RESEARCH COMMUNICATION

Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall

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Several platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) family members display C-terminal protein motifs that confer retention of the secreted factors within the pericellular space. To address the role of PDGF-B retention in vivo, we deleted the retention motif by gene targeting in mice. This resulted in defective investment of pericytes in the microvessel wall and delayed formation of the renal glomerulus mesangium. Long-term effects of lack of PDGF-B retention included severe retinal deterioration, glomerulosclerosis, and proteinuria. We conclude that retention of PDGF-B in microvessels is essential for proper recruitment and organization of pericytes and for renal and retinal function in adult mice.

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The control of cell migration and the formation of specific patterns during embryonic development are believed to depend, at least in part, on the precise spatial distribution of secreted growth and differentiation factors (GDFs). This is achieved by strictly localized and regulated synthesis and secretion of GDFs, but also by binding of the secreted GDFs to cell surface- and extracellular matrix molecules. One type of molecule strongly implicated in the regulation of GDF activities in vivo is the heparan sulphate proteoglycans (HSPGs; Baeg and Perrimon 2000; Gallagher 2001; Iozzo and San Antonio 2001). HSPG-binding properties have been demonstrated for a wide range of GDFs, including members of the FGF, TGF-β, EGF, IGF, PDGF, VEGF, Wnt, and hedgehog families, as well as many chemokines and cytokines (for review, see Gallagher 2001; Iozzo and San Antonio 2001). Most likely, the negatively charged sulfate groups on the disaccharide building blocks of heparan sulfate (HS) polysaccharide chains provide binding sites for positively charged amino acid sequence motifs present in the GDFs. By binding to HSPGs, specific gradients of the GDFs may be created, which may guide or restrict morphogenetic responses (Perrimon and Bernfield 2000; Ruhrberg et al. 2002; Gerhardt et al. 2003). HSPG binding may also lead to the formation of reservoirs of factors for use in wound repair. Finally, HSPGs may act as coreceptors by stabilizing active ligand-receptor complexes (Pellegrini et al. 2000).

Certain isoforms of platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) family members display positively charged stretches of amino acids residues at the C terminus. These stretches are included or excluded depending on alternative splicing or proteolytic processing (Eriksson and Alitalo 1999; Heldin and Westmark 1999). For VEGF-A, the long splice isoforms, which carry HSPG-binding domains, accumulate on the cell surface or in the extracellular matrix, whereas short VEGF-A is diffusible following cellular release (Park et al. 1993). The developmental role of HSPG binding of VEGF-A was recently addressed using mice in which the long VEGF-A splice isoforms were selectively ablated (Carmeliet et al. 1999; Ruhrberg et al. 2002). In these mice, extracellular VEGF-A distribution becomes more widespread, leading to changes in endothelial sprouting and branching, and to the formation of abnormal vascular patterns (Ruhrberg et al. 2002). In PDGF-A and PDGF-B, the HSPG-binding motifs do not affect receptor binding or biological activity of the recombinant proteins (Östman et al. 1989). However, in transfected cells, these motifs confer retention of the secreted growth factor to the surface of the producing cells. Conversely, absence of the retention motif leads to increased secretion of a diffusible protein that readily accumulates in the cell culture medium (LaRochelle et al. 1991; Ostman et al. 1991; Raines and Ross 1992, Andersson et al. 1994). The retention motif also appears to limit the action range of PDGF-B in vivo, as suggested from experiments with transplanted keratinocytes transfected with PDGF-B expression vectors (Eming et al. 1999). To achieve insight into the physiological role of PDGF-B retention, we deleted the PDGF-B retention motif in mice by targeted mutagenesis, and we analyzed the phenotypic consequences of this mutation.

