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Role of PDGF-B and PDGFR-β in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse

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SUMMARY

Development of a vascular system involves the assembly of two principal cell types – endothelial cells and vascular smooth muscle cells/pericytes (vSMC/PC) – into many different types of blood vessels. Most, if not all, vessels begin as endothelial tubes that subsequently acquire a vSMC/PC coating. We have previously shown that PDGF-B is critically involved in the recruitment of pericytes to brain capillaries and to the kidney glomerular capillary tuft. Here, we used desmin and α-smooth muscle actin (ASMA) as markers to analyze vSMC/PC development in *PDGF-B*$^{-/-}$ and *PDGFR-β*$^{-/-}$ embryos. Both mutants showed a site-specific reduction of desmin-positive pericytes and ASMA-positive vSMC. We found that endothelial expression of PDGF-B was restricted to immature capillary endothelial cells and to the endothelium of growing arteries. BrdU labeling showed that PDGFR-β-positive vSMC/PC progenitors normally proliferate at sites of endothelial PDGF-B expression. In *PDGF-B*$^{-/-}$ embryos, limb arterial vSMC showed a reduced BrdU-labeling index. This suggests a role of PDGF-B in vSMC/PC cell proliferation during vascular growth.

Two modes of vSMC recruitment to newly formed vessels have previously been suggested: (1) de novo formation of vSMC by induction of undifferentiated perivascular mesenchymal cells, and (2) co-migration of vSMC from a preexisting pool of vSMC. Our data support both modes of vSMC/PC development and lead to a model in which PDGFR-β-positive vSMC/PC progenitors initially form around certain vessels by PDGF-B-independent induction. Subsequent angiogenic sprouting and vessel enlargement involves PDGF-B-dependent vSMC/PC progenitor co-migration and proliferation, and/or PDGF-B-independent new induction of vSMC/PC, depending on tissue context.

Key words: PDGF, Pericyte, Vascular smooth muscle cell, Mouse, Angiogenesis

INTRODUCTION

The vertebrate vascular wall is composed of two principal cell types, endothelial cells (EC) and mural cells. Depending on the morphology and density, the latter cells are referred to as either pericytes (PC) or vascular smooth muscle cells (vSMC). Pericytes are solitary vSMC-like cells associated with the finest diameter blood vessels, i.e. arterioles, capillaries and venules, and share basement membrane with the endothelium (Sims, 1986). The pericyte density and morphology varies between capillary networks in different tissues. Certain specialized types of pericytes have acquired specific names, such as the mesangial cells in kidney glomeruli and the perisinusoidal fat storing cells (Ito-cells) in the liver. Vascular SMC form concentric layers around larger blood vessels, i.e. arteries and veins.

Communication between the EC and vSMC/PC compartments appears to be essential for normal blood vessel development. This is illustrated by the phenotypes caused by mutations in genes coding for signaling molecules in the vascular wall. Although the expression pattern of such molecules suggests uni-directional signaling, from EC to vSMC/PC, or vice versa, the mutant phenotypes often reveal defects in both cell compartments. Ablation of the endothelial tie-2 receptor, or its ligand angiopoietin-1 (ang-1), which is produced by periendothelial cells, leads to defects in angiogenesis, vascular remodeling and blood vessel maturation (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996; reviewed in Folkman and D'Amore, 1996). This is attributed to a primary defect in the EC, since only these cells express the tie-2 receptor. In addition, there is loss of vSMC/PC. This is conceivably secondary to the EC defect. The human genetic disease venous malformation is caused by a point mutation in the *tie-2 receptor* gene, which leads to an amino acid substitution activating the receptor kinase (Vikkula et al., 1996). Patients have regionally thin-walled and extremely dilated veins, indicating that the changes in endothelial tie-2 receptor activity have secondary effects on venous vSMC (Vikkula et al., 1996).

The above examples suggest that vSMC/PC defects can be secondary to signaling changes in EC. The reverse also appears to be true. Platelet-derived growth factor (PDGF) receptor-β (PDGFR-β) is expressed by developing vSMC/PC and lack of its signaling leads, not only to pericyte loss, but also to

endothelial changes followed by capillary dilation (microaneurysm) and rupture (Lindahl et al., 1997a). The EC changes associated with microaneurysm formation are likely secondary to the pericyte loss.

In addition to the genetic data mentioned above, the importance of reciprocal signaling between EC and mural cells in vascular development is highlighted by in vitro and in vivo experiments. The expression and release of PDGF by cultured EC has been well documented (Barrett et al., 1984; Collins et al., 1985, 1987; DiCorleto and Bowen-Pope, 1983) and EC-derived PDGF-BB has been shown to stimulate the proliferation of co-cultured mesenchymal cells (Hirschi et al., 1998). Conversely, vSMC/PC inhibit EC proliferation and migration in vitro (Orlidge and D'Amore, 1987; Sato and Rifkin, 1989). Co-culturing of vSMC/PC and EC leads to the activation of latent transforming growth factor-β (TGF-β) (Sato et al., 1990), which may have a role in promoting differentiation of vSMC from undifferentiated mesenchymal cells (Hirschi et al., 1998). In addition, contact co-cultures of EC and undifferentiated mesenchymal cells leads to the release of a soluble growth inhibitor for both cell types distinct from TGF-β (Hirschi et al., 1999). In vivo experiments suggest that blood vessels that have not recruited vSMC/PC need vascular endothelial growth factor (VEGF) for survival, whereas vSMC/PC covered vessels may survive in the absence of VEGF (Benjamin et al., 1998, 1999). Together, the observations that vSMC/PC promote EC growth arrest and survival suggest that vSMC/PC recruitment is required for the maturation of blood vessels.

