PDGF-D, a novel member of the platelet-derived growth factor family

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Academic dissertation

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To my Mother and Father

What is a scientist after all?
It is a curious man looking through a keyhole,
the keyhole of nature, trying to know what's going on.
-Jacques Cousteau-

"This is the strangest life I have ever known"
-Jim Morrison-

Hey-Ho, Let's Go
-The Ramones-

CONTENTS

ABBREVIATIONS 6

LIST OF ORIGINAL PUBLICATIONS 7

ABSTRACT 8

REVIEW OF THE LITERATURE 9

THE DEVELOPMENT OF BLOOD VESSELS 9

Vasculogenesis and angiogenesis 9
Endothelial cells 11
Pericytes and vascular smooth muscle cells 11

VASCULAR ENDOTHELIAL GROWTH FACTORS AND THEIR RECEPTORS 12

VEGF 13
VEGF-B 13
VEGF-C and VEGF-D 13
PlGF 13
VEGF-E 14
VEGFR-1 14
VEGFR-2 14
VEGFR-3 15

PLATELET-DERIVED GROWTH FACTORS 15

PDGF-A 16
PDGF-B 16
PDGF-C 16
Platelet-derived growth factor receptors 17
PDGFR-α 17
PDGFR-β 17
PDGFs in development 18
PDGFs in disease and treatment 19
PDGFs in tumors and tumor therapy 19
PDGFs in cardiovascular disease 19

AIMS OF THE STUDY 21

MATERIALS AND METHODS 22

RESULTS AND DISCUSSION 25

1. The cloning and characterization of human PDGF-D (I, IV) 25
2. The structure, chromosomal location and expression patterns of human *PDGFD* gene (I, II) 29
3. Overexpression of PDGF-D induces macrophage accumulation in normal skin and wound healing and increases the interstitial fluid pressure (III) 31
4. PDGF-D in blood vessel maturation during angiogenesis (III) 33

CONCLUDING REMARKS 35

ACKNOWLEDGEMENTS 36

REFERENCES 38

ABBREVIATIONS

AAV	Adeno associated virus
CUB	Clr/Cls, Urchin EGF-like protein and Bone morphogenic protein 1
E	embyonic day
EC	endothelial cell
ECM	extracellular matrix
Ig	immunoglobulin
IFP	interstitial fluid pressure
IHC	immunohistochemistry
K14	keratin 14
kb	kilo base pair
kDa	kilodalton
LYVE-1	lymphatic vessel endothelial hyaluronan receptor-1
mAb	monoclonal antibody
mRNA	messenger ribonucleid acid
PC	pericyte
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PECAM-1	platelet endothelial cell adhesion molecule-1
PlGF	placenta growth factor
SMA	smooth muscle actin
SMC	smooth muscle cell
TK	tyrosine kinase
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VSMC	vascular smooth muscle cell

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.

I Bergsten, E. *, **Uutela, M**. *, Li, X., Pietras, K., Östman, A., Heldin, C.-H., Alitalo, K., and Eriksson, U. PDGF-D is a specific and protease-activated ligand for the PDGF beta–receptor. *Nature Cell Biology* **3**(5):512-516 (2001).

II **Uutela, M.**, Laurén, J., Bergsten, E., Li, X., Horelli-Kuitunen, N., Eriksson, U. and Alitalo K. Chromosomal location, exon structure and vascular expression patterns of the human *PDGFC* and *PDGFD* genes. *Circulation* **103**: 2242 – 2247 (2001).

III **Uutela, M.**, Wirzenius, M.*, Paavonen, K.*, Rajantie, I., He, Y., Karpanen, T., Lohela, M., Wiig, H., Salven, P., Pajusola, K., Eriksson U. and Alitalo, K. PDGF-D induces macrophage recruitment, increased interstitial pressure and blood vessel maturation during angiogenesis. *Blood.* 104: 3198 - 3204 (2004).

IV **Uutela M.**, Jeltsch, M.*, Kunnapuu J.*, Hellberg C., Heldin C.H., Eriksson U. and Alitalo K. Proteolytically processed PDGF-D binds to both PDGF receptors. Manuscript.

* Equal contribution

ABSTRACT

Over the last decade or so, different angiogenic factors have been studied intensively. Several factors have been associated with cancer growth and vascular diseases, such as atherosclerosis. One of the most researched and important families of growth factors has been the PDGF/VEGF family. PDGFs are major mitogens for several cell types of mesenchymal origin, such as smooth muscle cells and fibroblasts. In vascular formation during vasculogenesis and angiogenesis, pericytes and vascular smooth muscle cells form a supporting structure around endothelial cell tubes to complete the formation of functional blood vessels. Without these cells the nacent vessels are leaky and vessels of both increased and decreased diameter are seen. PDGFs have been identified as some of the most important mitogens and chemoattractans to pericytes and vascular smooth muscle cells. For decades, only two chains of PDGF were known, PDGF-A and PDGF-B and only a few years ago two additional members of this family were discovered and named PDGF-C and PDGF-D.

This study presents the cloning and characterization one of these novel members of this family, PDGF-D. We have defined its receptor binding specifity, finding that it binds both PDGF receptors, α and β. We have also elucidated its domain structure, discovering that it and its closest relative PDGF-C have a CUB domain at the N-terminal end of the propeptide, a domain that is lacking from the two other members of the PDGF family. We have also solved the processing of PDGF-D, from a latent propeptide tothe active protein, which requires proteolytical removal of the CUB domain. We found out that PDGF-D is expressed in several normal and tumor tissues. Finally, we have used mouse models to find out that when overexpressed, PDGF-D is a strong recruiter of macrophages to tissues or wounded areas. We were able to use PDGF-D in combination with a known angiogenic factor, VEGF-E, to grow better functioning blood vasculature in mice.

This study addresses the basic questions concerning the nature of PDGF-D, and gives clues about its probable role in pathological conditions, such as atherosclerosis and wound healing. Our studies also suggest possible future uses of PDGF-D in e.g. wound healing therapy or for growing of new, functional blood vessels to ischemic tissues.

REVIEW OF THE LITERATURE

THE DEVELOPMENT OF BLOOD VESSELS

Vasculogenesis and angiogenesis

The vascular system provides oxygen and nutrients to all proliferating or developing tissues. During embryogenesis, the development of the vascular system occurs via two processes, vasculogenesis and angiogenesis (Figure 1). Vasculogenesis involves the differentiation of endothelial cells (ECs) from mesoderm derived precursor cells, the hemangioblasts (Risau and Flamme 1995). From these are derived the EC precursors (angioblasts) and the hematopoietic cell precursors. The hemangioblasts form primary blood islands, in which the cells are divided into interior and peripheral layers. Cells in the interior layer differentiate into hematopoietic stem cells and the cells in the periphery differentiate into angioblasts, which then cluster and reorganize to form capillary-like tubes (Asahara et al., 1997).

After the primary vascular plexus is formed, new capillaries form by sprouting or by splitting (intussusception) from pre-existing vessels. This process is called angiogenesis (Figure 1)(Risau, 1997). Further remodelling occurs when new vessels mature and form vessels of different sizes. Some vessels fuse to form larger ones while some regress. In the primary capillary plexus the ECs start to differentiate into arterial or venous type (Yancopoulos et al., 1998). Layers of pericytes and vascular smooth muscle cells (VSMCs) start to gather around newly formed EC tubes. Basal laminae form between the cells and provide further support to the new vessels. In pathological angiogenesis, the maturation and stabilization of the vessels occur improperly and the vessels remain immature (Hashizume 2000; Shunichi 2002). Angiogenesis is tightly regulated by intercellular signalling mechanisms, growth factors and cytokines. For some time now, the induction of angiogenesis has also been seen as a way of treating tissue ischemia (reviewed in Ferrara et al., 2003).

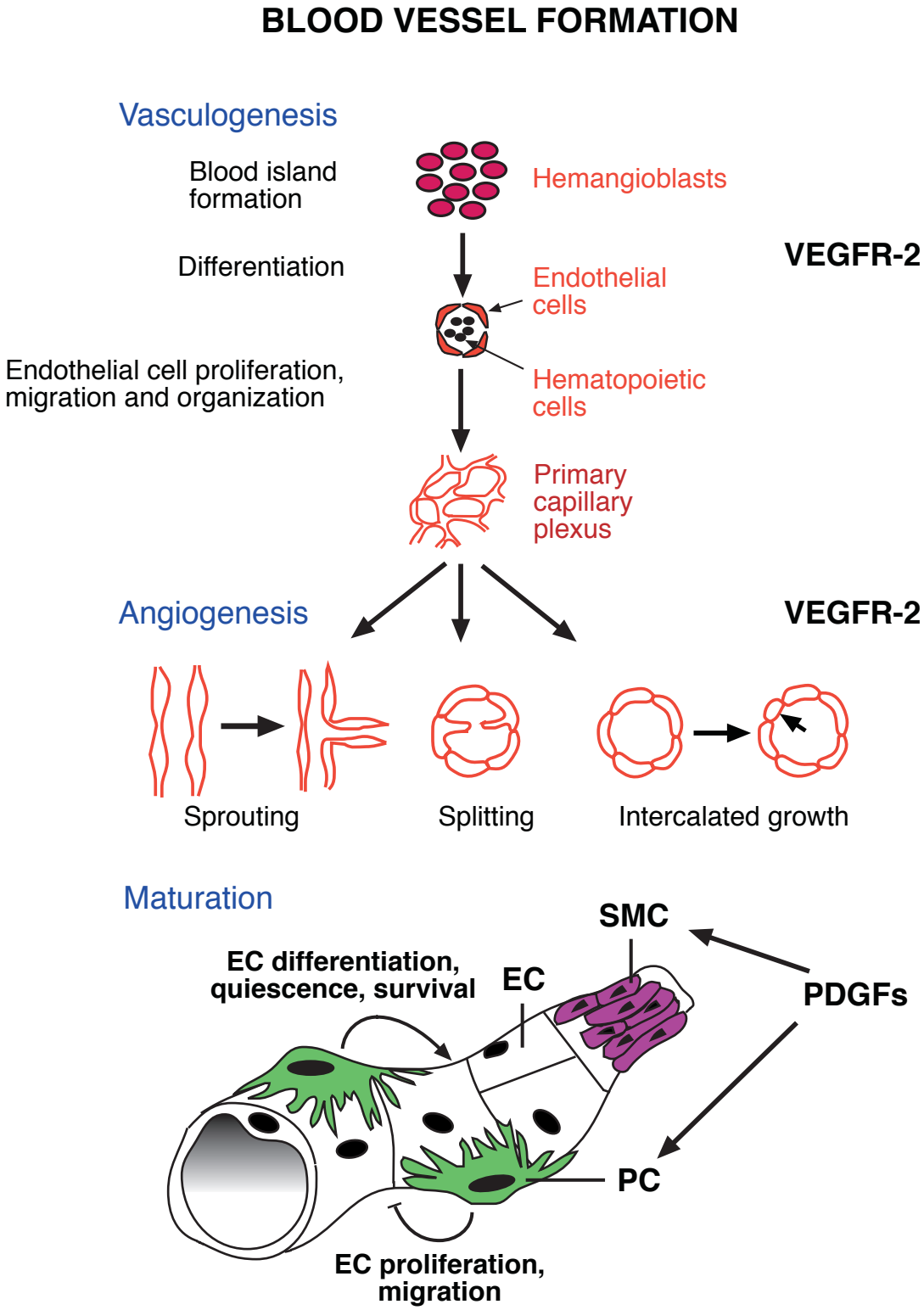

Figure 1. Schematic illustration of vasculogenic and angiogenic processes in developing embryos. In vasculogenesis, mesodermal cells first differentiate into hemangioblasts, whereafter endoderm-mesoderm interactions are required for further blood island differentiation (Risau and Flamme, 1995). After the formation