Results and Discussion

Generation and characterization of the pldf-b<sup>ret</sup> allele

To delete the PDGF-B retention motif, we targeted a loxp-flanked PGK-neo cassette into intron 5 and a premature translation stop codon/HindIII site into exon 6 of the pldf-b gene in mouse embryonic stem (ES) cells [Fig. 1]. Heterozygous mutants were produced and further crossed with protamine-Cre mice to delete the PGK-neo cassette, generating a pldf-b<sup>ret</sup> (retention motif knockout) allele. The pldf-b<sup>ret</sup> allele demonstrated Mendelian inheritance, and both pldf-b<sup>ret</sup> (+ indicates wild type)
and pdgf-bret/ret mice survived into adulthood. Pdgf-bret/ret mice were slightly growth retarded and showed reduced female fertility. Northern blot analysis of brain RNA showed similar size pdgf-b transcripts but approximately twofold reduced levels in pdgf-bret/ret mice compared to pdgf-b+/− mice (Fig. 1e). Expression of the pdgf-bret allele was also verified in the brain by reverse transcriptase PCR (RT–PCR) followed by diagnostic HindIII cleavage at the pdgf-bret premature stop codon (data not shown).

Recombinant PDGF-B lacking the C-terminal retention motif has full biological activity (Östman et al. 1989) and is more efficiently released from transfected cells than wild-type PDGF-B (LaRochelle et al. 1991; Ostman et al. 1991; Raines and Ross 1992; Andersson et al. 1994; Eming et al. 1999). We therefore expected a dominant effect of a potentially hyperfunctional pdgf-bret allele. However, genetic data suggested that the pdgf-bret allele was hypo-functional; pdgf-bret/+ mice were indistinguishable from pdgf-b+/− or pdgf-b−/− mice, and pdgf-bret/− mice were perinatal lethal like pdgf-b−/− mice [Levén et al. 1994]. Because the most important site for PDGF-B expression during development is the microvascular endothelium [Lindahl et al. 1997; Enge et al. 2002], we analyzed the expression of the pdgf-bret allele in endothelial cells. In situ hybridization of flattened retinas showed that expression of the pdgf-bret transcript was concentrated to endothelial cells situated at the tips of the vascular sprouts [Fig. 2a,c]. This confirmed the recent identification of pdgf-b mRNA as a marker for endothelial tip-cells [Gerhardt et al. 2003]. The pdgf-bret signal had a similar distribution [Fig. 2b,d], but was weaker than the pdgf-b+ signal, in agreement with the Northern data. To analyze the function and distribution of endogenous PDGF-Bret protein from endothelial cells, we established immortalized polyoma virus-transformed endothelium cells lines from wild-type and pdgf-bret/ret mice. Both lines expressed similar-sized pdgf-b transcripts, but the levels were threefold lower in pdgf-bret/ret cells [data not shown]. PDGF-B protein expression in the endothelium lines was too low to be detected by metabolic labeling or Western blotting [data not shown]. This was not unexpected, because prior attempts have failed to detect PDGF-B protein expressed from the endogenous pdgf-b gene by these methods; the only published examples of PDGF-B protein visualized by, for example, immunoprecipitation have utilized transfected cells. We therefore analyzed PDGF-B protein levels in conditioned media and cell lysates using a sensitive and specific method [Fredriksson et al. 2002] involving PDGF-B-binding aptamers and proximity-dependent DNA ligation (Fig. 2e). Conditioned media were also analyzed for activity in a PDGF β-receptor (PDGFR-β) tyrosine phosphorylation assay (Fig. 2g). Both assays demonstrated lower PDGF-B levels in pdgf-bret/ret cells compared to pdgf-b+/− cells. The two different assays yielded comparable results (0.1 or 0.15 ng/mL, respectively in pdgf-bret/ret samples, and 0.5 and 0.75 ng/mL in pdgf-b+/− samples), confirming that the PDGF-Bret protein had intact PDGF receptor-binding and activating properties. The ratio between the PDGF-B content in medium versus lysate was significantly higher for the pdgf-bret/ret than for the wild-type samples, suggesting that the PDGF-Bret protein is more efficiently released from the cells [Fig. 2f].
Pdgf-1ret/ret show impaired mice pericycle investment into the microvessel wall

