Cardiovascular disease, including atherosclerosis and cardiac fibrosis, is the most common cause of death in the Western world. The development of atherosclerosis involves a series of events, among them vSMC proliferation and migration.1 Cardiac fibrosis, which is a common feature in heart disease, involves a disproportionate accumulation of extracellular matrix between muscle fibers and around blood vessels.2 The mechanisms directing cardiac fibrosis are not completely understood, but it has been suggested that growth factors, cytokines, extracellular matrix–modulating enzymes, and components of the fibrinolytic system may contribute.3

It is well established that platelet-derived growth factors (PDGFs) are involved in several pathological settings, including tissue fibrosis, atherosclerosis, and tumor growth.1,4,5 However, it is not well investigated what role(s) the PDGFs may play in cardiac fibrosis.

PDGFs are a family of disulphide-bonded dimeric isoforms that are generated by 4 genes: the classical PDGF-A and PDGF-B chains and the 2 recently identified PDGF-C and PDGF-D chains.4,5–8 Both PDGF-C and PDGF-D chains display an N-terminal CUB domain in addition to the PDGF/vascular endothelial growth factor (VEGF) homology domain.5,7 The function(s) of the CUB domains in PDGF-C and PDGF-D is not fully known. However, proteolytic removal of the CUB domains by specific proteases is required for biological activity of both factors. PDGF-C is activated by tissue plasminogen activator.9 Recently, urokinase-type plasminogen activator (uPA) was identified as the enzyme that activates PDGF-D.10 All PDGF isoforms exert their biological functions by binding to and activating 2 receptor tyrosine kinases, PDGF receptor (PDGFR)-α and PDGFR-β. The PDGFs stimulate proliferation and direct migration, differentiation, and physiological functions of a variety of mesenchymal cell types. From knockout studies in mice, it is known that PDGF-B and PDGFR-β are essential for the development of support cells in the vasculature, whereas PDGF-A and PDGFR-α are more broadly required during embryogenesis.11

It is not established whether PDGF-C and PDGF-D are involved in cardiovascular disease. In a previous study, we have shown that transgenic overexpression of PDGF-C, a PDGFR-α ligand, induces cardiac fibrosis in mouse heart, followed by hypertrophy or dilated cardiomyopathy and vascular defects.12 PDGF-D is the most recently discovered member of the PDGF family and signals mainly through PDGFR-β,7 but others have also suggested activation of
PDGFR-α via receptor heterodimers. The expression pattern and biological effects of PDGF-D have not yet been studied in detail. Here we analyzed the expression of PDGF-D in the cardiovascular system. To investigate the pathological potential of excess PDGF-D signaling, we generated transgenic mice overexpressing the active growth factor domain (the core domain) of PDGF-D in the heart.

Materials and Methods

Generation of Transgenic Mice
A fragment encoding a signal sequence, the core domain of PDGF-D and human c-Myc epitope, was cloned into a vector containing the heart-specific α-myosin heavy chain promoter. The linearized vector was injected into male pronuclei of fertilized oocytes derived from B6CBA F1/Crl mice.

An expanded Materials and Methods section appears in the online data supplement available at http://circres.ahajournals.org.

Results

PDGF-D Is Expressed in Adult and Developing Tissues
The expression pattern of PDGF-D in the mouse was analyzed by investigating the expression of PDGF-D mRNAs in different tissues. The result showed that PDGF-D was encoded by a major 4.0-kb transcript with abundant expression in heart, kidney, and lung; lower expression in brain, liver, skeletal muscle, and testis; and very weak expression in spleen (Figure 1A). Expression of PDGF-D in adult heart was confirmed by analyzing protein extracts by immunoblotting using a specific rabbit Ig against human full-length PDGF-DD (Figure 1B). A species of 50 kDa, corresponding to full-length PDGF-D, was found in heart but not in spleen. Henceforth, we focused the expression study on the cardiovascular system.

