Platelet-derived growth factor D promotes the angiogenic capacity of endothelial progenitor cells

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Abstract. Neovascularization and re-endothelialization rely on endothelial progenitor cells (EPCs). However, the recruitment and angiogenic roles of EPCs are subject to regulation through the vascular microenvironment, which remains largely unknown. Platelet-derived growth factor D (PDGF-D) is a new member of the PDGF family that binds the PDGFR-β homodimer. However, it remains unknown whether and how it affects the angiogenic capacity of EPCs and participates in tube-like formation. EPCs were derived from rat bone marrow cells, and the gain-of-function approach was used to study the effects of PDGF-D on the biological activities of EPCs. EPCs that stably express PDGF-D were generated by lentiviral-mediated transduction, and the expression levels were evaluated by western blotting and reverse transcription, followed by real-time quantitative polymerase chain reaction (RT-qPCR). The biological activities of EPCs evaluated in the present study included proliferation, adhesion, migration, tube formation and senescence. Furthermore, the downstream signaling of PDGF-D was explored by western blot analysis. The results revealed that the lentiviral-mediated expression of PDGF-D in the microenvironment promoted the migration, proliferation, adhesion and tube formation of EPCs. In addition, PDGF-D suppressed the senescence of EPCs. Mechanistically, PDGF-D induced the phosphorylation of several signaling molecules, including STAT3, AKT, ERK1/2, mTOR and GSK-3β, suggesting that PDGF-D enhanced the angiogenic function of EPCs through PDGF receptor-dependent and -independent signaling pathways. In conclusion, PDGF-D promotes the angiogenic capacity of EPCs, including proliferation, migration, adhesion and tube formation, and thereby contributes to angiogenesis.

Introduction

Therapeutic angiogenesis designed to treat limb ischemic diseases has been extensively studied (1). Two major therapies for therapeutic angiogenesis include gene therapy, which uses proangiogenic growth factors to promote angiogenesis, and cell therapy, in which stem/progenitor cells are transplanted into the ischemic site and allowed to renew, adhere, migrate and differentiate into tissue-specific cells together with angiogenesis (2). Endothelial progenitor cells (EPCs), which may be derived from bone marrow cells or isolated from peripheral blood (3), can differentiate into mature endothelial cells and participate in the formation of new vessels in the ischemic area (4). Thus, EPC transplantation may ameliorate local ischemia in ischemic diseases. Moreover, the genetic modification of EPCs, such as the forced expression of certain angiogenic growth factors, has been shown to increase the angiogenic response and promote the bioactivity of EPCs (5-7). Taken together, the use of both gene therapy and cell transplantation has been considered to be an effective strategy for angiogenesis in an ischemic region (8).

The platelet-derived growth factor (PDGF) family is comprised of four genes: PDGF-A, PDGF-B, PDGF-C and PDGF-D (9,10). PDGFs bind to PDGF receptor α (PDGFRα) or β (PDGFRβ), and stimulate proliferation, migration and differentiation into many cell types in both developing and adult tissues (9,11). Compared with the well-studied roles of PDGF-A and -B in cardiovascular development and diseases, PDGF-D, as a newly discovered member of the PDGF family, has been much less studied. In the field of cancer, PDGF-D has been shown to promote the cell growth, aggressiveness, angiogenesis and endothelial-mesenchymal transformation (EMT) of colorectal cancer (12), and correspondingly, the inhibition of PDGF-D signaling was found to reduce angiogenesis in gastric cancer (13). PDGF-D was also demonstrated to contribute to neointimal hyperplasia in a rat model of vessel injury (14), to increase interstitial pressure, to induce macrophage recruitment and to promote blood vessel maturation (15). However, it remains unknown whether PDGF-D exerts any impact on the angiogenic activity of EPCs with regard to proliferation, migration, adhesion and differentiation.