In developing mouse embryos, PDGF-B expression is largely restricted to endothelial cells, whereas PDGFR-β expression occurs in vascular smooth muscle cells and pericytes [Lindahl et al. 1997]. Analyses of knock-out mice for PDGF-B and PDGFR-β have shown that PDGF-B signaling via PDGFR-β is critically involved in recruitment of pericytes and vascular smooth muscle cells (vSMCs) to blood vessels [Lindahl et al. 1997; Hellström et al. 1999, 2001]. To analyze pericycle densities in pdgf-bret/ret mice, we bred them onto the XlacZ background, which allows simple quantification of pericycle densities in whole-mount CNS preparations [Tidhar et al. 2001]. At embryonic day 15.5 [E15.5], the number of pericytes in the forebrain was reduced to ~50% of normal in pdgf-bret/ret mice, and even further in pdgf-bret/+ (~-25% of normal), Fig. 2h–k) and pdgf-b−/− mice (<5% of normal), data not shown). The pericycle deficiency in pdgf-bret/ret mice was confirmed using α-smooth muscle actin (SMA) and NG2 proteoglycan staining (data not shown). The reduction in brain pericycle density correlated with abnormal capillary morphology and focal reactive gliosis in postnatal mice, likely reflecting persistent microvascular dysfunction [Fig. 2l,m].

PDGF-B controls pericycle proliferation (Hellström et al. 1999), but may also stimulate pericycle migration along the developing microvessels. Because the pdgf-bret allele is hypomorphic at the levels of mRNA and protein expression, the observed reduction in pericycle numbers in pdgf-bret/ret mice was expected. Previously analyzed hypomorphic situations include Pdgf-b+/- mice, which show an ~30% reduction in retinal pericycle density [Hammes et al. 2002], and compound pdgf-b+/-, pdgfr-β−/− heterozygotes, which show an ~50% reduction [A. Lundkvist and C. Betsholtz, unpubl.]. We have also generated endothelium-restricted PDGF-B knockouts (pdgf-bret/+ mice, Enge et al. 2002), which vary in their degree of interindividual chimerism for the recombined PDGF-B allele, producing a range of PDGF-B-deficient states with up to 90% reduction in pericycle density in the most severely affected individuals [Enge et al. 2002]. Because these mice were viable, the lethality of pdgf-bret−/− mice and the severity of the pdgf-bret/ret phenotype [see below] appeared worse than expected from the observed reduction in pericycle density only. We therefore investigated in more detail the association between the pericytes and the endothelial cells in pdgf-bret/ret mice.

Pericytes normally extend dendritic processes that associate intimately with the abluminal endothelial surface [for review, see Allt and Lawrenson 2001]. High-resolution imaging of pericytes using NG2 staining and confocal microscopy confirmed such association in both sprouting and mature vessels in pdgf-b+/- mice [Fig. 3e]. In pdgf-bret/ret mice, however, the pericytes were partially dissociated from the abluminal endothelial surface and protruded cellular processes away from the vessel [Fig. 3f, arrows]. Similar dissociation of pericytes from the endothelial cells was observed in neovessels of tumors transplanted to pdgf-bret/ret mice [A. Abramsson, P. Lindblom, and C. Betsholtz, unpubl.]. However, the extensively hypomorphic situation created in pdgf-blox/− mice [Enge et al. 2002] did not result in apparent pericycle detachment. The sparse pericytes in these mice extended long and thin processes that remained tightly associated with the abluminal endothelial surface [Fig. 3b,d]. The inactivation of the floxed pdgf-b allele in these mice is, however, not complete, but rather results in a chimeric situation in which a variable proportion of the endothelial cells retain expression of PDGF-Bwt protein from a single unrecombined pdgf-b allele [Enge et al. 2002]. Apparently, this is sufficient to render the pericytes tightly associated with the abluminal endothelial surface.