Studies on vascular development have largely focused mainly on the origin of EC, their de novo assembly into vessel networks (vasculogenesis) and the sprouting of new endothelial tubes from preexisting ones (angiogenesis), (reviewed by Risau, 1997). Concerning the recruitment of vSMC/PC to newly formed blood vessels, it has been suggested that EC may elicit a signal(s) that trigger condensation and vSMC/PC differentiation of mesenchymal cells around the endothelial tube (Le Lievre and Le Douarin, 1975; Nehls et al., 1992; Hungerford et al., 1996; Beck and D'Amore, 1997; Hirschi et al., 1998; reviewed in Drake et al., 1998). Subsequently, vSMC/PC progenitors proliferate and migrate along new angiogenic sprouts (Nicosia and Villaschi, 1995; Benjamin et al., 1998). Our previous analysis of PDGF-B-deficient mice suggests that PDGF-B produced by capillary EC signals to neighboring vSMC/PC progenitors carrying PDGFR-β to promote the co-migration of pericytes along angiogenic sprouts (Lindahl et al. 1997a). Here, we present a detailed analysis of the vSMC/PC defects in *PDGF-B* and *PDGFR-β* null embryos suggesting that de novo formation of vSMC/PC occurs independently of PDGF-B and PDGFR-β. However, PDGF-B and PDGFR-β are critically involved in subsequent co-migration and proliferation of vSMC/PC.

MATERIALS AND METHODS

Animals

PDGF-B$^{+/-}$ mice (Levéen et al., 1994) and *PDGFR-β*$^{+/-}$ mice (Soriano, 1994) were bred as 129Ola/C57BL6J and 129Sv/C57BL6J hybrids, respectively. Heterozygotes were intercrossed and offspring of different age and genotype were killed and fixed for immunohistochemistry or in situ hybridization as described elsewhere (Boström et al., 1996; Lindahl et al., 1997a,b). Ages of mice subject to analysis are given as embryonic days (E) or postnatal days (P) where the vaginal plug was scored on E0.5 and birth usually took place on E19.

Histological analysis

Immunohistochemistry was performed using antibodies directed against α-smooth muscle actin (ASMA) (DAKO, U7033) and desmin (DAKO, U7023) according to protocols supplied by the manufacturer. Tissues were fixed in 4% paraformaldehyde (PFA) in PBS (Sigma, P-6148), embedded in paraffin and sectioned. After rehydration, antigen retrieval was accomplished by two 5-minute incubations at 95°C in 10 mM citric acid, pH 6.0. Endogenous peroxidase activity was blocked by incubation with PBS containing 0.6% H_2O_2 for 10 minutes. For B4 lectin staining of endothelial cells, the glass slides were incubated in PBS, pH 6.8, 1% Triton X-100, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM $MnCl_2$ for 15 minutes at room temperature. Following incubation of sections with 5 μg/ml of biotin-conjugated isolectin B4 from *Bandeiraea simplicifolia* (Sigma, L-2140) in a humid chamber for 2 hours, lectin binding was detected by streptavidin-conjugated alkaline phosphatase (DAKO, D0396), using standard immunohistochemical procedures. Desmin and ASMA stainings were performed on four individuals of each mutant genotype (mutants were taken from different litters) and littermate controls.

In situ hybridization

We applied a non-radioactive protocol for in situ hybridization using digoxigenin-labeled RNA probes (Boehringer Mannheim), and their detection on sections using alkaline phosphatase-conjugated antibodies (Boström et al., 1996). *PDGF-B* and *PDGFR-β* sense and antisense probes were generated as described previously (Lindahl et al., 1997a). The results shown were obtained using 14 μm thick sections and interference contrast microscopy. As negative controls, the corresponding sense probes were used.

BrdU labeling and quantification of vSMC

Cells replicating their DNA were labeled by intraperitoneal injection of BrdU (Sigma, B-9285, 100 μg/g body weight) to the pregnant mother. The embryos were delivered 2 hours later by Cesarean section, fixed in 4% PFA overnight, embedded in paraffin and sectioned at 5 μm. Antibodies against BrdU (Becton Dickinson, #347580), a secondary rabbit anti-mouse biotinylated antibody (DAKO, E0354), HRP-conjugated streptavidin (DAKO, P0397) were used and visualized with TSA-direct red (NEN, #NEL702) according to protocols supplied by the manufacturer. Anti-ASMA antibodies, either FITC-conjugated (Sigma, F-3777) or HRP-conjugated (DAKO, U7033) were used. In the latter case, these were visualized using TSA-direct green (NEN, #NEL701). Propidium iodide (1 μg/ml, Sigma, P4170) staining was done in PBS with 0.1% Tween-20 for 15 minutes. Anatomically distinct blood vessels in the digits were analyzed at E 17.5 in wild-type (n=2) and PDGF-B mutant embryos (n=2). Pictures were taken in a Nikon Eclipse 1000 microscope equipped with an Optronics CCD camera.