We investigated the expression of PDGF-D in tissue sections from mouse embryos (embryonic day [E] 12.5 to E16.5) and adult heart. We verified the specificity of the staining method using several controls (Figure 1K and online Materials and Methods section). By staining PDGF-C−/−embryos, which completely reproduced the results from the wild-type embryos, we also verified that the anti–PDGF-D Ig did not cross-react with PDGF-C (data not shown). We observed strong expression of PDGF-D in the myocardium at E12.5 (Figure 1C), and this expression continued throughout development (data not shown). PDGF-D was also detected in the adult epicardial and endocardial cardiomyocytes (Figure 1D through 1F). From E13.5 onward, PDGF-D expression was detected in larger blood vessels, such as the lumen of vessels, in liver, skin, lung, and skeletal muscle (Figure 1G and data not shown). By double-immunofluorescence staining, we showed that the expression of PDGF-D in developing blood vessels was restricted to arterial vascular smooth muscle cells (vSMCs) (Figure 1L through 1Q). To investigate whether PDGF-D and PDGFR-β were coexpressed, or expressed in adjacent cells, immunolocalization of PDGFR-β in embryonic and adult tissue was performed. In the heart, PDGFR-β expression was detected mainly in blood vessels (Figure 1H and 1I). Like PDGF-D, PDGFR-β was also expressed by blood vessels lining the vertebra (Figure 1J) and by blood vessels in skin, skeletal muscle, liver, and lung (data not shown). We further demonstrated that PDGF-D and PDGFR-β were coexpressed in the vSMCs of developing arteries (Figure 1R through 1T). These results indicate that PDGF-D may provide an autocrine stimulatory loop in vSMCs and paracrine signals to PDGFR-β expressing cells in surrounding tissues.

Core PDGF-DD Activates PDGFR-β In Vitro
To analyze the biological effects of PDGF-D, without the need for proteolytic activation of the latent full-length protein, a construct containing the core domain of PDGF-D was generated (Figure 2A). Transfected Cos-1 cells expressed core PDGF-D as a 21-kDa species under reducing conditions (Figure 2B). The biological activity of core PDGF-DD was verified by its ability to activate PDGFR-β expressed in PAE cells using PDGF-BB as a positive control (Figure 2C).

PDGF-D Activates PDGFR-β in Heart-Specific Transgenic Mice
To investigate the biological activities of PDGF-DD in vivo, we generated transgenic mice with heart-specific overexpression of the PDGF-D core domain using the α-myosin heavy chain promoter (Figure 2D). Twelve transgenic founders were obtained. The expression of c-Myc–tagged transgenic PDGF-D was confirmed by immunoblotting using rabbit Ig against the human c-Myc epitope and was detected as a 21-kDa species (Figure 2E).

To demonstrate in vivo activity of transgenic PDGF-DD, heart lysates were immunoprecipitated using an antiserum to PDGFR-β and immunoblotted using anti-phosphotyrosine antibodies. Phosphorylation of PDGFR-β was only observed in transgenic hearts (Figure 2F). This suggests that transgenic PDGF-DD is able to stimulate PDGFR-β signaling in vivo.

Transgenic Mice Expressing PDGF-D Develop Cardiac Fibrosis
Transgenic mice displayed grossly enlarged hearts with abnormal atrial regions (Figure 3A). The ventricle wall thickness in these animals was disproportionately reduced to the chamber volume (Figure 3B). There was a variation in the penetration of the phenotype among different founders, which was probably attributable to differences in copy number and integration sites of the transgene. Three founders experienced an early postnatal death caused by heart failure within 5 weeks. Of the remaining founders, 1 female was mated but failed to complete pregnancy. Because of ethical considerations, all remaining transgenic mice were euthanized at the age of 6 to 8 weeks. Consequently, we could not establish a transgenic mouse line.

Histological analyses were performed on age-matched transgenic founders and wild-type littermates. Immunolocalization of c-Myc–tagged PDGF-D confirmed the presence of transgenic PDGF-D in the myocardium (Figure 3C through 3E). In most transgenic mice, expression of PDGF-D was restricted to distinct areas, wherein a strong local accumulation of interstitial fibroblasts was observed (Figure 3D and 3E). This expansion of fibroblasts was confirmed with hematoxylin/eosin staining (Figure 3F through 3H) and re-
sulted in cardiac fibrosis, as shown by collagen-specific staining (Figure 3I through 3K). Excess collagen deposition was also seen around large arteries (tunica adventitia), at sites of PDGF-D expression (Figure 3J). The action of PDGF-D was local, because fibrosis was observed only at sites of transgenic expression. In the 3 founders that died from heart failure, the fibrosis was more widespread (Figure 3K). Proliferation of fibroblasts was shown by immunohistochemical staining for proliferating-cell nuclear antigen (PCNA) (Figure 3L and 3O). These findings suggest that core PDGF-DD is able to initiate cardiac fibrosis.