of the primary capillary plexus, new vessels are generated via angiogenesis (Risau, 1997). During sprouting angiogenesis, ECs degrade the underlying basement membrane, migrate, proliferate and reassemble into tubes. In non-sprouting angiogenesis, new vessels are formed by intussusceptive growth or the existing vessels increase in size through intercalated growth. The formed vasculature is remodeled into a tree-like hierarchy, containing vessels of different sizes. When excess branches are pruned, some vessels regress and others fuse to form larger vessels. The vessels further mature by recruitment of pericytes and smooth muscle cells. Formation of the extracellular matrix and particularly the basal lamina gives support to the vessels. The most important growth factors or their receptors mediating blood vessel growth and maturation are indicated on the right. **EC**= endothelial cell, **PC**= pericyte, **SMC**= smooth muscle cell. Modified from (Saaristo et al., 2002) and (Carmeliet and Collen, 2000).

Endothelial cells

Endothelial cells are very diverse in their morphology, function and gene-expression profiles. Morphologically, they differ in size, shape, thickness, number of microvilli, and the position of the nucleus. For example, aortic endothelial cells are generally thicker but cover a smaller area than those lining the pulmonary artery (Conway and Carmeliet, 2004). ECs function in a variety of physiological situations, and therefore the capillary endothelium of each individual normal tissue is highly specialized. There are also results which show some specific EC markers that are present only in tumor vasculature (Arap et al., 1998).

Pericytes and vascular smooth muscle cells

Pericytes and vascular smooth muscle cells (VSCMs) form a supporting structure around EC tubes to complete the formation of functional blood vessels. Without these cells the forming vessels are leaky and vessel diameter varies extensively (Hellstrom et al., 2001). In the mature vascular system, arteries are surrounded by VSMCs but smallest capillaries are only partially covered by pericytes. Vessels of intermediate size, arterioles and venules, have cells that have properties of both pericytes and VSMCs.

Pericytes and VSMCs originate from multiple sources including mesenchymal cells, neural crest cells and epicardial cells (Hungerford and Little, 1999). In embryonic mesodermal cells they have common progenitors with the ECs (Flk-1 positive embryonic stem cells), which can differentiate into ECs in presence of vascular endothelial factor (VEGF) or into VSMCs in the presence of platelet-derived growth factor B (PDGF-B) (Yamashita et al., 2000). Pericytes and VSMCs are closely related and they can interconvert into each other (Nehls and Drenckhahn, 1993; Nicosia and Villaschi, 1995). There are however, some major differences between the two. Pericytes are adjacent to and surround the endothelium, share a common basement membrane with the endothelium, and have gap-junction connections with the endothelial cells. VSMCs also surround ECs, but they do not share the basal layer with them and do not have direct connection with ECs as pericytes do.

The location of pericytes and VSMCs at the interface between the endothelium and the surrounding tissue makes them ideally positioned to take an active part in the

angiogenic process (Allt and Lawrenson, 2001). In many diseases involving angiogenesis, such as tumour growth and diabetic retinopathy, inhibition of pathological neovascularization is one possible treatment and recent results now indicate pericytes as possible antiangiogenic targets (Benjamin et al., 1999; Gee et al., 2003). On the other hand, pericytes and VSMCs are also coming into focus in proangiogenic therapies, especially when there is a need to stabilize newly formed vessels after successful neovascularization (Cao et al., 2003; Dor et al., 2002; Richardson et al., 2001).

VASCULAR ENDOTHELIAL GROWTH FACTORS AND THEIR RECEPTORS

The VEGF family of growth factors consists of five polypetides (VEGF-A, -B, -C, -D and placenta growth factor, PlGF) which are able to bind variably to vascular endothelial growth factor receptors (VEGFR) 1, 2 and 3.

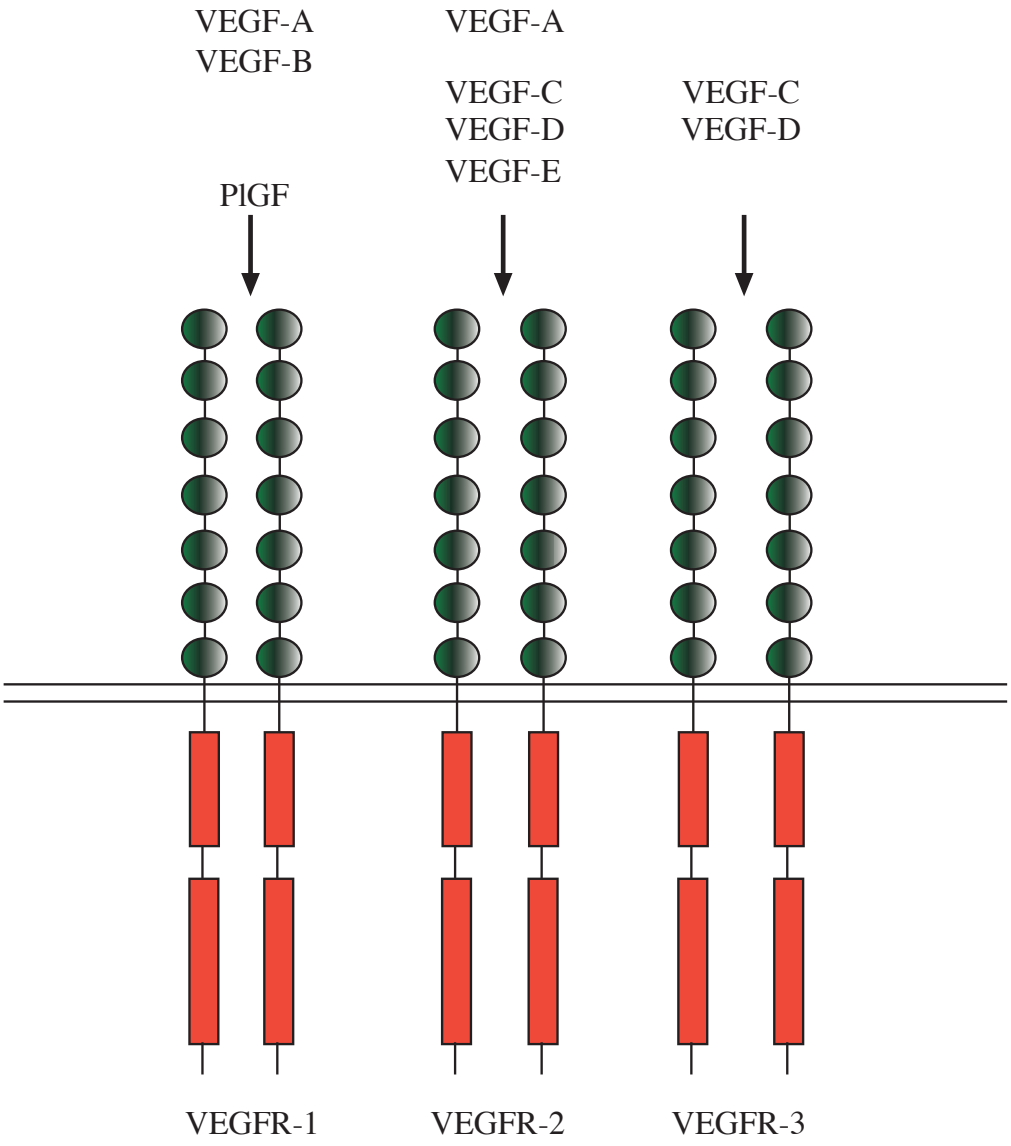

Figure 2. Receptor binding of VEGF family members. VEGFR-1 and VEGFR-2 are predominantly expressed in the endothelium of blood vessels, and VEGFR-3 in the endothelium of lymphatic vessels.

VEGF

VEGF (or VEGF-A) is a major regulator of vasculogenesis and angiogenesis and binds to and activates VEGFR-1 and VEGFR–2. VEGF is also known to induce vascular permeability (Senger et al., 1983) (Dvorak et al., 1995). VEGF is expressed as several isoforms of different amino acid chain lengths ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$)(Ferrara et al., 2003). Of these, $VEGF_{165}$ seems to be most efficent inductor of angiogenesis (Stalmans et al., 2002).VEGF is a highly specific mitogen for vascular ECs (Connolly et al., 1989; Ferrara and Davis-Smyth, 1997; Ferrara and Henzel, 1989; Leung et al., 1989; Plouet et al., 1989) and it is also a survival factor for ECs, both *in vitro* and *in vivo* (Alon et al., 1995; Gerber et al., 1998). Pericyte coverage of the new vessels has been proposed to be one of the critical factors that determine the sensitivity of ECs to VEGF (Benjamin et al., 1999). VEGF has also an effect on the blood cells; it can promote monocyte chemotaxis and induce colony formation by mature granulocyte-macrophage progenitor cells (Clauss et al., 1990)(Broxmeyer et al., 1995). In the ischemic brain VEGF exerts an acute neuroprotective effect, as well as longer latency effects on the survival of new neurons (Sun et al., 2003).