Given the importance of EPCs in angiogenesis and the above-mentioned studies, it was hypothesized that PDGF-D may play a role in the mediation of the biological properties...
of EPCs, particularly those required for EPC-assisted angiogenesis, such as proliferation, migration, adhesion and differentiation. In the present study, a gain-of-function model was used to evaluate the effects of PDGF-D on the activity of EPCs. The findings of the present study support the premise that PDGF-D is a potentially promising target for therapeutic angiogenesis through the potentiating of the angiogenic activity of EPCs.

Materials and methods

EPCs isolation, culture and characterization. The isolation of EPCs from rat bone marrow cells was carried out, as previously detailed (6), with slight modifications. In brief, female Sprague-Dawley rats (4 weeks of age) were sacrificed by cervical dislocation. Then, bone marrow mononuclear cells were separated from the femurs and tibiae using Ficoll density gradient centrifugation (Amersham Biosciences, Freiburg, Baden-Württemberg, Germany), and cultured in Endothelial Cell Basal Medium-2 (Lonza Group, Ltd., Basel, Switzerland) supplemented with EGM-2 MV SingleQuots (Lonza Group, Ltd.) and 5% fetal bovine serum (FBS). EPCs at passages 3-8 were used for all studies. For the immunofluorescence experiments, cells were prepared and analyzed under a fluorescence microscope (Carl Zeiss, Jena, Thuringia, Germany), following previously described procedures (6). Briefly, cells were incubated with primary antibodies against CD133 (Ab2839; Abcam, Cambridge, MA, USA) at a dilution of 1:150, VEGFR2 (Ab2349; Abcam) at a dilution of 1:200 and CD34 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a dilution of 1:50; and Dil-AcLDL (Biomedical Technologies, Baden-Württemberg, Germany), and cultured in Endothelial Growth Medium-2 (Lonza Group, Ltd.) and 5% fetal bovine serum (FBS). EPCs at passages 3-8 were used for all studies. For the immunofluorescence experiments, cells were prepared and analyzed under a fluorescence microscope (Carl Zeiss, Jena, Thuringia, Germany), following previously described procedures (6). Briefly, cells were incubated with primary antibodies against CD133 (Ab2839; Abcam, Cambridge, MA, USA) at a dilution of 1:150, VEGFR2 (Ab2349; Abcam) at a dilution of 1:200 and CD34 (ab2839; Abcam) at a dilution of 1:150, FITC-UEA-lectin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a dilution of 1:150 and Dil-AcLDL (Biomedical Technologies, Madrid, Spain) at a dilution of 1:150, followed by incubation with anti-Alexa Fluor 647-FITC (Cwbio Tech, Beijing, China) secondary antibody at a dilution of 1:200 as referred to our previous research (11). Then, cells were counterstained with DAPI (Roche Diagnostics, Basel, Switzerland) and imaged with a fluorescence microscope. The experimental protocol using animals for research was approved by the Ethics Committee of Chongqing Medical University.

Lentivirus generation. The complementary DNA encoding the full-length PDGF-D was obtained by PCR in RNA purified from buffalo rat liver (BRL) cells (ATCC; American Type Culture Collection, Manassas, VA, USA), and subcloned into the lentiviral-based EGFP vector LV5 (Biosettia, San Diego, CA, USA) via the NotI and BamHI sites. The sequences of the oligos used for this subcloning were: 5'-ATAAGAATGCGG CGGCCGACCATGCAAGCTATCTTTA-3' (forward) and 5'-GGGATCCCTTATCGAGTTGCTTGGAC-3' (reverse). The lentiviruses were generated by transfection subconfluent 293T cells with lentiviral expression vectors and packaging plasmids using calcium phosphate precipitate. The viral supernatants were collected at 48 h after transfection, centrifuged at 75,000 x g for 90 min, re-suspended and filtered through 0.45 μm filters (EMD Millipore, Billerica, MA, USA). Infection efficiency was determined by GFP immunofluorescence. The PDGF-D expressed in EPCs (PDGF-D-EPCs) was selected by puromycin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) after lentivirus infection. EPCs that expressed lentiviral-mediated GFP were used as controls (GFP-EPCs). EPCs without any treatment were defined as wild-type EPCs (wt-EPCs).