**Overexpression of PDGF-D Induces Vascular Remodeling**

The expansion of interstitial connective tissue caused a disorganized capillary network, with areas of lower capillary density than normally found in the heart. Endothelial-specific staining (platelet-endothelial cell adhesion molecule-1)
arterial SMCs was shown by PCNA staining (Figure 3L through 3N). Reexpression of SMA in cardiac myocytes, which is normally restricted to the fetal myocardium, was also observed (Figure 4H) (described in the article by Pontén et al.12 and references therein). We demonstrated a 70% upregulation of matrix metalloproteinase (MMP)-9 in the transgenic heart by immunoblot analysis (Figure 4I). MMP-9 activity has been shown to correlate with proliferation/migration of SMCs in arterial remodeling16 and supports our data that PDGF-D induced arterial remodeling. These observations suggest that PDGF-DD is a potent mitogen for vSMCs in vivo.

Comparison Between PDGF-D and PDGF-C Overexpression in Mouse Heart

We compared PDGF-D transgenic hearts with those overexpressing PDGF-C. The phenotypes were similar to a large extent, but, overall, the phenotype was more severe in the PDGF-D transgenic mice.

Visualization of cardiac myofibrils and collagen showed that PDGF-D transgenic mice with a failing heart displayed elongated and slightly dilated myofibers (Figure 5A and 5B), similar to the female PDGF-C transgenic mice that also died from heart failure12 (data not shown). In contrast, male PDGF-C transgenic mice developed a compensatory hypertrophy of myofibers (Figure 5C).12

Both PDGF-C and PDGF-D transgenic mice displayed an altered remodeling of extracellular matrix, shown by the extensive collagen deposition (Figure 5A through 5C). We also confirmed upregulation of collagen-I in transgenic mice by immunoblot analysis (data not shown). We were unable to detect any changes of MMP-9 expression in PDGF-C transgenic hearts (data not shown), in contrast to mice overexpressing PDGF-D (see Figure 4I). This emphasizes the absence of vSMC proliferation and thickening of arterial walls in those animals (Figure 5G).

To demonstrate localization of PDGF receptors, we performed immunohistochemical staining of heart sections (Figure 5H through 5N). In wild-type mice, PDGFR-β expression was detected in blood vessels and in the interstitium, probably corresponding to both cells of microvessels and scattered fibroblasts (Figure 5H). In PDGF-D transgenic mice, PDGFR-β was expressed by vSMCs in enlarged arterial walls (Figure 5I) and in accumulating interstitial fibroblasts (Figure 5J). PDGFR-β was also detected in fibroblasts of PDGF-C transgenic hearts (Figure 5K). PDGFR-α was normally expressed by scattered interstitial fibroblasts and around a small subset of blood vessels (Figure 5L). Similar to PDGFR-β, PDGFR-α was expressed by proliferating fibroblasts in both PDGF-D and PDGF-C transgenic hearts (Figure 5M and 5N). However, PDGFR-α expression was not seen in the enlarged arterial walls of PDGF-D transgenic mice (data not shown).

PDGF-D Stimulates Proliferation of Primary Cardiac Fibroblasts

To demonstrate that proliferation of cardiac interstitial fibroblasts in the transgenic mice was a direct effect of PDGF-DD,
primary cardiac fibroblasts were isolated and stimulated with serum-free conditioned medium containing core PDGF-DD or PDGF-BB (positive control) or serum-free medium alone (negative control) in the presence of 5-bromo-2′-deoxyuridine (BrdUrd). Quantification of BrdUrd-labeled cells (Figure 6A) showed that cells stimulated with PDGF-DD incorporated significantly more BrdUrd than did unstimulated cells, and cells stimulated with PDGF-BB strongly incorporated BrdUrd (Figure 6B). Conditioned medium from mock transfected Cos-1 cells was unable to stimulate PDGFR-α expressed by PAE cells above background (data not shown). We investigated the ability of PDGF-DD to activate PDGFR-α or PDGFR-β expressed by cardiac fibroblasts. Conditioned media from core PDGF-DD–expressing Cos-1 cells or recombinant core PDGF-CC were applied onto the cardiac fibroblasts, and phosphorylation of PDGFR-α (Figure 6C) and PDGFR-β (Figure 6D) were measured. PDGF-DD was able to stimulate PDGFR-β but not PDGFR-α, whereas PDGF-CC was able to stimulate both PDGF receptors. These results verify that PDGF-DD is able to induce proliferation of fibroblasts and that PDGF-DD is a specific PDGFR-β agonist in cardiac fibroblasts.