VEGF-B

VEGF-B has two, differentially spliced isoforms, $VEGF\text{-}B_{167}$ and $VEGF_{186}$. The more abundant *in vivo* form is $VEGF\text{-}B_{167}$ (Li and Eriksson, 2001; Olofsson et al., 1996). Its highest expression levels are found in brown fat, skeletal muscle and the myocardium (Olofsson et al., 1999). The only VEGFR that VEGF-B activates is VEGFR-1 (Olofsson et al., 1998). The function of VEGF-B has long been unclear, and although it is abundant in some tumor types (Salven et al., 1998) there has been no conclusive evidence of its effects.

VEGF-C and VEGF-D

VEGF-C and VEGF-D are the two members of the VEGF family which are capable of inducing the proliferation and migration of lymphatic endothelial cells *in vitro* and growth of new lymphatic vessels *in viv*o (Achen et al., 1998; Jeltsch et al., 1997; Joukov et al., 1996; Makinen et al., 2001). Unprocessed VEGF-C binds to VEGFR-3 but only the fully processed form can bind to and activate VEGFR-2 (Joukov et al., 1997). Simnilarly, human VEGF-D can bind to and activate both VEGFR-2 and VEGFR-3 when fully processed (Achen et al., 1998). At high concentrations these fully processed factors can promote angiogenesis and increase vascular permeability, although not as much as VEGF-A (Joukov et al., 1997; Oh et al., 1997; Witzenbichler et al., 1998).

PlGF

Placenta growth factor (PlGF) has three isoforms, PlGF-1, –2 and –3; all bind specifically to VEGFR-1 (Cao et al., 1997; Maglione et al., 1991; Maglione et al., 1993;

Park et al., 1994). PlGF can also form heterodimers with VEGF, and the VEGF/PlGF heterodimers have been shown to bind to VEGFR-2 (DiSalvo et al., 1995). PlGF homodimers are chemotactic for monocytes and ECs *in vitro* (Clauss et al., 1996) and PlGF is angiogenic and arteriogenic *in vivo* (Pipp et al., 2003). PlGF may induce myocardial revascularization by amplifying the angiogenic activity of VEGF, which is upregulated in the ischemic myocardium. In addition, PlGF may stimulate myocardial angiogenesis by increasing VEGF expression by fibroblasts, which are abundant in the myocardial stroma (Luttun et al., 2002).

VEGF-E

VEGF-E is sometimes included as a sixth member of the VEGF protein family. It is a viral protein discovered from the genome of the Orf virus (OV), which is an epitheliotropic parapoxvirus of cattle and sheep (Cottone et al., 1998; Lyttle et al., 1994). Twenty four different forms of VEGF-E are known so far (Mercer et al., 2002). Of these VEGF-E $_{NZ-2}$, VEGF-E $_{NZ-7}$ and VEGF-E$_{D170I}$ are the best characterized. These three forms all bind to VEGFR-2, all with essentially the same activity (Lyttle et al., 1994; Meyer et al., 1999; Wise et al., 1999). Of these forms VEGF-E $_{NZ-7}$ has been most used in research so far. Overexpression of this factor induces strong angiogenesis, although the quality of the newly made blood vessels is poor (Kiba et al., 2003) (III).

VEGFR-1

VEGFR-1 has seven immunoglobulin (Ig) homology domains in the extracellular part, a trans-membrane region and an intracellular tyrosine kinase (TK) domain, which is interrupted by a kinase-insert domain (Matthews et al., 1991; Shibuya et al., 1990; Terman et al., 1991). It is present mostly in ECs and binds VEGF, PlGF (Park et al., 1994) and VEGF-B (Olofsson et al., 1998). An alternatively spliced form producing soluble form of VEGFR-1 has been shown to inhibit VEGF activity (Kendall and Thomas, 1993). The precise function of VEGFR-1 is still debated, but it has been implicated in negative regulation of VEGF activity (Fong et al., 1999). VEGFR-1 phosphorylation is very weak *in vitro* after VEGF stimulation (Seetharam et al., 1995; Waltenberger et al., 1994), and high concentrations of PlGF that saturate the VEGFR-1 sites for binding, have been shown to potentiate the activity of VEGF both *in vivo* and *in vitro*, suggesting that VEGFR-1 may act as a decoy receptor for VEGF in the ECs, not primarily transmitting a mitogenic signal, but rather binding VEGF to make it less available for VEGFR-2 (Park et al., 1994). However, other results have indicated that VEGFR-1 is able to interact with various signal-transducing proteins and generate a mitogenic signal (Landgren et al., 1998; Zeng et al., 2001). In addition to ECs, VEGFR-1 is expressed in monocytes, macrophages, pericytes, placental trophoblasts, renal mesangial cells and in some bone marrow derived hematopoietic stem cells (Barleon et al., 1994; Clauss et al., 1996; Hattori et al., 2002; Ziegler et al., 1993).

VEGFR-2

The structure of VEGFR-2 is similar to VEGFR-1, but VEGFR-2 binds VEGF, VEGF-C, VEGF-D and viral VEGF-E. It is primarily present in ECs, but also expressed in megakaryocytes, platelets, retinal progenitor cells, some hematopoietic

stem cells and in circulating endothelial precursor cells (Katoh et al., 1995; Ziegler et al., 1999). Although VEGFR-2 binds VEGF with lower affinity than VEGFR-1, there is a general agreement that most of the biological effects of VEGF are mediated via VEGFR-2. Receptor specific mutant forms of VEGF have been used to show that VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability- enhancing effects of VEGF and that EC survival is dependent on VEGFR-2 (Gerber et al., 1998; Gille et al., 2001; Keyt et al., 1996).

VEGFR-3

VEGFR-3 is a receptor that binds VEGF-C and VEGF-D (Achen et al., 1998; Joukov et al., 1996). Furthermore, the lymphangiogenic effects of VEGF-C and VEGF-D are thought to be mediated via VEGFR-3 (Veikkola et al., 2001). VEGFR-3 has only six immunoglobulin (Ig)–homology domains in the extracellular domain, a transmembrane region and a tyrosine kinase (TK) domain. Two different forms of VEGFR-3 are expressed in humans. The long form is more abundant, the short form having 65 amino acids less in its C-terminus (Hughes 2001). During early vascular development VEGFR-3 is expressed in the endothelium of the developing vasculature, but when lymphatic vessels form, it is expressed almost exclusively in the lymphatic endothelium (Kaipainen et al., 1995).

PLATELET-DERIVED GROWTH FACTORS

Platelet-derived growth factors (PDGFs) are major mitogens for several cell types of mesenchymal origin, such as smooth muscle cells and fibroblasts. Originally, PDGF was identified as a constituent of whole blood serum that was absent in cell-free plasma-derived serum (Kohler and Lipton, 1974; Ross et al., 1974; Westermark and Wasteson, 1976). Not very long afterwards, PDGF was purified from human platelets, thus gaining its name (Antoniades et al., 1979; Heldin et al., 1979; Kaplan et al., 1979). Later it was discovered that PDGFs are synthesized by a number of different cell types. PDGFs are composed of homo- and heterodimeric polypetide chains. For a long time only two different chains were known, PDGF-A and PDGF-B, the so called classic PDGFs. Later two additional chains were discovered, PDGF-C (Li et al., 2000) and PDGF-D (I), (LaRochelle et al., 2001). These four members of the family bind to and activate two PDGF receptors, PDGFR-α and PDGFR-β, which also form either homo- or heterodimeric complexes on the cell surface after ligand binding.

PDGFs have a knot with eight cysteine residues that are conserved and present in all four chains. It is known that in the two classical PDGFs, two of the cysteine residues (second and fourth) are involved in cysteine bonds between the two subunits in the PDGF dimer, the other six are engaged in intrachain disulfide bonds (Haniu et al., 1994; Haniu et al., 1993). In the "new" PDGFs, there are four extra cysteine residues in PDGF-C and two extra ones in PDGF-D. At the moment it is unclear what kind of effect these extra cysteins have on the three dimensional structure of PDGF-C and PDGF-D.

PDGF-A

The gene producing the PDGF-A chain is located on chromosome 7 (Betsholtz et al., 1986). PDGF-A occurs as two different splice forms, producing mRNAs of 2.1 and 2.4 kb in length and encoding protein chains of 196 and 211 amino acid residues. The gene has 7 exons, exon 1 encoding the signal sequence, exons 2 and 3 precursor sequences that are removed during processing, while exons 4 and 5 encode most of the mature PDGF-A protein. Exon 6 encodes a C-terminal sequence, which is spliced out when the 211 aa splice form is produced. Exon 7 is mainly noncoding, but has the stop-codon for the 196 aa form (Bonthron et al., 1988). PDGF-A is synthesized and assembled into disulphide-linked dimers in the endoplasmic reticulum, but to make it active, the N-terminal extension is cleaved and the mature form activated in the exocytic pathway and then released into extracellular matrix (Ostman et al., 1992). PDGF-AA binds to and activates only the PDGFR-α homodimer. Several cell types express PDGF-A, e.g. fibroblasts (Paulsson et al., 1987), VSMCs (Nilsson et al., 1985; Paulsson et al., 1987), ECs (Collins et al., 1987), macrophages (Nagaoka et al., 1992) and astrocytes (Noble et al., 1988; Richardson et al., 1988). At the tissue level the highest expression levels are found in the heart, pancreas and skeletal muscle (Fredriksson et al., 2004).

Notably, PDGF-A also forms dimers with PDGF-B. PDGF-AB actually produces a strong mitogenic and chemotactic effect on cells, especially in cells expressing both α and β receptors (Heidaran et al., 1990).

PDGF-B

The gene producing the PDGF-B chain is located on chromosome 22 (Dalla-Favera et al., 1982; Swan et al., 1982). PDGF-B gene produces mRNA of 3.5 kb in length and encodes a protein chain 241 amino acids long. The structure of the gene is similar to PDGF-A, except that exon 7 is completely noncoding. Exons 2 and 3 encode an N-terminal part and exon 6 a C-terminal part that have to be proteolytically removed before PDGF-B is activated (Johnsson et al., 1984). PDGF-B is produced and cleaved in the exocytic pathway (Ostman et al., 1992). PDGF-Bs expression pattern in different cell types is much like PDGF-As, it is expressed in several different cell types, e.g. VSMCs (Majesky et al., 1988), ECs (Hellstrom et al., 1999), macrophages (Ross et al., 1990) and fibroblasts (Raines, 1991). During development PDGF-B is mostly expressed in the developing vasculature, where PDGF-B is produced by endothelial cells (Hellstrom et al., 1999). At the tissue level in adults, most abundant expression of PDGF-B is found from heart and placenta (Fredriksson et al., 2004).