Cell viability assay. EPCs (2.0x10^4 cells/ml) were respectively transplanted in 96-well plates and co-cultured with PDGF (vehicle, 50 and 100 ng/ml, respectively). At the indicated time points, cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, according to the manufacturer's protocol. Dimethyl sulfoxide was added to each well to dissolve the formazan precipitate. Absorption was measured at a wavelength of 570 nm using Gloma Multi Detection System (Promega Corporation, Madison, WI, USA). Data were compiled from six independent experiments, and each was carried out in duplicate.

Cell adhesion assay. EPCs co-cultured with PDGF (vehicle, 50 and 100 ng/ml, respectively) were respectively seeded on collagen type I and fibronectin-coated 96-well plates (1x10^4 cells/well), and incubated for 1 h at 37°C. Non-adherent cells were removed by washing with PBS. The remaining adherent cells were measured by MTT assay, according to the manufacturer's protocol. Data were collected from six independent experiments, and each was carried out in duplicate.

Transwell assay. In vitro Transwell invasion was assayed on 24-well chambers (Corning Incorporated, Corning, NY, USA), according to the manufacturer's protocols. In brief, the Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) pre-coated filter was solidified for 1 h at 37°C. Then, EPCs co-cultured with PDGF (vehicle, 50 and 100 ng/ml, respectively, 4.0x10^4 cells/well) were respectively positioned in the upper chambers, and allowed to migrate for 72 h. Non-migrated cells were removed by scraping, while migrated cells were fixed in 20% methanol and stained with 0.5% crystal violet. These cells were scored from five randomly selected fields, and images were captured under a light microscope (Olympus Corporation, Tokyo, Japan) with ×200 magnification. The cell migration rate was presented as a percentage of the migration in the presence of the vehicle.

Tube formation assay. A pre-coated 24-well plate with Matrigel (BD Biosciences) was used for the tube formation assay. EPCs co-cultured with PDGF (vehicle, 50 and 100 ng/ml, respectively, 4.0x10^4 and 2x10^4 cells/well, respectively) were respectively seeded on the Matrigel. After incubation for 72 h at 37°C, five randomly chosen fields were counted, and images were captured under a light microscope (Olympus Corporation) with ×400 magnification.

Cell senescence assay. EPCs were co-cultured with PDGF-D (vehicle, 50 and 100 ng/ml, respectively) in a 24-well plate for 24 h. Cell senescence was measured using a β-galactosidase staining kit (Beyotime Institute of Biotechnology, Suzhou, Jiangsu, China), according to the manufacturer's protocol. Data were compiled from six independent experiments, and each was carried out in duplicate.

Immunoblotting. Western blot analysis was performed as previously detailed (11). The following antibodies (Abs)
(Cell Signaling Technology, Inc., Danvers, MA, USA) were used (all dilutions, 1:1,000): GSK-3β Ab (cat no. 9315), phospho-GSK-3β Ab (cat no. 9323), STAT3 Ab (cat no. 8232), phospho-STAT3 Ab (cat no. 9134), mTOR Ab (cat no. 2972), phospho-mTOR Ab (cat no. 2971), ERK1/2 Ab (cat no. 4696), phospho-ERK1/2 Ab (cat no. 8544) and PDGF-D Ab (SCBT, Dallas, TX, USA). The signals were visualized by chemiluminescence (UVP, Upland, CA, USA) based on the manufacturer’s instructions and grayscale values were calculated by ImageJ version 1.80 [National Institutes of Health (NIH) Bethesda, MD, USA].