Activated PDGF-DD Is Upregulated in Apolipoprotein E Knockout Mice

To strengthen the pathological relevance of the transgenic model, we investigated the expression of PDGF-D in hearts from apolipoprotein E knockout (apoE−/−) mice. These animals spontaneously develop atherosclerotic plaques resembling human lesions,19 followed by subsequent hypertension and cardiac hypertrophy.20 Histological analysis of hearts from 6-month-old animals showed progressed lesions of the aortic root (data not shown). Analysis of PDGF-D and uPA (identified as the enzyme that activates PDGF-D10) mRNAs by quantitative PCR showed upregulation of PDGF-D mRNA expression of ~80% in apoE−/− mice compared with wild-type controls (P<0.05) (Figure 7A). The expression of uPA appeared to be higher in apoE−/− mice compared with wild-type animals, although the differences did not reach statistical significance (P=0.11) (Figure 7B). Others have reported upregulation of uPA expression in 20-week-old apoE−/− mice.21 The expression of active PDGF-D was investigated by analyzing protein extracts from the hearts by immunoblotting using an affinity-purified rabbit anti-peptide Ig. Three different PDGF-D species were found both in

Figure 3. Cardiac fibrosis in PDGF-D transgenic mice. A, Hearts from a wild-type (wt) (left) and a transgenic (tg) (right) mouse that died from heart failure. The heart from the transgenic mouse showed an abnormal size/shape, especially in the atrial region. B, Cross-sectioned heart from wild-type (left) and transgenic (right) mice showing dilation of ventricles as compared with chamber volume. C through E, Immunohistochemical staining using antibodies to human c-Myc. Staining was absent in wild-type animals (C). In most transgenic hearts, the expression of PDGF-D was restricted to certain areas at which an expansion of interstitial cells was observed (D and E) (arrows). F through H, Hematoxylin/eosin staining of normal myocardium (F) and accumulation of interstitial fibroblasts in transgenic hearts (G and H). The fibroblast expansion was localized to the area of transgenic expression (arrow). I through K, Masson’s trichrome staining of a wild-type animal (l) compared with a transgenic animal with thickened arterial wall and fibrosis (arrow) locally at site of transgenic PDGF-D expression (J) and another with widespread fibrosis that died from heart failure (K). L through O, PCNA staining. Myocardium with artery from a wild-type mouse (L), proliferation of SMCs (arrows) in arterial walls (M and N), and interstitial fibroblasts (O) in transgenic animals. Scale bars: 1 mm (A); 20 μm (C through O).
apoE−/− and wild-type mice, ≈50, ≈20, and ≈16 kDa, under reducing conditions (Figure 7C). The ≈16-kDa species has been described as the active fragment following uPA-mediated activation.10 Quantification of the amounts of active PDGF-D (≈16-kDa species) showed an upregulation of ≈45% in apoE−/− mice compared with wild-type control (samples normalized to calnexin). The mean relative expression of active PDGF-D (≈16-kDa species) versus full-length PDGF-D was 12% in wild type (range, 9.5% to 16%) and 26% in apoE−/− (range, 15% to 42%). These observations suggest that PDGF-D may have an important role in cardiovascular pathologies involving cardiac fibrosis and hypertrophy.