PDGF-C

PDGF-C was the first of the two so called new PDGFs to be discovered. As with the classical PDGFs, PDGF-C forms dimers, but only homodimers, which bind primarily toPDGFR-α homodimer (Li et al., 2000), but there are reports showing that it can activate also the PDGFR-αβ heterodimer (Cao et al., 2002; Gilbertson et al., 2001). The gene is located in the human chromosome 4, and, uniquely among the PDGFs, has only 6 exons (II). The first exon encodes a signal sequence, while exons 2 and 3 encode an N-terminal CUB domain (Clr/Cls, Urchin EGF-like protein and bone morphogenic protein 1 (Bork, 1991). Exon 4 encodes the so called hinge area in

front of the PDGF-homology domain, which is encoded by exons 5 and 6 (II). Full length PDGF-C is 345 amino acids long and to make PDGF-C active, the N-terminal CUB domain must be cleaved off proteolytically (Li et al., 2000). PDGF-C mRNA is expressed in a variety of tissue types, most strongly in the heart, pancreas and kidney (Gilbertson et al., 2001; Li et al., 2000). In human cell types PDGF-C expression has been detected in VSMCs, ECs and fibroblasts (II)(Gilbertson et al., 2001).

Platelet-derived growth factor receptors

PDGF receptors are proteins which have five immunoglobulin-like extracellular domains, a transmembrane domain and an intracellular tyrosine kinase domain (Claesson-Welsh, 1989; Matsui et al., 1989; Yarden et al., 1986). Since PDGFs are dimeric molecules, they bind to two receptors simultaneously and thus dimerize the receptors and become activated (Bishayee et al., 1989; Heldin et al., 1989; Seifert, 1989). The structures of the PDGFRs are similar to those of the colony stimulating factor-1 (CSF-1) receptor (Coussens et al., 1986) and the stem cell factor (SCF) receptor (Yarden et al., 1987). The level of PDGF receptor expression on cell surfaces is not constant, for instance during inflammation the levels of PDGFR-β on connective tissue cells goes radically up (Rubin et al., 1988), and stimulation with basic fibroblast growth factor or tumor necrosis factor α selectively increases the expression of PDGFR-α in VSMCs (Schollmann et al., 1992; Sihvola et al., 1999).

PDGFR-α

The human PDGFR-α gene is located on chromosome 4 (Spritz et al., 1994). It binds and is activated by any of the following combinations of PDGF dimers; PDGF-AA, PDGF-AB, PDGF-BB or PDGF-CC. Activation of PDGFR-α transduces powerful mitogenic signals, and stimulates chemotaxis (Hosang et al., 1989; Shure et al., 1992) in certain cell types but is also claimed to inhibit chemotaxis in other types, such as SMCs (Siegbahn et al., 1990) (Koyama et al., 1996)(Yokote et al., 1996). The classic target cells for PDGFs such as fibroblasts and SMCs express both receptor types, but there are some cell types which express only the other one. O-2A glial precursor cells, human platelets and rat liver endothelial cells are cell types known to express only PDGFR-α (Hart et al., 1989; Heldin et al., 1991; Vassbotn et al., 1994).

PDGFR-β

The human PDGFR-β gene is located in chromosome 5 (Yarden et al., 1986). It binds to and is activated only by PDGF-BB and PDGF-DD. As is the case with PDGFR-α, activation of PDGFR-β is also source of powerful mitogenic signals and chemotaxis (Heldin and Westermark, 1996; Siegbahn et al., 1990). PDGFR-β also mediates the formation of circular actin structures on the dorsal cell surface (Eriksson et al., 1992), whereas receptor α does not. Fibroblasts and SMCs express both receptors, but usually they express PDGFR-β at higher levels (Heldin and

Westermark, 1996). The cellular expression pattern of PDGFR-β is mostly similar to PDGFR-α, but some types of cells express only receptor β. Such kinds of cell types are, for example, certain capillary ECs, pericytes and mammary epithelial cells (Bar et al., 1989; Smits et al., 1989; Sundberg et al., 1993; Taverna et al., 1991).

PDGFs in development

Deletion of PDGF-A chain in mice leads to defective development of alveoli of the lung, giving an emphysema-like phenotype. This is caused by the lack of distal spreading of alveolar smooth muscle cell progenitors during lung development, leading to death of the mice around 3 wk of age (Boström, 1996; Lindahl et al., 1997b). PDGFR-α deficient mice die during embryogenesis, the embryos displaying cranial malformations and defiency of myotome formation (Soriano, 1997). Genetically partially rescued PDGFR-α null mouse chimeras display similar phenotype to PDGF-A null mice (Sun et al., 2000). The expression patterns of PDGF-A and PDGFR-α during lung development suggest a paracrine pattern, where PDGF-A is expressed by the epithelium and PDGFR-α by populations of adjacent mesenchymal cells (Boström, 1996; Lindahl et al., 1997b). PDGF-A deficient mice display also a reduced number of intestinal villi, showing abnormaily in their length and thickness (Karlsson et al., 2000). During intestinal development PDGF-A is initially expressed in the intestinal epithelium whereas PDGFR-α is expressed in proliferating meenchymal cells immediately subjacent to the epithelium (Karlsson et al., 2000). The loss of PDGF-A results in failure of PDGFR-α positive cells to localize properly in the gut (Karlsson et al., 2000). This would indicate that the role of PDGF-A in the development of the gut would be to secure the placement of the PDGFR-α positive mesenchymal cells. The PDGF-A null mice also developed a tremor due to severe hypomyelination of the neuronal projections within the central neural system (CNS) (Fruttiger et al., 1999).

Although PDGF-B binds to all three receptor-dimers, during development it seems to act mostly via PDGFR-β homodimers. This conclusion has been made mostly by comparing the knock-out models where the deletion of PDGF-B or PDGFR-β has quite similar effects. The knock-out mice die at embryonic stage from widespread microvascular bleedings (Levéen, 1994). This is caused by a severe shortage of VSMCs and pericytes (Lindahl et al., 1997a). In both types of mice the mesangial cells are almost completely missing (Levéen, 1994; Soriano, 1994). This leads to poor filtration in the glomeruli. In the process of mesangial cell recruitment, ECs express PDGF-B and mesangial cells PDGFR-β, suggesting paracrine signaling similar to that in capillary formation elsewhere (Lindahl et al., 1998). In contrast to PDGFR-β null mice, the PDGF-B null mice had also heart defects with an increased size and trabeculation of the myocardium. The fact that this is not detected in the PDGFR-β null mice indicates that in the heart PDGF-BB may act via PDGFR-α.

The roles of the PDGF-C and PDGF-D in embryonic development are still not known, although the close similarities between the PDGF-B and PDGFR-β null phenotypes make it difficult to envision a role for PDGF-D as a developmental ligand for PDGFR-β. Such is not the case with PDGF-A and PDGFR-α. As PDGF-B does not explain the differences either, the remaining candidate is PDGF-C (Li et al., 2000). Some still unpublished results from PDGF-C null mice have indeed given

an indication that PDGF-C has partially overlapping functions with these two other ligands (Betsholtz, 2004).

PDGFs in disease and treatment

PDGFs in tumors and tumor therapy

Two decades ago, the transforming gene in Simian Sarcoma Virus, *sis*, was found to encode PDGF-B (Doolittle et al., 1983; Waterfield et al., 1983). Since then PDGF overactivity has been associated with several cancer types. In gliomas and astrocytomas PDGFs and PDGFRs are upregulated in an autocrine manner and their expression levels are higher in advanced forms of gliomas than in less malignant tumors (Hermanson et al., 1992; Hermanson et al., 1996). On the other hand, ininvasive gastric carcinomas PDGF-A has been found to provide an effective prognostic marker because high levels of PDGF-A correlate with high grade carcinomas (Nakamura et al., 1997)(Katano et al., 1998). The new PDGFs have also been associated with some tumors, PDGF-C with Ewing carcinomas (Zwerner and May, 2001) and PDGF-D with e.g. ovarian and lung cancer (LaRochelle et al., 2002).

In development of anti-cancer therapies PDGFs and their receptors are beginning to draw more attention. Especially PDGFR-β in pericytes constitutes a relevant target for anti-cancer therapy. This based on results, according to which the lowering of the the interstitial pressure of the tumor helps the access of chemotherapeutic drugs to the tumors and thus enhances their effects (Pietras et al., 2002). This was obtained mostly by inhibiting the function of PDGFR-β inside the tumor using the new PDGF receptor blocker, STU571 i.eGlivec (Pietras et al., 2001). Such therapy, combined with for example the new anti-angiogenic drugs, like Bevacizumab (Avastin™, Genentech) which is an anti-VEGF monoclonal antibody (Yang et al., 2003), can prove to be very effective in treatment of solid tumors.

PDGFs in cardiovascular disease

Atherosclerosis is a complex, largely inflammatory response in large and medium arteries to a number of different risk factors that results in an accumulation of cells and extracellular matrix in the intimal space (Ross, 1999). One central player in the forming of the atherosclerosis is the macrophage. Accumulation of macrophages is the first cellular event in lesion initiation, and they are present throughout the stages of lesion development. In fact, reducing macrophage recruitment can decrease lesion development (Boring et al., 1998; Ni et al., 2001).

PDGFs and their receptors are expressed at low levels in normal vessels, but the situation changes when atherosclerotic lesions start to form. The lesions demonstrate increased levels of PDGF-B compared to normal vessels (Barrett and Benditt, 1987), and the macrophages at the scene express both PDGF-A and PDGF-B (Evanko et al., 1998). Expression of PDGF-C and PDGF-D by macrophages has not been reported. PDGF receptor β expression is also increased in atherosclerotic lesions, most of the expression being in SMCs (Rubin et al., 1988) with more localized expression in macrophages (Tanizawa et al., 1996). Elevated PDGF receptor α expression has been detected in atherosclerosis, and also from renal

arterosclerosis (Floege et al., 1998; Giese et al., 1999; Sihvola et al., 1999). The involvement of PDGFs has been tested in several animal models, for example by using balloon catheterization of rat carotid arteries. In the denuded artery, there is an increase of activated PDGF receptors in the vessel wall (Abe et al., 1997; Panek et al., 1997). The initial thickening of the vessel wall that follows this treatment was inhibited with PDGF antibodies (Ferns et al., 1991). Furthermore, infused PDGF-BB into the rat carotid artery following a denudation caused significant thickening of the lesion area compared to non-treated animals (Jawien et al., 1992).