Real-time qPCR. TRizol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to purify the total RNA from cells. One milligram of total RNA was used for reverse transcription using a reverse transcription kit (TransGen Biotech, Beijing, China). Specific products of rat PDGF-D, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and PDGF-B were amplified by qPCR using the following primers: PDGF-D, 5'-TCC TCGCTTCCCCAAGC‐3' (forward) and 5'-TTTCTCTGCAACCGTGTC‐3' (reverse); VEGF, 5'-CAAAGCCAGCATACTAGGAGAGA‐3' (forward) and 5'-VTATTCATTCTCTTGTTGGTCACTGCCATTCAC‐3' (reverse); HGF, 5'-TTTCCGGTTGTAAGAGGAGATAC3' (forward) and 5'-TTCAAATACCCATCCTGTC-3' (reverse); PDGF-B, 5'-TGCTGTCTACGCCTGCTTGG3' (forward) and 5'-GCTCACGCCCCATCTTTGC‐3' (reverse); GAPDH, 5'-CCCATCTTGAAGGTTACGC‐3' (forward) and 5'-TATTGATGATCACGAGATTTC‐3' (reverse). The expression levels of these genes were evaluated by qPCR using a TransStart Green Q-PCR SuperMix kit (TransGen Biotech) with the following thermocycling conditions: Denaturation at 95°C for 10 min, followed by 95°C for 30 sec, and 60°C for 20 sec for 40 cycles. The relative expression of each targeted gene was determined using the \[2^{-\Delta\Delta Cq}\] comparative method (16) and normalized against that of GAPDH. Each sample was run as triplicate and repeated in three independent experiments.

ELISA. According to the manufacturer’s protocol, an ELISA kit (cat no. DL-PDGF-D-RA; DiLdevelop, Jiangsu, China) was used to determine the level of PDGF-D present in the extracellular medium. Following the adsorption of Detection Reagent A, the plates were incubated with a fixed amount of total protein from the medium. Thereafter, these plates were incubated with biotinylated Detection Reagent B and washed, followed by incubation with streptavidin-horseradish peroxidase. Then, chromogen tetramethylbenzidine was added, and the readings at an absorbance of 450 nm were recorded using a microplate reader (SpectraMax M2; Molecular Devices, LLC, Sunnyvale, CA, USA).

Statistical analysis. The statistical significance of differences among groups was tested using analysis of variance (ANOVA), followed by Tukey’s honestly significant difference (HSD) test after a normality test. GraphPad Prism v.6.01 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for analysis. P-values <0.05 and <0.01 were considered to indicate statistically significant differences.

Results

Characterization of EPCs. The biological properties of EPCs derived from rat bone marrow cells were first characterized using a previously described system, in which the cells were cultured in the endothelial cell selection medium and isolated (6). The obtained cells had a spindle shape with an endothelial cell-like morphology at day 7 (Fig. 1A). In addition, 90% of the attached cells exhibited an uptake of Dil-acLDL and the concurrent binding to FITC-UEA-lectin (Fig. 1A), which are the important characteristics of EPCs (17). Furthermore, it was found that these cells expressed VEGFR2, CD34 and CD133 (Fig. 1B), which are well-known endothelial markers (18). Taken together, this demonstrates that the cells derived from the rat bone marrow cells were EPCs. EPCs were used at passages 3-8 for the subsequent experiments.

Verification of EPCs stably expressing PDGF-D. In order to generate EPCs stably expressing GFP as controls (GFP-EPCs) or PDGF-D (PDGF-D-EPCs), EPCs were infected with lentiviruses encoding GFP alone or encoding both PDGF-D and GFP, followed by puromycin selection for one week. Infection efficiency was first evaluated by observing GFP using a fluorescence microscope. As shown in Fig. 2A, the infection efficiency of EPCs by lentiviruses was >90%. Next, the expression levels of PDGF-D in the stably expressing EPCs after drug selection by RT-qPCR and western blot analysis were assessed, and it was found that the mRNA expression in the PDGF-D-EPCs was 0.5-fold more than that in the GFP-EPCs (Fig. 2B). Consistent with the RT-qPCR results, western blot analysis also revealed a significantly elevated PDGF-D expression in PDGF-D-EPCs, when compared with that in the GFP-EPCs (Fig. 2C). In addition, it was found that the secretion of PDGF-D in the culture medium of PDGF-D-EPCs was significantly increased within 14 days of examination, when compared with the GFP-EPCs (Fig. 2D).