Discussion

The biological function of the recently identified PDGFR-β agonist PDGF-D remains unclear. To investigate this, we performed an expression analysis in mouse tissues. Based on our findings that PDGF-D is highly expressed in the heart, we decided to focus our study on the cardiovascular system. In embryonic and adult heart, we observed that PDGFR-β was expressed by blood vessels and microvessels, an observation consistent with the previously reported expression of PDGFR-β.22 PDGF-D was expressed in the adjacent myocardium, suggesting a paracrine mode of action of PDGF-D. Interestingly, PDGF-D was also expressed by developing arterial vSMCs and colocalized with PDGFR-β. This indicates that PDGF-D may also provide autocrine signaling in PDGFR-β–expressing cells. PDGF-B, the other PDGFR-β ligand, is restricted to endothelial cells of capillaries and small arteries during mouse development.22 The differences in expression patterns suggest that PDGF-B and PDGF-D provide distinct signals to PDGFR-β–expressing cells. Both PDGF-B and PDGFR-β knockout embryos fail to recruit vSMCs and pericytes and die from vascular bleedings.11 Because of the similar phenotypes of those 2 knockouts, it is difficult to argue for a critical role of PDGF-D during mouse development. However, previous observations in PDGF-B deficient embryos indicate that vSMC/pericyte progenitors were properly recruited to several arteries and to microvessels in some organs, such as skeletal muscle and skin.22 This is interesting, as high PDGF-D expression was observed at these sites (E.F.B., A.P., U.E., unpublished observations, 2005).

To investigate the pathological consequences induced by excess PDGFR-β signaling, we overexpressed the core domain of PDGF-D in mouse heart. PDGF-D induced proliferation of interstitial fibroblasts, leading to extensive deposition of collagen. This caused a progressive dilation of the ventricles compared with chamber volume, leading to heart failure and early postnatal death. The phenotype observed in PDGF-D core transgenic mice resembled the previously reported animals overexpressing full-length PDGF-C in the heart, as well as those phenotypes described for human hypertrophy and dilated cardiomyopathy (described in the article by Pontén et al12 and references therein). Cardiac fibrosis generally develops as a response to cardiac hypertrophy. Eventually, a transition from hypertrophy to dilation occurs, and this decompensated state leads to heart failure, as seen in the PDGF-D transgenic mice.
We also demonstrated that PDGF-DD is a potent mitogen for primary cardiac fibroblasts and that PDGF-DD was able to specifically stimulate PDGFR-\(\beta\)-bearing vSMCs. Others have reported that PDGF-DD induces migration/proliferation of primary fibroblasts from rat.\(^{23}\) PDGF-D transgenic mice displayed vascular remodeling, including vessel dilation, locally decreased capillary density, and increased number of SMC-coated vessels, suggesting arteriolarization of microvessels. In addition, proliferation of arterial vSMCs caused thickening of the vessel wall. Similar vascular changes were observed in the PDGF-C transgenic mice,\(^ {12}\) with the exception of vSMC proliferation, which was found exclusively in hearts overexpressing PDGF-D. We also detected increased levels of MMP-9 in PDGF-D transgenic mice, indicating arterial remodeling. The results suggest that PDGF-DD is a potent in vivo mitogen for PDGFR-\(\beta\)-bearing vSMCs. Our data are supported by in vitro studies showing that PDGF-DD promotes vSMC proliferation/survival.\(^ {23,24}\) The absence of vSMC proliferation in PDGF-C transgenic mice was supported by the observation that whereas PDGFR-\(\beta\) was found around both arteries and microvessels, PDGFR-\(\alpha\) was found only around a small subset of vessels. It has been described that PDGF-CC mainly stimulates PDGFR-\(\alpha\) but can activate PDGFR-\(\beta\) via \(\alpha\beta\) heterodimers.\(^ {6,25}\)