The blocking of PDGFs or their receptors as a form of treatment in atherosclerosis was tested some time ago and inhibitors of PDGF-B or PDGFR-β to stop SMC/pericyte accumulation gave some positive results (Maresta et al., 1994).

Another side to the story is the possible use of PDGFs to help create new blood vessels after atherosclerosis has created an ischemic area. PDGFs are not very angiogenic, but they are very important factors in recruiting SMCs and pericytes around new vessels, therefore the combinations of PDGFs with some effective angiogenic factors may turn out to be very useful.

AIMS OF THE STUDY

Specific aims of the study:

I To clone and characterize a novel growth factor, PDGF-D, to find out its amino acid sequence, domain structure, receptor binding and activating properties as well as tissue expression.

II To find the exon-intron structure, chromosomal localization of PDGF-D and its expression in normal cells and various types of tumor cells.

III To investigate the potential of PDGF-D in treating certain diseases; in wound healing and growing of new, functional blood vessels in combination with angiogenic factors.

IV To verify the receptor binding properties of PDGF-D, to clarify the processing site and to explore the function and significance of the CUB domain in PDGF-D.

MATERIALS AND METHODS

The materials and methods are described in detail in the original publications, which are here referred to using Roman numerals.

The cloning of PDGF-D cDNA (I)

The PDGF-D cDNA was originally identified from NCBI EST database, cloned with PCR from a human fetal lung 5'-STRETCH PLUS λgt10 cDNA library (Clontech). The PCR fragment was labelled to high specific activity by random priming (Amersham Inc.) and the same cDNA library was screened by plaque hybridisation to isolate several partial cDNA clones. The longest subcloned insert was 1934 bp in length and encoded the C-terminal of PDGF-D while the 5´-part was missing. The 5´-part of the cDNA was then amplified by Rapid Amplification of cDNA Ends (RACE) using human heart cDNA as the template (Clontech).

mRNA expression of PDGF-D in human tissues, cell lines and tumor cell lines (I, II)

To test the mRNA expression levels of PDGF-D in human tissues, a human Multiple Tissue Northern blot (Clontech) was hybridised with the ^{32}P-labeled 327 bp PCR fragment of PDGF-D cDNA. A full length PDGF-B cDNA was used as control probe.

Human umbilical vein and microvascular endothelial cells and coronary artery SMCs were obtained from Promocell and cultured in passage 2-5, as recommended by the supplier. The Wi-38 fetal lung fibroblasts and human tumor cell lines were obtained from American Type Culture Collection. The isolation of polyadenylated RNA was by oligo-dT cellulose chromatography; 5 μg was electrophoresed in agarose gels containing formaldehyde, blotted onto Hybond-N filters (Amersham), which were hybridized with *PDGFC* and *PDGFD* cDNA fragments. These were used to find the expression levels of PDGF-D mRNA in endothelial- and smooth muscle cells and in different tumor cell lines.

PDGF-D expression in mouse sections (I, II)

For immunohistochemistry, affinity-purified rabbit antibodies to human PDGF-D were applied onto tissue sections prepared from paraformaldehyde-fixed and paraffin-embedded mouse embryos.

Production and receptor binding properties of PDGF-D protein (I, II, IV)

Several different methods for producing the PDGF-D protein were used. For the receptor-Ig binding experiments only a small amount of metabolically labelled protein was needed, so PDGF-D expression plasmid was transfected into 293T cells

and the conditioned medium was collected and used for immunoprecipitation with receptor-Ig proteins (PDGFR-α-Ig and PDGFR-β-Ig, R&D).

To test the receptor competition assay a large amount of purified PDGF-D protein was needed. This was produced in baculoviral system and checked with SDS-PAGE under reducing conditions, followed by western blot analysis using the antipeptide antiserum. The ligand competition binding experiments were carried out using porcine aortic endothelial (PAE) cells expressing human PDGFR-α or PDGFR-β.

Analysis of genetic structure and chromosomal location of *PDGFD* (II)

To analyze the exon-intron organization of *PDGFC* and *PDGFD*, human genomic clones for the gene*s* were isolated by PCR-based screening from P1 bacteriophage and P1 artificial chromosome (PAC) human diploid genome libraries (Genome Systems). Genomic organization of *PDGFC* and *PDGFD* was determined by sequencing the whole coding sequence and identifying the exon-intron junctions from the genomic clones.

To find out the chromosomal location of *PDGFD* gene, fluorescense in situ hybridization (FISH) was used. Genomic DNA clones were nick-translated with biotin 11-dUTP (Sigma) and hybridized on metaphase chromosomes derived from normal human peripheral blood lymphocytes, which were treated with5-bromodeoxyuridine (BrdU) at early replicating phase to induce banding pattern (10). A multicolor image analysis was used for the acquisition, display and quantification of the hybridization signals. The identification of the chromosomes was based on 4',6'-diamino-2-phenylindole banding pattern which resembles G-bands after BrdU incorporation at the early replicating phase.

Generation and analysis of transgenic K14-PDGF-D mice (III)

Human PDGF-D cDNA (bp 176 - 1285; Genbank sequence number: AF336376) was inserted into the K14 promoter expression vector. The resulting construct was digested to separate the expression cassette from the rest of the plasmid and purified. A 5 ng/ml solution of the DNA was injected into fertilized eggs of the FVB/n-strain of mice and the resulting transgenic mice were maintained in this strain. For analysis of PDGF-D mRNA expression in the skin of transgenic and wild type littermate mice, tissues were snap-frozen in liquid nitrogen and homogenized with a dismembrator. Total RNA was extracted with the RNEasy Kit (QIAGEN GmbH, http://www1.qiagen.com). Using the RNA a northern blot was made and hybridized with a human PDGF-D probe. Protein expression was verified by immunohistochemistry, by staining with antibodies against human PDGF-D. Two transgenic lines were used for the analysis, with similar results.

The skin of the transgenic mice and their wild type littermates was removed, fixed in paraformaldehyde and paraffin embedded. Some skin samples were snap-frozen in liquid nitrogen and embedded in Tissue-Tek OCT- compound (Sakura-Finetek Europe BV, http://www.sakuraeu.com). Sections from these samples were stained with markers for blood vessels (PECAM-1), lymphatic vessels (VEGFR-3 and LYVE-1), hematopoietic cells (CD45), T-lymphocytes (CD3), B-lymphocytes (B-220), granulocytes (Ly-6G) and macrophages (F4/80). Measurement of interstitial fluid pressure from the skin of seven transgenic and seven wild type mice was carried out by using the modified Wick technique. To analyse the vessels from a wider area

of the skin, the ears of the transgenic and wild type littermate mice were also whole mount stained with antibodies against PECAM-1, LYVE-1 and smooth muscle actin.

Wound healing experiments on K14-PDGF-D mice (III)

One reason for creating the K14-PDGF-D transgenic mouse line was to investigate the possible participation and effect of PDGF-D in the wound healing process. Two circular wounds were made on both sides of the back with a 5 mm punch-biopsy tool. The wounds were allowed to heal for up to 10 days, after which the mice were sacrificed and the wounds were collected. Sections were stained with haematoxylin and eosin. The wound area was quantified by measuring the distance between the edges of the migrating epidermis and dividing it with that of the original wound, measured here as the distance between the edges of the *panniculus carnosus* muscle layer. Sections were stained for blood- and lymphatic vessels and hemtopoietic cells. To evaluate connective tissue amount from the wounds, sections were also stained with Van Gieson's stain and Masson's Trichrome stain.

Production and use of AAVs (III)

To investigate the effect of strong local overexpression of PDGF-D alone and when combined with a known angiogenic factor, we created adeno-associated viruses producing various growth factors. The full length VEGF-E (bp 1- 399, Genbank AF106020), the full length PDGF-D (PDGF-DFL), and a short form (PDGF-DΔN, bp 917-1285) as well as full length PDGF-B (bp 1023-2368, GenBank NM_002608) and human serum albumin (HSA, bp 112- 1866, Genbank NM_000477) cDNAs were cloned into the psub-CMV-WPRE plasmid. The recombinant AAVs were produced as described before (Karkkainen et al., 2001). 50 µl of purified AAV (5 x10^{11} genomic particles/ml) was injected into the subcutis of the ear or gastrocnemius muscle of FVB or NMRI nude mice. Four weeks later the mice were sacrificed and the tissues were analyzed similarily to the samples from K14-PDGF-D transgenic mice.

The AAV-injected mice were also used in the vessel permeability test, where FITC-Dextran was injected intravenously into the mouse tail vein. The ears were monitored under a fluorescence microscope and pictures exposed for equal time periods were taken after 1, 2 and 4 minutes.

RESULTS AND DISCUSSION

1. The cloning and characterization of human PDGF-D (I, IV)

We identified PDGF-D as a human expressed sequence tag (EST) in a BLAST search of the National Center for Biotechnology Information EST database. The EST sequence was compared to the sequence of PDGF-C and an identity of approximately 50% between them was detected. Several cDNA clones were isolated but the 5´end of the complete coding region was lacking and using rapid amplification of cDNA ends (RACE) I was able to generate the missing part. The full length cDNA of PDGF-D encodes a polypetide which is 370 amino acids long. It has a signal sequence (residues 1-22) and two domains, an N-terminal CUB domain (residues 56-167) and a C-terminal PDGF/VEGF-homology domain (residues 272-362), which also has the highest identity with the other members of the PDGF/VEGF-family (Figure 3), especially PDGF-C (~50% identity). Only seven of the eight invariant cysteine residues found in other PDGF/VEGF domains are present in PDGF-D, the fifth being replaced by glycine residue. Like PDGF-C, PDGF-D also has an insertion of three amino acid residues between the conserved cysteine residues, three and four, being the residues NCG in the case of PDGF-D. This feature among others shows that PDGF-C and PDGF-D form their own closely related subgroup in the PDGF/VEGF family (Figure 4).