PDGF-D in the microenvironment promotes EPCs proliferation, migration, adhesion and tube formation and inhibits senescence. Next, the effects of PDGF-D in the microenvironment on the biological activities of EPCs in vitro were examined. MTT assays revealed that PDGF-D treatment significantly promoted EPCs proliferation, when compared with that noted in the vehicle (Fig. 3A). Given that the migration and adhesion of EPCs are required for vasculature regeneration (19), Transwell assays were performed to examine the effects of PDGF-D on EPC migration. The migration ability of EPCs stimulated by PDGF-D (100 ng/ml) was ~2.5-fold higher than that of EPCs stimulated by the vehicle (Fig. 3B). In addition, PDGF-D significantly enhanced the adhesion of EPCs, when compared with the vehicle (Fig. 3C). Tube formation in EPCs has been shown to be one of the key steps to promote angiogenesis (20). EPCs were co-cultured with different concentrations of PDGF-D (vehicle, 50 and 100 ng/ml, respectively) on Matrigel, and their capability to form capillary tubes was evaluated. EPCs co-cultured with PDGF-D (100 ng/ml) had significantly increased tube formation, when compared with those co-cultured with the vehicle (Fig. 3D). Furthermore, analysis of senescence was carried out to investigate the effect of PDGF-D on EPC senescence, as it was shown that delayed
senescence is helpful for EPCs to migrate and adhere to ischemic sites and excise angiogenesis (21). It was found that the number of senescent EPCs was significantly lower in EPCs treated with 100 ng/ml of PDGF-D than those treated with the vehicle (Fig. 3E). Collectively, these results indicate that PDGF-D has proangiogenic effects on EPCs.

**PDGF-D acts in a PDGFR-dependent and -independent manner.** In order to ascertain whether PDGF-D activates PDGFR and the downstream signaling, the expression of essential kinases involved in the PDGFR signaling pathway was examined by western blot analysis. As shown in Fig. 4A and E, PDGF-D overexpression promoted the phosphorylation...
of STAT3, AKT, ERK1/2, mTOR and GSK-3β. Interestingly, PDGF-D altered the expression and secretion of several PDGFR-independent regulators, including VEGF, HGF and PDGF-B (Fig. 4F). Collectively, these observations indicate that PDGF-D mediates endothelial angiogenesis in a PDGFR-dependent and -independent manner.

**Discussion**

In the present study, the gain-of-functional approach was used to examine the impact of PDGF-D on the biological activities of EPCs obtained from rat bone marrow cells. The major findings from this study were as follows: i) PDGF-D in the microenvironment promotes EPC proliferation, migration, adhesion and tube formation; ii) PDGF-D suppresses EPC senescence; iii) PDGF-D mediates the biological activities of EPCs through PDGFR-dependent and -independent mechanisms.

Cell based gene therapy, which has a proangiogenic effect, appears to be a powerful approach for the treatment of limb ischemic diseases (5,22,23). For instance, VEGF-transfected macrophages (5) and reprogrammed human postnatal dermal fibroblasts with EC transcription factors ER71/ETV2 (23) have been shown to be particularly effective in thrombus recanalization and resolution. In contrast to the previous belief that angioblasts are only implicated in vasculogenesis in pre-natal life, it is presently a general consensus that EPCs are generated in both pre- and post-natal life (24). Mesodermal cells produced during embryogenesis give rise to hemangioblasts, which differentiate into EPCs or hematopoietic stem cells (HSCs). The former exhibits the capacity to differentiate into ECs, and HSCs generate all blood cell lineages (25), which contributes significantly to angiogenesis. In the present study, it was found that PDGF-D substantially enhanced the angiogenic function of EPCs, including proliferation, adhesion, migration and tube formation. In addition, angiogenesis is an organized series of events that lead to the maturation of a mature vascular network, which needs the precise temporal and spatial regulation of numerous angiogenic factors, including VEGF, basic fibroblast growth factor-2 (FGF-2) and PDGF. Furthermore, it was found in the present study that the overexpression of PDGF-D increased the release of VEGF, HGF and PDGF-B into the culture medium. Together, it was postulated that PDGF-D may promote angiogenesis by enhancing the angiogenic activity of EPCs and increase the paracrine release of angiogenic factors.