Cardiac-specific overexpression of PDGF-A and PDGF-B has also been studied (P. Lindblom, C. Bondjers, and C. Betsholtz, personal communication, 2005). PDGF-A transgenic mice develop severe fibrosis with early lethality, whereas overexpression of PDGF-B gives rise to a milder nonlethal phenotype, with focal fibrosis and no obvious vascular changes. This was unexpected, because PDGF-B is considered to be one of the most potent mitogens for mesenchymal cells in human disease.\(^5\) Compared with other growth factors that have been overexpressed in the mouse, at least PDGF-A, -C, and -D are more potent to induce cardiac fibrosis. For example, insulin-like growth factor-I induced a nonlethal hypertrophic response followed by fibrosis;\(^ {26}\) fibroblast growth factor-2 induced more blood vessels but no fibrosis;\(^ {27}\) and transforming growth factor-\(\beta\) promoted atrial, but not ventricular, fibrosis.\(^ {28}\) Whereas tumor necrosis factor-\(\alpha\) induced a severe inflammatory response but not fibrosis.\(^ {29}\) Taken together, these results suggest that PDGFs may play an important role in cardiac fibrogenesis and left ventricle remodeling. Furthermore, in a recent study,
PDGF-D has been reported to induce a severe mesangial proliferative glomerulopathy in mice.\textsuperscript{17} Based on our results showing that PDGF-DD strongly induces proliferation of vSMCs, leading to thickening of the vessel wall, it appears likely that this growth factor may also play an important role in other vascular diseases, such as atherosclerosis. This hypothesis is supported by our observation that PDGF-D expression is upregulated in apoE\textsuperscript{−/−} mice.\textsuperscript{23} There is a strong link between PDGF activity and atherosclerosis, where vSMCs accumulate in the subendothelial zone of larger vessels.\textsuperscript{1} In particular, PDGFR-β expression and activation is increased in atherosclerotic lesions, which is of interest because PDGF-DD is a PDGFR-β-selective ligand.\textsuperscript{7} In addition, we showed an increased amount of active PDGF-DD in apoE\textsuperscript{−/−} mice. This is noteworthy because uPA recently has been described as the enzyme that activates latent PDGF-DD.\textsuperscript{10}

**Figure 6.** PDGF-D stimulates proliferation of primary cardiac fibroblasts. A, Microphotographs showing BrdUrd (BrdU) incorporation of cardiac fibroblasts (in red) that were untreated, stimulated with conditioned media containing core PDGF-DD, or stimulated with PDGF-BB. Cell nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI) (blue). B, Quantification of cells incorporating BrdUrd showing that PDGF-DD stimulated cardiac fibroblasts to incorporate more BrdUrd compared with control cells (\(*P<0.01\)). C, PDGF-C, but not PDGF-D, was able to induce tyrosine phosphorylation of PDGFR-α expressed by cardiac fibroblasts (top). The amount of precipitated PDGFR-α was monitored using antibodies to PDGFR-α (bottom). D, Both PDGF-C and PDGF-D were able to induce tyrosine phosphorylation of PDGFR-β expressed by cardiac fibroblasts (top). The amount of precipitated PDGFR-β was monitored using antibodies to PDGFR-β (bottom). IP:R indicates immunoprecipitation of PDGFR (α or β).
has been reported that uPA is upregulated in apoE-/− mice,21 as well as in human atherosclerotic lesions.30 Furthermore, uPA has been shown to induce cardiac fibrosis in transgenic mice31 and accelerate the progression of atherosclerosis in apoE-/− mice.32 Interestingly, uPA-deficient mice were shown to develop less cardiac hypertrophy and fibrosis at pressure overload compared with wild-type animals.33

In conclusion, we have shown that PDGF-D is expressed in the cardiovascular system and may provide both autocrine and paracrine signaling through PDGFR-β. Furthermore, we demonstrate that PDGF-DD is a potent mitogen for fibroblasts and vSMCs in transgenic mice. In addition, we show enhanced expression and activation of PDGF-DD in a mouse model of cardiovascular disease. Our results provide novel insight into the functional significance of PDGF-D and suggest a role for PDGF-DD in vascular development as well as in cardiovascular and fibrotic diseases.

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PDGF-D induces cardiac fibrosis and proliferation of vascular smooth muscle cells in heart-specific transgenic mice
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Materials and Methods

Northern blot analysis

A 363-bp PCR fragment (primers: forward-5’-GGCAGGTCATAACCATGAT-3’ and reverse-5’-TCGAGGTGTCCTTGGAGCT-3’) corresponding to part of the mouse PDGF-D cDNA was generated in the presence of $^{32}$P dCTP (3000 Ci/mmol, Amersham Biosciences). The probe was used to hybridize a mouse multiple tissue Northern blot according to the manufacturer (BD Biosciences).

Immunoblot analysis

Total protein (20-50 µg) from mouse tissue extracts (wild-type, core PDGF-D transgenic or apoE/- mice (n=3) was subjected to SDS-PAGE, and immunoblotted using an affinity-purified rabbit Ig to human full-length PDGF-DD (1 µg/ml), an affinity-purified rabbit Ig against a synthetic peptide derived from the mouse PDGF-D sequence (amino acid residues 254-272, CRKSKVLDRLNDDAKRYS) (1 µg/ml), a rabbit Ig against human c-Myc epitope (1/500, Santa Cruz), a rabbit serum against mouse
collagen-1 (1/400, Calbiochem), a goat polyclonal Ig against mouse MMP-9 (1 µg/ml, R&D systems), and a rabbit serum against human calnexin (a kind gift from E. Raschperger).