Severe postnatal glomerular and retinal defects in pdgf-bret/ret mice

PDGF-B and PDGFRβ are critically involved in mesangial cell recruitment to kidney glomeruli [Levén et al. 1994; Soriano 1994]. Pdgf-bret/ret embryos displayed a marked reduction in the number of mesangial cells in glomeruli at late gestation, leading to the formation of ballooning glomerular capillaries [Fig. 4a,b]. This defect was similar to, but slightly milder than, those seen in pdgf-b−/− and pdgfr-β−/− embryos [data not shown]. The mesangial cell deficiency was temporary, and by 1 mo of age, most glomeruli had normalized [Fig. 4c,d, data not shown]. By 6 mo of age, however, the picture was again pathological; extensive accumulation of extracellular matrix was revealed by PAS [Fig. 4e,f] and collagen [data not shown] staining. Albuminuria was apparent in most
pdgf-bret/ret mice at 3 mo (Fig. 4g), implicating renal dysfunction before the development of advanced glomerulosclerosis.

Macroscopic examination revealed white ocular opacities in -10%, and small eyes in 100%, of adult pdgf-bret/ret mice. Histological analysis revealed extensive retinal changes in all pdgf-bret/ret individuals, including degeneration of nuclear and photoreceptor layers, invasion of retinal pigment epithelial cells (RPEs), fibrosis, and traction (Fig. 4h,i). The retinal vasculature was severely disorganized. SMA- and XlacZ4-positive cells did not invest the vascular walls appropriately (Fig. 4j–o), but instead formed cellular sheets on the retinal surface (Fig. 4k,o arrowheads). Vessel-associated vSMCs were present, but abnormally organized (Fig. 4m), and pericytes were partially detached from the endothelium (Fig. 4p). The latter coincided with up-regulated SMA expression, which is normally undetectable in the retinal pericytes, implicating altered cell differentiation.

To explore the development of the retinal pathology, we analyzed newborn pdgf-bret/ret mice. Retinal vessels normally begin to sprout from the optic disc on postnatal day 1 (P1) and reach the retinal margin on P7. Astrocytes appear in the retina before vasculization, and are known to regulate retinal angiogenesis (Gerhardt et al. 2003). Pdgf-bret/ret mice showed normal astrocyte patterns (data not shown), but the retinal vessels were abnormal from the onset of sprouting. Plexus formation was delayed and asymmetric at P2 [data not shown]. The vascularplexuses at P5 were highly irregular and displayed fewer sprouts [Fig. 5c,k] and sites with increased [Fig. 5g] as well as decreased [Fig. 5c,k] vascular density. Vascular remodeling occurred, but the pattern of remodeled vessels was abnormal, with a notable shortage of branch points (Fig. 5g). In pdgf-bret/ret and pdgf-bret/+ mice, pericytes distributed across the entire vascular plexus at all ages analyzed (Fig. 5b,i,j,n). In pdgf-bret/ret mice, however, the abnormal retinal plexuses showed significant reduction in pericyte density, as revealed by both XlacZ4 and NG2 staining (Fig. 5d,h,l,p).

PDGF/VEGF retention and migration of vascular cells

Because it is unlikely that the retention motif in PDGF-B has a direct role in PDGF receptor binding, a more plausible explanation for the observed effects, also supported by published in vitro data, is that the retention motif helps localizing secreted PDGF-B to proteins or proteoglycans on the endothelial cell surface or in the periendothelial matrix, thereby promoting its recognition by neighboring receptor-carrying cells, that is, the PDGF-β-positive pericytes. PDGF receptor activation by immobilized or less readily diffusible PDGF-B protein may also potentially limit receptor down-regulation, thereby producing a more sustained signal. Such a scenario has been implicated for FGFs [Dechelle et al. 2000], and indic
rectly also for PDGF-stimulated cell proliferation [Westermark and Heldin 1985] in vitro.

The effects observed in pdgf-b<sup>ret/ret</sup> mice go beyond those seen in the hypomorphic situations obtained in pdgf-b<sup>−/−</sup>, pdgfr-<sup>−/−</sup>, and pdgf-b<sup>Δ exon−</sup> mice. The finding that pericytes were partially detached and often extended their dendritic processes away from the vessels suggests that PDGF-B retention is required for the formation of depots or gradients of the factor, which help confining pericyte migration to restricted paths—the abluminal microvessel surfaces. An analogous scenario has been proposed for VEGF-A, for which extracellular retention shapes depots or gradients that stimulate directed endothelial cell migration along astrocytes [Gerhardt et al. 2003].