During the process of angiogenesis and vasculogenesis, EPCs are activated to reach the proliferative sites, allowing these cells to enter the circulation (26). Thereafter, EPCs go to their target tissue, attach to the endothelia of the vessel, and penetrate through the basement membrane and ECM. After arriving at the remodeling site, EPCs start their differentiation into or interact with ECs. Although the exact function of EPCs remain unclear, it has been widely considered that EPC differentiation goes through the adhesion to extracellular matrix components, proliferation, maturation, and the possession of an endothelial phenotype (27). During this process, PDGF family members, such as PDGF-A and -B, play important roles (28). The present study provides evidence that PDGF-D also potentiates the angiogenic ability of EPCs, including proliferation, migration and tube formation, and inhibits their senescence in vitro. Given that decreased angiogenesis capacity is also an important feature of aging blood vessels (29,30), mainly
manifested as EPCs senescence, and that the EPCs activity in the circulatory system is critical for endothelial cell renewal, vascular repair and neovascularization (31), we believe that the anti-senescence effect of PDGF-D on EPCs can reduce vascular aging and enhance their function (32). All these increase the capability of EPCs to integrate into the major capillary plexus that constitutes the fundamental vascular network, which is required for angiogenesis. Thus, the present study suggests that PDGF-D plays a crucial part in angiogenesis and vasculogenesis by activating EPCs.
PDGF isomers play biological roles by binding to two specific cell surface receptors with different affinities. The α-receptor binds all three isomers with high affinity, whereas the β-receptor (PDGFR-β) binds only to PDGF-B1. PDGFRα and PDGFRβ have partly different functions, because these are differentially expressed in different cell types. However, these two PDGF receptors can regulate different signal transduction pathways in cells that have both receptor types (33). As mentioned above, in the present study, it was observed that PDGF-D promoted angiogenesis partially through the direct potentiation of biological activities of EPCs. On the other hand, previous research suggests that angiogenic signaling induced by PDGF-D is PDGFRα-independent (34). PDGFR could bind to scaffold molecules, which act as bridges to other signaling molecules. For example, the p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase) physically interacts with the p110 catalytic subunit (35) and Grb2 complexes with the nucleotide exchange factor Sos1 (36), which in turn stimulates Ras and its downstream signaling Erk1/2 MAP-kinase (37). Similarly, PDGF activation has been shown to indirectly activate several kinase signaling, including the Rho, Cdc42 and MAP-kinase pathways (38). Thus, it was speculated that PDGF-D may also have a paracrine function. Indeed, it was found that the overexpression of PDGF-D not only potentiates the phosphorylation of STAT3, AKT, ERK1/2, GSK-3β and mTOR, but also increases the paracrine release of angiogenic factors, including VEGF, HGF and PDGF-B. Thus, the investigators consider that PDGF-D promotes the angiogenic capacity of EPCs through both PDGF signaling-dependent and -independent pathways, while the exact molecular basis underpinning the latter, that is, the activation of PDGF-independent pathways, remains to be elucidated.

Although the enhancement of angiogenic capability of EPCs by PDGF-D in vitro was observed, it was noted that the in vivo situation may be different. For example, new vessel formation in an adult environment requires the process of neovascularization, which includes not only EPCs formation in the primitive vascular network, but also ECs activation, in vitro observations.

In summary, it was demonstrated in the present study that the newly identified PDGF family member, PDGF-D, boosts the in vitro angiogenic activity of EPCs, such as proliferation, adhesion, migration and tube formation. This also provides evidence that PDGF may exert its positive impact on angiogenesis through both PDGF signaling-dependent and -independent pathways, while detailed relevant networks remain to be deciphered. These findings support the premise that PDGF-D may be potentially used as a therapeutic target for the treatment of ischemic disease.

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