**Generation of heart-specific transgenic mice**

Human PDGF-D cDNA was subjected to PCR (primers: forward-5’-GATAGTCGACGCCACCATGGAGACAGACACA-3’ and reverse-5’-GATAGTCGACTTACAGATCCTCTTCTGAGAT-3’). The product, encoding the core domain of PDGF-D (amino acid residues 248-370) was cloned into a pSecTag2 vector (modified by removal of the sequence between Sfi I and Kpn I). The resulting construct, pSecTag2-corePDGF-D, was again subjected to PCR (primers: forward-5’-GATAGTCGACGCCACCATGGAGACAGACACA-3’ and reverse-5’-GATAGTCGACTTACAGATCCTCTTCTGAGAT-3’). The generated fragment, encoding a signal sequence, core domain of PDGF-D and human c-Myc epitope was cloned into a vector containing the α-myosin heavy chain (α-MHC) promoter\(^1\). Linearized vector was injected into male pronuclei of fertilized oocytes derived from B6CBA F1/Crl mice (Karolinska Center for Transgene Technologies, Karolinska Institute, Stockholm). Twelve PDGF-D transgenic founders were obtained, five males and seven females. Transgenic mice with cardiac-specific over-expression of full-length PDGF-C were previously generated\(^2\). The Animal Welfare Agency in Sweden approved the animal experiments in this study.
**In vitro** expression of PDGF-D

Cos-1 cells were transfected with the pSecTag2-corePDGF-D vector. Recombinant His-tagged core PDGF-DD protein was purified from conditioned serum-free medium using Ni-NTA-agarose (Qiagen), and analyzed by SDS-PAGE, and stained by Coomassie brilliant blue.

**PDGF receptor analysis**

PAE cells over-expressing PDGF receptors were stimulated with PDGF-BB (20 ng/ml), or core PDGF-DD (20 ng/ml), and primary cardiac fibroblasts were stimulated with conditioned media from Cos-1 cells transfected with the pSecTag2-corePDGF-D vector, or core PDGF-CC (300 ng/ml), and analyzed for PDGF receptor activation as previously described\(^3\). Similarly, hearts were analysed for PDGF receptor activation as described\(^4\). The amounts of precipitated PDGFR-\(\alpha\), or PDGFR-\(\beta\) were monitored using a goat anti-mouse PDGFR-\(\alpha\) or PDGFR-\(\beta\) antibody (R&D systems).

**Histology and immunostaining**

Paraffin-sections (6 \(\mu\)m) were prepared. Hematoxylin-eosin (H&E), or Masson’s tricrome staining was performed using standard procedures. Phosphotungstic acid hematoxylin (PTAH) staining was performed according to the manufacturer (Bio Optica).

Immunohistochemical staining of PDGF-D was performed using the Ig against human full-length PDGF-DD (5 \(\mu\)g Ig/ml). Antigen retrieval was obtained by heating the tissue slides in 0.01 M citrate buffer, pH 6.0, at 95°C for 20 min. PDGF-D staining on embryonic tissue was performed as above but without antigen retrieval. To verify the
specificity of the method, control sections were incubated with anti-PDGF-D Ig, preincubated with a ten-fold molar excess of full-length PDGF-DD, or with rabbit pre-immune serum (1/500), or with rabbit IgG (5 or 10 µg/ml). In all cases only background stainings was observed. As another specificity control, sections from PDGF-C -/- embryos\(^5\) were stained.

Transgenic PDGF-D in the hearts was detected by using a rabbit polyclonal Ig against the human c-Myc epitope (1/500, Santa Cruz Biotechnology).