**Critical roles of PDGF-B and PDGFR-β in the retina and kidney glomeruli**

The grossly abnormal retinal and glomerular vascular patterns in pdgf-b<sup>ret/ret</sup> mice reported here and in other genetic or pharmacological models of PDGF-B and PDGFR-β signaling deficiency [Levén et al. 1994; Soriano 1994; Klinghoffer et al. 2001; Enge et al. 2002; Sano et al. 2002; Uemura et al. 2002] suggest a crucial function for PDGF-B/PDGFRβ signaling at these sites. In some of these models, pericyte recruitment is also abrogated at other sites, with less dramatic consequences for organ histology and function. This pattern of organ sensitivity to pericyte deficiency may be relevant in the context of diabetes. Defects in retinal pericytes and renal mesangial cells are hallmarks of diabetic microangiopathy, but their cause(s) and consequence(s) are unclear. Further studies using pdgf-b<sup>ret/ret</sup> mice and other PDGF-B and PDGFR-β-deficient models may help clarify the roles of pericytes and mesangial cells in this disease.

**Materials and methods**

**Generation and analysis of pdgf-b<sup>ret/ret</sup>-deficient mice**

A targeting construct encompassing the 3′ region of the pdgf-b gene was generated with a premature translation stop codon inserted in exon 6 (amino acid position 211; see Fig. 1) by site-directed mutagenesis using the Altered Sites II In Vitro Mutagenesis System Q6210 (Promega). After sequence verification, the targeting construct was linearized with NotI and transfected into R1 ES cells. Correctly targeted ES cell clones were identified (Fig. 1) and used to generate germline pdgf-b<sup>−/−</sup> mutants using previously described protocols [Levén et al. 1994]. PolyaRNA isolation and Northern blotting were done using RNeasy midiprep, oligotex mRNA (QIAGEN), and Northern Max (Ambion) kits. For in situ hybridization, eyes were dissected in ice-cold 4% paraformaldehyde (PFA) and processed as described [Fruittiger 2002]. Immunohistochemistry on retinas was performed as described [Gerhardt et al. 2003]. Other mouse tissues were fixed in 4% PFA, paraffin-embedded, sectioned, and stained using standard protocols. For urine sampling, mice were placed in metabolic cages and urine collected during a 24-h period (volume range 1–3 ml). Cell-lysates were generated (Pietras et al. 2001). PDGF-B concentrations were analyzed by homogenous proximity ligation assay (EMD). PDGF-B binding properties of novel vascular endothelial growth factors. EMBO J. 21: 4307–4316.

**Endothelioma cultures**

Endothelial cells were collected from lungs of adult pdgf-b<sup>ret/ret</sup> and control mice and cultured as described [Allavena et al. 1995]. After 2–3 d, the cells were infected with polyoma virus [Bussolino et al. 1991]. Cells were routinely cultured in MCDB131 [Nitrogen] supplemented with 15% fetal calf serum [HyClone], endothelial cell growth supplement 50 µg/ml (Sigma), heparin 100 µg/ml [Sigma], glutamine, and antibiotics on gelatin-coated tissue culture dishes. Serum-free media conditioned for 24 h by subconfluent cultures of pdgf-b<sup>−/−</sup> and pdgf-b<sup>Δ exon−</sup> endothelioma cells were collected and concentrated to 10% of the original volume using Centriplus filter devices [Amicon]. Cell-lysates were generated [Pietras et al. 2002], and receptor phosphorylation was determined using cells stably transfected with PDGF β-receptor as described [Pietras et al. 2001]. PDGF-B concentrations were analyzed by homogeneous proximity ligation assay (EMD). PDGF-B binding properties of novel vascular endothelial growth factors. EMBO J. 21: 4307–4316.

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