGFR-α and -β in newly formed corneal blood vessels

To localize the PDGF receptors in the newly formed blood vessels, a radiolabeling in situ hybridization method was performed on corneal microvessels using specific probes for PDGFR-α and PDGFR-β (29, 30). Expression of PDGFR-α mRNA was detectable in FGF-2, PDGF-AA, PDGF-BB-, and PDGF-CC-induced vessels (Fig. 5A–D). Similarly, PDGFR-β expression was also found on blood vessels (Fig. 5E–H), albeit PDGF-AA-induced vessels gave rise to only weak signals. Both PDGFR-α and -β positive signals were distributed in the same cell populations of blood vessels as those revealed by colocalization of anti-CD-31 staining (data not shown). However, it was difficult to distinguish whether these receptors were expressed on endothelial cells or on mural cells. These data provide a molecular basis for PDGFR-α and -β to form heterodimers in addition to their homodimers. Thus, the angiogenic response induced by PDGF-CC is most likely to be mediated by PDGFR-α and -αβ.

Receptor activation by PDGF-CC in endothelial cells coexpressing PDGFR-α and -β

To identify what receptors were responsible for mediating the PDGF-CC-induced angiogenesis, PAE cells cotransfected with PDGFR-α and -β receptors were analyzed for receptor phosphorylation after stimulation with different ligands. As expected, PDGF-AA, PDGF-BB, and PDGF-CC all induced heavy phosphorylation of PDGFR-α in PAE cells (Fig. 6A, lanes 2–4). In contrast, only a small fraction of PDGFR-α receptor became phosphorylated without ligand stimulation (Fig. 6A, lane 1). In addition to activation of PDGFR-α receptor, PDGF-BB efficiently induced phosphorylation of PDGFR-β receptor in PAE cells expressing α and β receptors (Fig. 6B, lane 3). Surprisingly, PDGF-CC but not PDGF-AA was able to induce phosphorylation of PDGFR-β in PDGFR-α and PDGFR-β coexpressing PAE cells (Fig. 6B, lane 4). These data are consistent with a recent report that PDGF-CC activates PDGFR-αβ heterodimers (17). In contrast, PDGF-AA failed to induce phosphorylation of PDGFR-β (Fig. 6B, lane 2).

DISCUSSION

Our results demonstrate that PDGF-CC is a novel angiogenic factor in vivo. The angiogenic activity of
PDGF-CC is one of the first examples of the in vivo functional property described for this molecule. The potential roles of the PDGF family in regulation of angiogenesis have not been well studied. Earlier studies have focused only on the role of PDGFs on endothelial cells in vitro or assayed in the chick chorioallantoic membrane system (21, 22). For example, it has been showed that PDGF-BB can directly induce endothelial cell proliferation, migration, and tube formation in vitro whereas PDGF-AA lacks such an effect on endothelial cells (36–40). In agreement with these in vitro studies, PDGF-BB has been found to induce new blood vessel growth in the CAM (21, 22). PDGF-AA and PDGF-AB are able to stimulate angiogenesis in the CAM, albeit less potently than PDGF-BB (21, 22).

Recently, gene targeting studies in mice demonstrated that PDGF-AA and PDGF-BB are important regulators for multiple systems during embryonic development (2–7, 41–43). Elimination of PDGF-A gene leads to a lethal phenotype in a majority of animals before the E10 stage; surviving animals have pulmonary, central nervous system, and dermal abnormalities (4–6). The phenotype from inactivation of the PDGFR-α is even more severe, with additional defects found in cardiovascular and skeletal systems, leading to embryonic death ~ E8–E16 (7). Mice lacking PDGF-BB or PDGFR-β receptor develop similar phenotypes with characteristics of leaky and hemorrhagic blood vessels (41–43). This defect is due at least in part to improper recruitment of supportive cells to the blood vessels (41, 42, 44). Thus, these genetic studies suggest that PDGF-BB plays a critical role in the recruitment of mural cells to blood vessel endothelial cells during embryogenesis.

PDGF receptors have been localized on microvascular endothelium, suggesting a direct role for PDGFs on endothelial cells and smooth muscle cells (22, 37, 40). Consistent with these previous reports, we have found that PDGFR-α and -β are expressed on newly formed blood vessels induced by various angiogenic factors. However, in our in vitro studies we have not been able to observe proliferative or chemotactic effects of PDGF-CC or other PDGFs on bovine capillary endothelial or human umbilical vein endothelial cells (unpublished data). These in vitro results agree with other published observations of negative effects of PDGFs on these endothelial cells in vitro (45). The explanation for these in vitro results is most likely the alterations of endothelial cell features in cell culture. In vivo, it appears that PDGFR-β receptor is the major type of PDGF receptor expressed on endothelial cells (22). However, a more recent study shows that PDGFR-β was only expressed on vascular smooth muscle cells (VSMC) and pericytes but not on endothelial cells (42). Thus, PDGF-BB, a PDGFR-β receptor ligand, may directly or indirectly induce the growth of capillary endothelial cells. In our present study, it is somewhat surprising that PDGF-AA, an activator specific for PDGFR-α, was able to induce angiogenesis in vivo. The underlying mechanisms of the angiogenic effect induced by PDGF-AA could be due to the association of VSMC to blood vessels. Contributions of mural cells to the neovascularization induced by PDGF-AA may occur at several steps. PDGF-AA is a direct mitogen for VSMC through activation of PDGFR-α (46). The activated VSMC may secrete angiogenic factors such as FGF-2 that promote endothelial cell growth. This hypothesis seems to be valid in the case of IL-1β-stimulated smooth muscle cells that release angiogenic factors (47). The outgrowth of VSMC may regulate their attachment and detachment to the basement membrane of endothelial cells in vivo and thus may allow endothelial cells to
sprout with guidance of VSMC. This hypothesis is speculative and needs to be validated. In addition to VSMC, it has been reported that the PDGFR-α receptor is expressed on microvascular endothelial cells (37). In this case, PDGF-AA may directly induce endothelial cell growth.