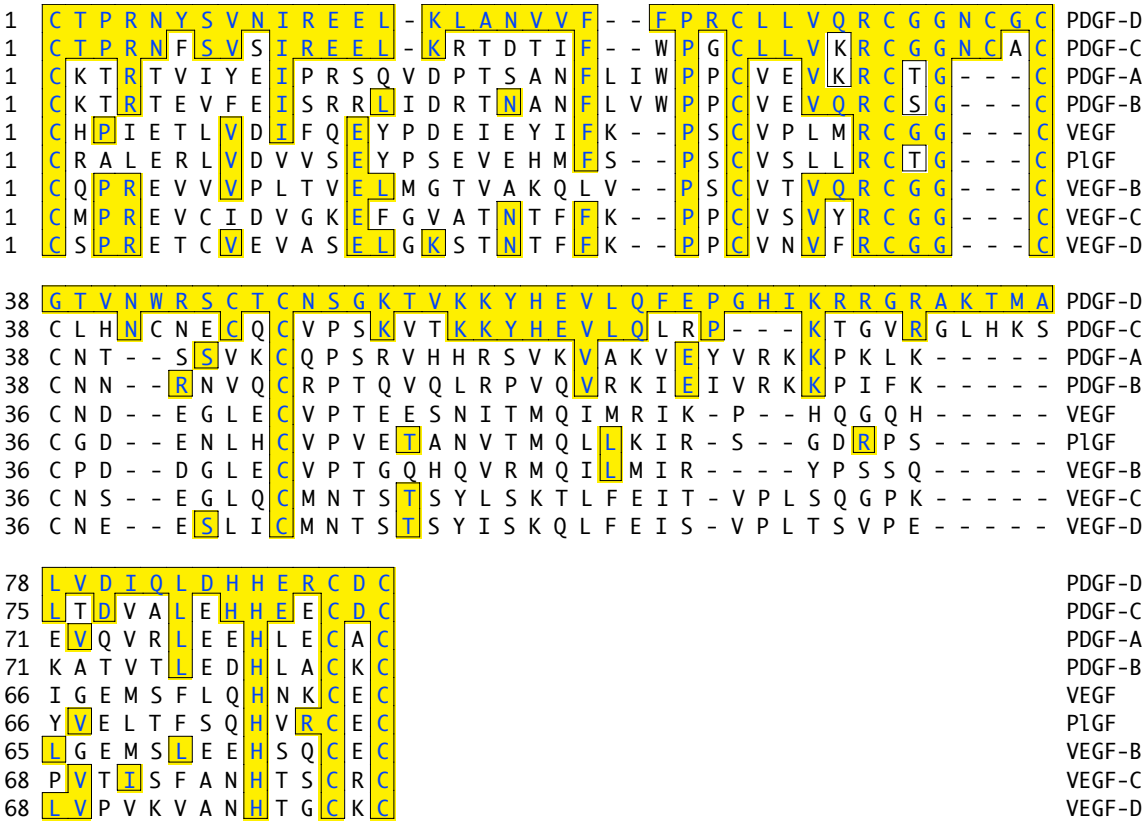

Figure 3. Alignment of the amino-acid sequences of members of the PDGF/VEGF family, in which the regions that encompass the conserved cysteine rich domain are shown. Identical residues to those in PDGF-D are boxed. The fifth invariant cysteine residue in PDGF-D is replaced by glycine.

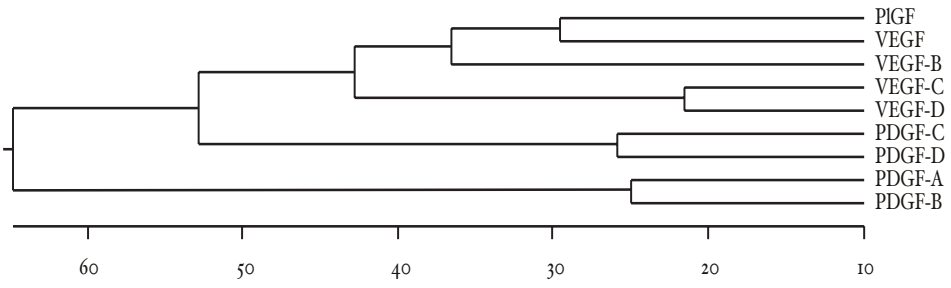

Figure 4. The dendrogram comparison of the growth-factor homology domains in the PDGF/VEGF family. PDGF-C and PDGF-D form a subgroup of the PDGFs. Numbers show substitutions per 100 residues.

We expressed the full length form of PDGF-D containing a C-terminal six-histidine tag, the purified protein was shown to be 55 kDa under reducing and 90 kDa under non-reducing conditions, therefore showing that it is composed of homodimers. It is well documented that full length PDGF-C needs proteolytic processing to remove the CUB domain and release the active PDGF/VEGF homology domain (core domain) which then interacts with the receptor. We could not identify a natural enzyme which would remove the CUB domain from PDGF-D, but in the presence of serum, PDGF-D underwent proteolytic processing to generate the active growth factor dimer of the 23 kD PDGF homology domains, but lacking the N-terminal CUB domains. To produce enough active protein for our receptor binding experiments, we generated a mutated form of PDGF-D. This form included factor Xa cleavage sites (IEGRx2, replacing residues 251-258, including the predicted cleavage site, RKS/K). When produced in insect cells, this form showed similar properties in SDS-PAGE to wild-type PDGF-D, under both reducing and non-reducing conditions. This protein was used in PDGF receptor binding experiment, which showed that PDGF-D activates only PDGFR-β. However, other publications claimed that PDGF-D would also bind and activate PDGFRα/β heterodimers (LaRochelle et al., 2001).

When PDGFR-α-Ig fusion protein became available, we decided to test this binding again. This time we produced PDGF-D in mammalian cells in the presence of serum. Suprisingly we noticed that the processed growth factor domain cleaved from the full-length PDGF-D bound to both PDGFR-α-Ig and PDGFR-β-Ig. We produced also another PDGF-D protein called PDGF-DΔN, starting from the amino acid 248, 9 amino acids before the predicted cleavage site (RKS/K). This PDGF-DΔN polypeptide lacking the CUB domain but containing part of the intervening sequences was not proteolytically processed anymore and bound to PDGFR-β but only very weakly to the PDGFR-α. Another produced protein was the CUB domain with the whole intervening sequence before PDGF-homology domain. This did not bind to either PDGF receptor, as expected. When co-expressed, CUB domain formed a dimer with the naturally matured PDGF-D and almost totally prevented its binding to PDGFR-α, but did not have any effect on the binding of PDGF-D to PDGFR-β.

Because the ΔN form of PDGF-D lacking the CUB domain was not proteolytically processed at the cleavage site, we wanted to see if the CUB domain can transfer the cleavage function to another homologous protein. We therefore made a PDGF-D-VEGF chimeric protein, where the growth factor domain of VEGF was fused to the N-terminal part of the PDGF-D polypeptide containing the CUB domain so that the presumed cleavage site was preserved. The chimeric protein was cleaved in between the CUB domain and VEGF domain and detailed analysis of the various fusions showed that the cleavage was strongly inhibited when the N-terminal PDGF-D sequence was further truncated so that the tribasic sequence RKSK was partly deleted. This suggested that the major cleavage site in the chimeric protein is located in this sequence.

When the chimeric proteins were analysed in nonreducing conditions, very little of the homodimeric cleaved VEGF protein could be detected in the gels. Instead, the major species of the CUB-VEGF protein were the full-length dimer and a heterodimer formed between the full-length and cleaved CUB-VEGF chimera. This suggests that the first cleavage separating the N-terminal CUB domain of PDGF-D

and the VEGF domain is efficient, but the second cleavage is much more inefficient after the loss of the first CUB domain. All forms bound to VEGFR-1 and VEGFR-2, indicating that the CUB domain does not transfer the latency to VEGF. The co-transfection of the PDGF-D and CUB-VEGF vectors led to heterodimerization of the polypeptides. This co-transfection also had similar effect as co-transfection with just the CUB domain, namely the binding of mature PDGF-D to PDGFR-α was mostly prevented while there was no effect on the PDGFR-β binding.

These results show that PDGF-D is indeed a dimer forming member of the PDGF/VEGF protein family, and that it is a very close relative to PDGF-C, described only one year earlier (Li et al., 2000). We show also that the role of the CUB domain is of great importance in activation of the PDGF-D; it has to be removed before the PDGF-D dimer can bind to and activate its receptor. We do not yet know which enzymes are responsible for the processing *in vivo*. The discovery that the fully processed PDGF-D binds to both PDGFR-α and -β, although PDGF-D was first identified as PDGFR-β specific ligand with no affinity towards PDGFR-α suggests new possible functions for PDGF-D, as PDGFR-α is expressed in some cell types that do not contain PDGFR-β, such as astrocytes.

Although full length PDGF-D is unable to activate PDGFR-β, there is the interesting possibility of formation of the full length PDGF-D-core domain PDGF-D dimer, which could bind to the receptor as a monovalent ligand and act as an antagonist. The tissue expression pattern seems to be similar to that of PDGF-B, but there are also some differences, suggesting the possibility that in some tissues PDGF-D might even be the primary ligand for PDGFR-β.

When searching the more exact role for the CUB-domain in PDGF-D, our results indicated that the CUB domain does not mask receptor binding epitopes in a CUB-VEGF chimeric factor, although such chimera blocked PDGF-D binding to PDGFR-α and formed heterodimers with PDGF-D. The chimeras formed dimers not only with each other, but also with full length PDGF-D, demonstrating that CUB-domains regulate dimer formation specificity. Interestingly, a small amount of heterodimers was also detected, which included one processed polypeptide and another one, which was full length.

While previous studies showed that unprocessed PDGF-D has no receptor binding activity, our experiments employing the various recombinant PDGF-D proteins indicate that the ability of the N-terminally fused CUB domain to inhibit receptor binding cannot be transferred to the related VEGF growth factor. CUB-VEGFs as well as CUB domain alone seems to block PDGF-D binding to PDGFR-α but not to PDGFR-β. This specificity may reflect the fact that the affinity of PDGF-D to PDGFR-α is lower than to PDGFR-β, and thus more easily disturbed. This raises also the question of the role of the CUB-domain cut loose during the processing of PDGF-D. Could it have some kind of blocking effect, binding to full length PDGF-D, forming heterodimers and blocking further processing? This would also provide some explanation to the functional role of an alternatively spliced form of PDGF-D which has no PDGF-homology domain. This splice form has been cloned from mice, but not yet from humans ((Zhuo et al., 2003), our unpublished data). This would also explain the fact that when expressed in mammalian cells, always almost exactly half of the full length PDGF-D is processed to mature form and the other half stays intact.