Endothelial-specific staining was performed using TSA indirect (NEN Life Science) with a rat anti-mouse CD31/platelet-endothelial cell adhesion molecule (PECAM-1) antibody (1/500, BD Biosciences). Smooth muscle actin staining was performed using a monoclonal anti-human \(\alpha\)-smooth muscle actin antibody 1A4 (1/500, DAKO). Antigen retrieval was obtained as above. VEGF staining was performed using a rabbit polyclonal anti-VEGF antibody A 20 (1/100, Santa Cruz Biotechnology). Antigen retrieval was obtained as above. Staining for proliferating cell nuclear antigen (PCNA) was performed using a monoclonal anti-human PCNA antibody (1/1000, Chemicon). PDGFR-\(\beta\) specific staining was performed using a goat polyclonal antibody (2 µg/ml, R&D systems). Antigen retrieval was obtained as above and by trypsin digestion (0.25 mg/ml, 37°C, 20 min). PDGFR-\(\alpha\) specific staining was performed using a goat polyclonal antibody (5 µg/ml, R&D systems). Antigen retrieval was obtained as for PDGFR-\(\beta\). Staining of macrophages/monocytes was performed using a rabbit polyclonal antibody F4/80 (1/300, Serotech). Antigen retrieval was obtained by trypsin digestion (see above). For smooth muscle actin, VEGF, PCNA c-Myc, PDGFR-\(\beta\), PDGFR-\(\alpha\) and macrophage stainings, Elite ABC Vectastain (Vector laboratories) was used. In all
experiments listed above, blocking of non-specific binding was performed using TNB blocking buffer (TSA indirect, NEN Life Science), supplemented with 20% serum (from the same species as the secondary biotinylated antibody).

For statistical analysis of SMA-positive vessel density, 7 wild-type and 8 transgenic animals were used. The means for 10 visual fields in left ventricle, at 40x magnification, were calculated for each mouse and used for Students t-test.

Frozen sections (20 µm) from mouse embryos (E17.5) were post-fixed with 4% paraformaldehyde and permeabilized with 1 % Triton X-100 in PBS. PDGF-D specific staining combined with endothelial, smooth muscle cell-, or PDGFR-β was performed by incubating the tissue in TNB blocking buffer supplemented with 10% horse or goat serum, and then incubated with primary antibodies: an affinity purified rabbit fragment to human PDGF-D peptide, corresponding to a part of the core domain (10 µg/ml), rat anti-mouse PECAM-1 (see above), or goat anti-mouse PDGFR-β (see above). Secondary antibodies: anti-rabbit-Alexa 594/Alexa 488 (1/200-1/1000, Molecular probes), anti-rat-Alexa 594 (1/200, Molecular probes), anti-goat-Alexa 488 (1/200, Molecular probes), and anti-SMA-FITC antibody (1/100, Sigma). Bound antibodies were detected using a fluorescence microscope (ZEISS Axiophot).

**Isolation of primary cardiac fibroblasts and proliferation analysis.**

Primary cardiac fibroblasts from C57/Bl6 mouse hearts were isolated essentially as described for the isolation of kidney fibroblasts. Fibroblasts, at passages 2-6, were stimulated with conditioned media from Cos-1 cells transfected with the pSecTag2-
corePDGF-D vector, or with 50 ng/ml of PDGF-BB. BrdU incorporation was measured as described\(^6\). Students t-test was used for statistical analysis of the data.

**Transcript analysis by quantitative PCR**

Six months old female apoE\(^--\) and wild type mice on C57BL/6 background were used. The mice were fed a normal chow diet. The hearts were excised and snap frozen in liquid nitrogen (n=4 in each group). Subsequently, the heart tissue was ground into a powder and 10 mg from each heart was put into RLT buffer (Rneasy, Qiagen) and homogenized. Following purification of total RNA according to the manufacturer’s instructions for heart tissue, 1 \(\mu\)g of total RNA was reverse transcribed into cDNA using iScript (Bio-Rad). Quantitative PCR was performed on a Corbett Research RG-3000A with primers to mouse PDGF-D (forward-5’-GGCAGGTCATACCATGAT-3’; reverse-5’TGCAGGTGGTGCTTCTAGCT), mouse uPA (forward-5’-GCATGCTGACTGCTCCTTC-3’; reverse-5’-GTAGGCCAGGCTGTCTTCCC -3’), and the housekeeping gene GAPDH (forward-5’-TGTTATCTGGAAGGACTCATGAC-3’; reverse-5’-ATGCCAGTGAGCTTCCCGTTCAGC -3’). Data are expressed as % expression of GAPDH. Mann-Whitney U-test was used for statistical analysis of the data.
References