Our present results show that the angiogenic activity of PDGF-CC in vivo is more potent than PDGF-AA, whereas the angiogenic effect and neovascularization pattern stimulated by PDGF-CC is nearly indistinguishable from those induced by FGF-2, PDGF-AB, and -BB. These data imply that PDGF-CC may have differential effects compared with PDGF-AA on blood vessels. In support of our findings, it has been reported that PDGF-CC exhibits more potent mitogenic effect on VSMC than PDGF-AA (17). Similarly, such a differential biological effect between PDGF-CC and PDGF-AA has been observed with their transforming capacity. Whereas PDGF-CC is able to transform NIH3T3 cells and induce tumorigenicity, PDGF-AA has only a weak effect on transformation of these cells (18, 22, 48). Taken together, these findings suggest that PDGF-CC may activate PDGFR-α and -β receptors. A recent study demonstrates that PDGF-CC is indeed able to activate PDGFR-αβ heterodimers (8, 17). In fact, our results show the expression of PDGFR-α and -β in newly formed blood vessels. These data provide a molecular basis for the formation of PDGFR-α and PDGFR-β heterodimers in vivo. Thus, PDGF-CC but not PDGF-AA could activate PDGFR-αβ heterodimers to trigger an angiogenic response. Our additional results support the finding that PDGF-CC, but not PDGF-AA, is able to phosphorylate PDGFR-β in endothelial cells coexpressing α and β receptors. Although the activation of the PDGFR-β receptor by PDGF-CC is most likely through the PDGFR-αβ heterodimers, the possibility that activation of PDGFR-α by PDGF-CC may subsequently transactivate PDGFR-β should not be excluded. In any of these events, the activation of PDGFR-β by PDGF-CC requires coexpression of PDGFR-β and PDGFR-α in the same cells. Besides PDGF-CC, another novel member of the PDGF family, PDGF-DD, has been identified as a specific ligand for PDGFR-β but not for PDGFR-α (15, 16). The biological function of PDGF-DD in regulation of angiogenesis is not known. Our data suggest that the PDGF family has a complex role in regulation of blood vessel formation.

Figure 5. In situ detection of PDGFR-α and -β on newly formed blood vessels Mouse corneas implanted with FGF-2 (A, E, I), PDGF-AA (B, F), PDGF-BB (C, G), or PDGF-CC (D, H) were removed at day 5 after implantation. Bright-field photomicrographs of emulsion autoradiograms of corneal tissue sections hybridized with the oligonucleotide probes for mouse PDGFR-α (A–D) and -β (E–H). Vascular endothelial cells and smooth muscle cells were labeled (arrows). A 50-mer random probe was used as a negative control in detection of FGF-2-induced corneal vessels (I).

Figure 6. Tyrosine phosphorylation of PDGFR-α and -β in PAE cells PAE cells transfected with PDGFR-α and -β were untreated (lane 1) or treated with PDGF-AA (lane 2), PDGF-BB (lane 3), or PDGF-CC (lane 4), and cell lysates immunoprecipitated with anti-PDGFR-α (A) or anti-PDGFR-β (B) antibodies. The immunocomplexes were separated onto a SDS gel and blotted with an anti-phosphotyrosine antibody. The protein bands represent phosphorylated PDGFR-α (A) and PDGFR-β (B).
giostatin and endostatin are also proteolytic fragments (50–52). Therefore, proteases have both positive and negative influences on angiogenesis.

Therapeutic angiogenesis with growth factors such as VEGF and FGF-2 has become an attractive approach for the treatment of ischemic heart and limb disorders. However, animal studies and early clinical trials with a single angiogenic factor including VEGF have both encountered severe problems (53–57). It is highly plausible that the establishment of functional and stable vessels requires a combination of several growth factors. For example, the families of PDGF and angiopoietins may play critical roles in recruiting mural cells (smooth muscle cells and pericytes) to endothelial cells (58, 59). The mural cells can stabilize and perhaps maintain the newly formed blood vessels. PDGF-CC could complement the vascular functions of other angiogenic factors such as VEGF. Taken together, our results demonstrate that PDGF-CC induces angiogenesis in vivo. Treatments with PDGF-CC alone or in combination with other angiogenic factors could provide new approaches for therapeutic angiogenesis.

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