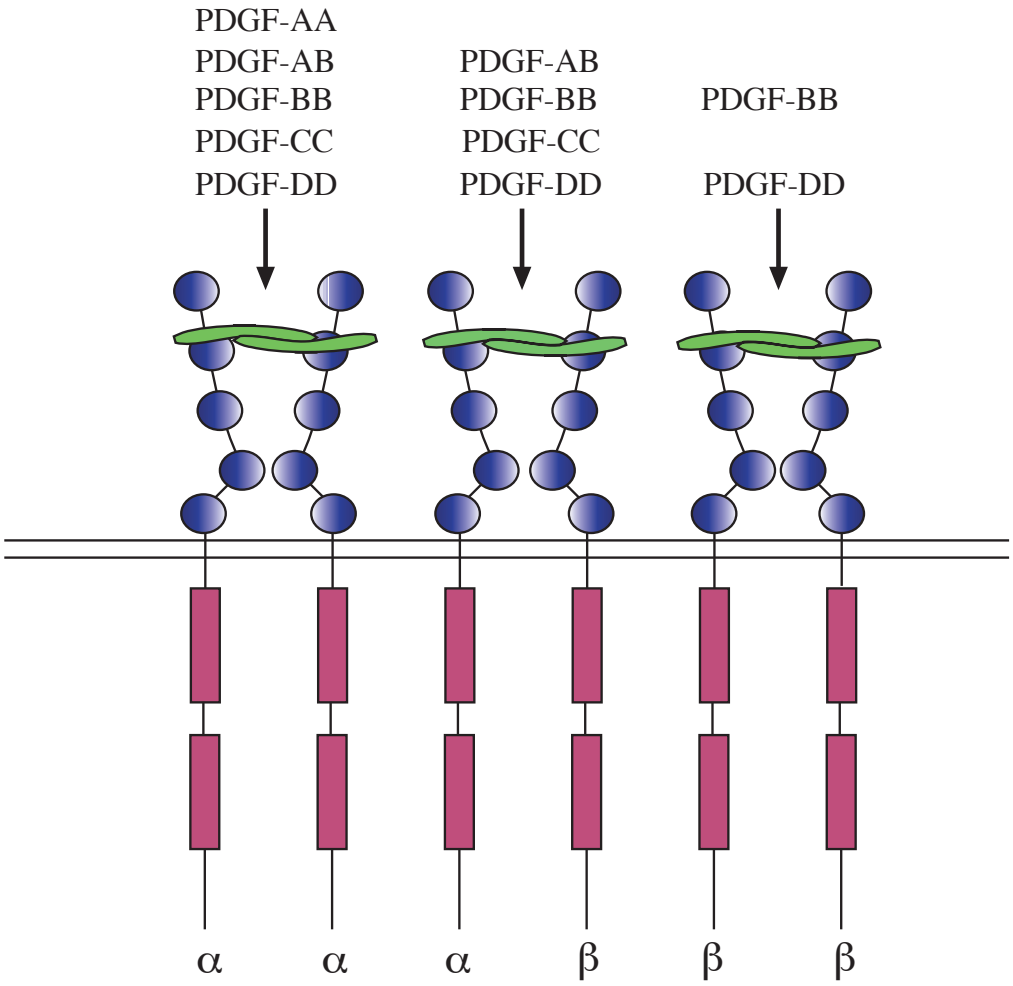

Figure 5. **Binding of PDGFs to PDGFRs.** PDGFRs can form homo- and heterodimers. PDGFs form five different dimeric isoforms.

2. The structure, chromosomal location and expression patterns of human *PDGFD* gene (I, II)

We obtained human genomic clones of *PDGFC* and *PDGFD* from PCR based screening of P1 artificial chromosome libraries. By sequencing these clones we were able to determine the exon/intron structure of both genes (Figure 6). *PDGFD* consists of 7 exons, *PDGFC* of 6. Both genes start with a long untranslated sequence in exon 1, which also encodes the signal peptide. Exons 2 and 3 encode the CUB domain and are ~ 58% identical. Exons 6 and 7 of PDGFD encode the PDGF/VEGF homology domain and are ~ 57% identical with *PDGFC* exons 5 and 6, which encode the homology domain. The main difference between the otherwise closely related sequences is exon 4 of *PDGFD*, which contains sequences that

cannot be identified in *PDGFC*. This exon encodes residues which are part of the hinge area between the CUB domain and the PDGF/VEGF homology domain. The rest of the hinge region is encoded by exon 5 (exon 4 in *PDGFC*). This part of the hinge region contains a conserved basic sequence motif RKS/K, which may be one of the targets of proteolytic cleavage needed for releasing of the active form of PDGF-D.

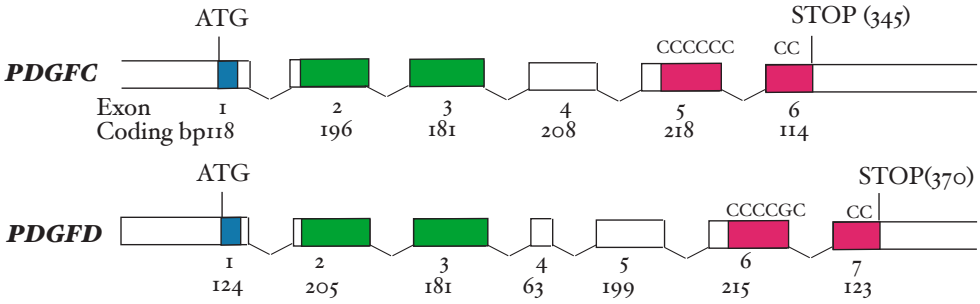

Figure 6. The genomic organization of *PDGFC* and *PDGFD*. Exons shown are numbered and the length of each coding sequence is marked in bp. Start (ATG) and stop codons and polypetide lengths are marked. Signal sequences (SS) are shown in blue, CUB domains in green and PDGF/VEGF homology domains in red.

Genomic clones were also used to find the chromosomal locations of *PDGFD* and *PDGFC* genes. *PDGFD* was localized to human chromosome 11q22.3 to 23.1 and *PDGFC* to 4q32.

The vascular expression patterns of these novel genes were investigated using antibodiesgenerated against them, and signals from PDGF-D were detected in the advetitial connective tissue layer of the suprarenal artery, while PDGF-C was present in the smooth muscle cell (SMC) layer. This result was consistent with the expression patterns in cultured SMCs and fibroblasts.

We analysed the expression of PDGF-D mRNA transcripts in several human tissues by northern blotting. This showed an approximately 4.0 kb transcript, highest expression levels occurring in heart, pancreas and ovary. Slightly lower expression levels were present in the placenta, liver, kidney, prostate, testis, small intestine, spleen, thymus, colon and peripheral blood leucocytes. No expression was detected in the brain, lung or skeletal muscle.

Tissue expression of PDGF-D was also investigated immunohistochemically in developing mouse embryos during mid-gestation (E14.5). This showed PDGF-D expression in several tissues, including the developing heart and kidneys. In the developing kidney we observed a strong staining of the fibrous capsule that surrounds the embryonic kidney and the metanephric mesenchyme of the cortex.

We also found that these novel PDGFs are expressed in several tumor cell lines. The 4.0 kb transcript of PDGF-D was present most strongly in a lung alveolar carcinoma and in breast and prostate carcinoma cell lines.

Because of the involvement of the PDGFs in blood vessel development and in the pathogenesis of arteriosclerosis (Betsholtz and Raines, 1997; Ross, 1993), it was of interest to find expression of the PDGF-C and PDGF-D mRNAs in cultured microvascular endothelial cells. PDGF-D was found in the adventitial connective tissue surrounding the suprarenal artery, suggesting that it provides a paracrine ligand for the arterial SMCs. The biological activity of recombinant PDGF-C and PDGF-D was confirmed in human coronary artery SMC cultures, where both of these factors stimulated cell proliferation/survival. In previous studies, both PDGF-A, which transduces signals via PDGFR-α, and PDGF-B, which transduces signals via both PDGFR-α, PDGFR-β and via their heterodimers have been shown to mediate SMC proliferation stimuli. Several antagonists specific for PDGF or its receptors have recently been developed and shown to inhibit intimal hyperplasia formation in various animal models, predominantly via increased SMC apoptosis and possibly also via interference with SMC migration (Leppanen et al., 2000). Thus it can be speculated that via their ability to stimulate the PDGFRs, PDGF-C and PDGF-D could also be involved in the intimal SMC accumulation in arteriosclerosis.

The role of PDGF-D in the course of embryogenesis is still to be deduced, and will remain so until the knock-out model is complete and analysed. Our results show that it is expressed during embryogenesis and has a likely a role during the development of the heart and kidney.

The PDGF-C and PDGF-D mRNAs were also expressed, along with PDGF-A and PDGF-B mRNAs, in several tumor cell lines, but the variable expression patterns seen in a subset of the cell lines indicates that these genes are differently regulated. The PDGFs secreted by tumor cells could be responsible for some of the stromal proliferative or so-called desmoplastic reactions around tumors. Others have obtained similar results regarding the possible involvement of PDGF-D in tumors, its expression has been detected from a variety of tumors other than those studied by us (LaRochelle et al., 2002). There has results suggesting involvement of PDGF-D in prostate cancer, with PDGF-D acting as a potential oncogene (Ustach et al., 2004).

3. Overexpression of PDGF-D induces macrophage accumulation in normal skin and wound healing and increases the interstitial fluid pressure (III)

No obvious differences were detected by macroscopic inspection of the skin of K14-PDGF-D transgenic mice and their wild type littermates. Epidermal thickness, dermal cellularity, and blood and lymphatic vessel numbers were similar in transgenic and wild type skins. Whole mount immunohistochemistry for smooth muscle actin (arteries and larger veins) and microlymphangiography using fluoresecent dextran (lymphatic vessels) showed no differences between the transgenic and wild type mice. Instead, large numbers of macrophages accumulated under the skin of transgenic mice. On average the transgenic mice had 3.7 ± 0.4 fold more macrophages in the skin than their wild-type littermates. No differences were found in the numbers of granulocytes or B- and T-lymphocytes in the skin or in the different leukocyte populations in the peripheral blood, neither was there a difference in the total number of mononuclear cells or monocytes in the circulation.

There was no difference in the re-epithelialization of the skin punch biopsy wounds between the transgenic and wild type mice. When the number of cells in the granulation tissue were counted from identical surface areas under the hyperproliferative epithelium in corresponding areas of the wounds, the cellular density was in general greater in the K14-PDGF-D positive mice. Total cell influx into the wound area was greatest in the transgenic mice during the first four days after wounding, being maximally a 39% increased in the transgenic mice, but the difference did not reach statistical significance during the later stages of wound healing.

The most significant difference between the wounds of the transgenic and wild type mice was the number of macrophages. During the first four days after wounding, there was no difference in macrophage influx, but between days five and seven the macrophage numbers started to decrease in the granulation tissue of wild type mice, while they continued to increase in the transgenic mice. The number of macrophages peaked on day seven, being about twofold greater in the transgenic mice, and this difference persisted until day ten. No endogenous PDGF-D mRNA was found in the wounds by RT-PCR. This is consistent with the recent report that PDGF-D is not present in platelets (Fang et al., 2004). In the wound granulation tissue there was no difference detected in number of blood- or lymphatic vessels between the transgenic and wild-type mice.

We analyzed the effects of acut e overexpression of PDGF -D in adult skin and muscle. For this analysis, AAV vectors encoding the full-length PDGF-D (DFL) or the activated form (ΔN) lacking the CUB domain were generated and first tested *in vitro*. AAV vectors encoding PDGF-B and HSA were used as controls.

No difference in blood vessel numbers or the amount of c onnective tissue could be detected in the injected region. However, when the viruses were injected into mice transplanted with GFP-marked bone marrow cells from a donor of the same mouse strain, a strong accumulation of GFP positive cells was detected in the ears injected with AAV-PDGF-DFL or AAV-PDGF-DΔN and a weaker accumulation in the ears injected with AAV-PDGF-B. Such an accumulation did not take place in ears injected with AAV encoding HSA or with PBS alone. Immunohistochemical analysis indicated the presence of numerous macrophages in the AAV-PDGF-D or AAV-PDGF-DΔN injected muscle, but much less in AAV-PDGF-B or AAV-HSA injected muscles.

PDGF-B has been shown to raise dermal IFP to a normal level after it has been lowered for example by anaphylaxis (Rodt et al., 1996). The inhibition of the PDGFR-β signaling lowers interstitial hypertension in tumors (Pietras et al., 2001), and increases the efficacy of chemotherapy (Salnikov et al., 2003). In the K-14-PDGF-D mice the IFP measured from the dermis was between -1.0 and -1.5 (± 0.136) in transgenic mice and -1.1 and -2.1 mmHg (± 0.065) in wild type mice. This increase in skin IFP of the transgenic mice was statistically significant.

Our results show that enforced PDGF-D expression in skin increases macrophage recruitment into the unperturbed skin, and that this effect was enhanced during the wound healing process. We also observed extensive macrophage accumulation in skeletal muscle injected with AAV-PDGF-D, whereas much less accumulation was observed in AAV-PDGF-B injected muscle. These results are consistent with studies showing that PDGF-B induces macrophage migration via the PDGFR-β

receptor (Siegbahn et al., 1990), although PDGF-D seems to be a more potent chemoattractant for these cells.

Macrophages are known to play an important role in wound healing by producing a variety of growth factors and cytokines and by phagocytosing cellular and matrix debris (Rappolee et al., 1988). PDGF purified from platelets accelerates wound healing by stimulating the chemotaxis and proliferation of fibroblasts, smooth muscle cells, neutrophils and macrophages (Pierce et al., 1991). These effects are mediated via the activation of PDGFR-β, which is upregulated in connective tissue cells and epithelial cells during the repair process, explaining the higher accumulation of cells to wounded areas of the transgenic mice (Antoniades et al., 1991; Reuterdahl et al., 1993). In addition to smooth muscle cells and macrophages also fibroblasts, which form a major part of the granulation tissue, express PDGFR-β (Heldin and Westermark, 1999). The recombinant soluble PDGF-D could provide another tool to modulate wound healing via this receptor.

IFP affects capillary fluid filtration and the filling of lymphatic vessels. Many solid tumors demonstrate interstitial hypertension, thus making the delivery of many anticancer drugs more difficult. Our results are consistent with the fact that PDGFR-β is essential for the maintenance of steady-state IFP (Wiig, 1990). Many solid tumors demonstrate interstitial hypertension, thus making the delivery of many anticancer drugs more difficult. Our results are consistent with the fact that PDGFR-β is essential for the maintenance of steady-state IFP (Pietras et al., 2001). Together with earlier results, these define the role of the PDGFR-β and its ligands in maintaining and controlling IFP.

4. PDGF-D in blood vessel maturation during angiogenesis (III)

To investigate the contribution of PDGF-D to vessel stabilization, we tested AAV-PDGF-D in combination with AAV producing the angiogenic endothelial mitogen VEGF-E. We observed that AAV-VEGF-E induced a strong angiogenic response and that the AAV-VEGF-E infected ears had enlarged vessels. SMA stained vessels showed a loose, irregular coating by smooth muscle cells (arrows in Figure 7) in comparison to AAV-HSA infected ears. Ears injected with a combination of AAV-PDGF-D and AAV-VEGF-E displayed the normal tight structure of the smooth muscle layer, this is similar to when AAV-PDGF-B was used with AAV-VEGF-E.

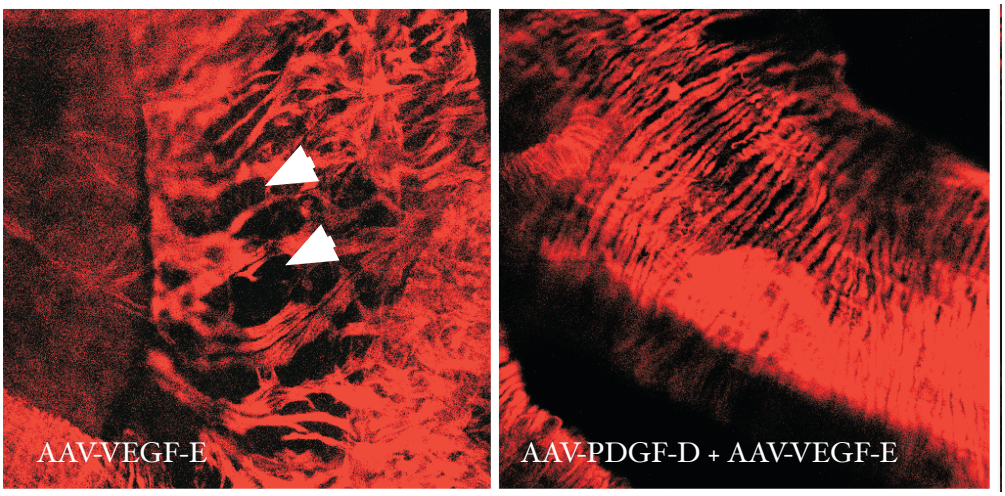

Figure 7. Whole mount staining for SMA of AAV-infected ears. The arteries from AAV-VEGF-E infected ears show an irregular coating by SMCs. When AAV-PDGF-D is included the vessels appear normal, a tight SMC layer structure.

In mouse gastrocnemius muscle infected with AAV-VEGF-E alone the vessels were enlarged, and, compared to the vasculature of the untreated mice, the SMC coating was irregular. When AAV-VEGF-E was administered in combination with AAV-PDGF-D or with AAV-PDGF-B, the vessels were still enlarged but the SMC coating was thick and regular.

When tested for vascular leakiness by injecting FITC-Dextran into the tail vein, the vessels in the ears treated with the combination of AAV-PDGF-D or AAV-PDGF-B and AAV-VEGF-E had reduced leakiness in comparison to the vessels formed in the ears injected with AAV-VEGF-E alone. By contrast, a VEGF-E induced increase in blood capillaries was unaffected by AAV-PDGF-D or AAV-PDGF-B, which, when used alone, did not appear to have any effect on the smooth muscle cell coating or permeability of the vessels.

There has been a recent report on the angiogenic potential of PDGF-D (Li et al., 2003). In our experiments PDGF-D alone was not angiogenic in the ear or skeletal muscle, but when expressed together with VEGF-E, which can induce a strong angiogenic response, it promoted stabilization of the newly generated, enlarged and leaky vessels induced by VEGF-E alone. This effect may be due to the PDGF-D induced stimulation of the proliferation and migration of SMCs, which we have shown for coronary artery SMCs *in vitro* (II). Since our results indicate that PDGF-D has a significant ability to regulate macrophage recruitment, and that both PDGF-D and PDGF-B improve the SMC coating of angiogenic blood vessels and decrease their permeability, it follows that PDGF-D may prove useful in the development of therapeutic tools for the treatment of wounds and for blood vessel stabilization in tissue engineering and various proangiogenic therapies to counteract tissue ischemia.

CONCLUDING REMARKS

Angiogenesis and the molecules involved in it have been under investigation for many years now. Over the years several published results have shown how significantly angiogenesis and angiogenetic factors are involved in tumorigenesis and vascular diseases. It was therefore particularly interesting to discover a new member of the growth factor family that has a central role in this field.

Our first results confirmed that PDGF-D is a close relative of the other members of the PDGF/VEGF family of growth factors, and we were able to analyse the structure and organization of the *PDGFD* gene and its receptor activation capability, and, after later results somewhat completed the picture, we were really able to start exploring its function *in vivo,* and any possible uses in proangiogenic therapies.

Our results indicate that mature PDGF-D has similar receptor binding properties as PDGF-B and that the major difference between the two growth factors is the CUB domain and its possible role in controlling the cleavage of PDGF-D. We were also able to clarify the role and significance of the CUB domain in PDGF-D and its closest relative, PDGF-C. The removal of the CUB domain is necessary for PDGF-D to become active, and we showed that its removal process controls how much active PDGF-D there is available.

Considering the major role of macrophages in the formation of atherosclerotic lesions, and the ability of PDGF-D to recruit them, it would be very interesting to investigate the role of PDGF-D in the formation of these lesions and atherosclerosis in general, to see if the blocking of PDGF-D possibly have any therapeutic effect.

This study gives first answers of the involvement of PDGF-D in some pathological conditions, such as wound healing process, and possible future usage as part of some therapies such as wound healing, growing of new, functional, blood vessels or chemotherapy.

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