BrdU labeling of *PDGFR-β*-positive vSMC/PC progenitors

PDGFR-β in situ hybridization was done on E11.5, E14.5 and E18.5 embryos delivered from mothers that had received single intraperitoneal injections of BrdU 2 hours earlier. Sections were subsequently stained with anti-BrdU antibodies, as described above, with the exception that the development was performed using 3,3′-diaminobenzidine (Sigma, D-5905). Pericapillary *PDGFR-β*-positive cells in developing brain tissue were scored as BrdU positive or negative.

RESULTS

Vascular *PDGF-B* and *PDGFR-β* expression

PDGF-B is expressed in capillary and arterial or arteriolar EC

during embryonic and early postnatal development (E11.5-P10) (Fig. 1 and data not shown). Venous EC lack *PDGF-B* expression during the embryonic stages (data not shown). A detailed analysis of brain vessels revealed a heterogeneous endothelial *PDGF-B* expression pattern. At E11.5 and E14.5, *PDGF-B* expression occurred in most vascular EC recognized in the brain (Fig. 1A-D). At E18.5, P4 and P10, this pattern was changed in that *PDGF-B* expression became restricted to short capillary segments without or with irregular lumina containing few erythrocytes. Such capillary segments were composed of EC with abundant cytoplasm and appeared immature. Thus, these EC might represent angiogenic sprouts. Their density was decreased at P4 compared with E18.5 and was even further decreased by P10. In contrast, the *PDGF-B*-negative capillaries had a regular diameter lumen containing abundant erythrocytes and appeared more mature than the *PDGF-B*-positive capillaries (Fig. 1E,F). Similar differences in *PDGF-B* expression between immature and mature capillaries were also seen outside the brain, e.g. in lung, heart, muscle and skin (data not shown). In late embryogenesis (E18.5), when *PDGF-B* was downregulated in mature capillaries (Fig. 1E,F), its expression continued in developing intracerebral arteries/arterioles (Fig. 1G). The liver was the only site where no endothelial *PDGF-B* expression could be found (data not shown).

In mid-to-late gestational embryos, developing pericytes are normally seen as solitary *PDGFR-β*-positive cells in close proximity to capillaries (Lindahl et al, 1997a,b, 1998). Developing vSMC of arteries/arterioles are likewise *PDGFR-β* positive, and occur in single or multiple layers around the endothelial tube (Lindahl et al, 1997a, 1998). Here we confirmed the presence of abundant *PDGFR-β*-positive vSMC/PC progenitors in all wild-type organs examined with the exception of the liver, which only contained rare *PDGFR-β*-positive cells that did not localize to the liver sinusoids (porta circulation), but might represent developing mural cells of the systemic circulation (data not shown). In E14.5-16.5 *PDGF-B*$^{-/-}$ embryos, *PDGFR-β*-positive PC progenitors were essentially lacking in several tissues, such as the brain, heart, brown adipose tissue, peripheral lung parenchyma and gastrointestinal villi. However, such cells were present, but less abundant, in several other tissues, such as skeletal muscle, skin and the adrenal gland in *PDGF-B*$^{-/-}$ embryos. There were also sites at which no apparent reduction in the abundance of *PDGFR-β*-positive cells was seen in *PDGF-B*$^{-/-}$ embryos, notably vascular plexa, such as the perineural vascular plexus, plexus choroideus and vascular plexa of the gastrointestinal tract (data summarized in Table 1).

At most sites studied, developing arteries were surrounded by similar amounts of *PDGFR-β*-positive cells in wild-type and *PDGF-B*$^{-/-}$ embryos (data not shown). As discussed in more detail below, we hypothesize that this reflects sites at which *PDGFR-β*-positive cells were induced in a PDGF-B-independent manner.

Desmin and ASMA as markers for pericytes and vSMC

PDGFR-β expression may be limited to a subset of developing vSMC or might be downregulated in conjunction with vSMC/PC differentiation and proliferative quiescence. In addition, whereas *PDGFR-β* has been a useful marker for the cellular defects in *PDGF-B*$^{-/-}$ mice, it cannot be applied to *PDGFR-β* null mice. To further characterize the vSMC/PC defects in *PDGF-B*$^{-/-}$ mice, and to compare the vascular phenotypes of *PDGF-B*$^{-/-}$ and *PDGFR-β*$^{-/-}$ mice, we therefore used desmin and α-smooth muscle actin (ASMA) as additional markers for developing vascular mural cells. It has previously been reported that mid-capillary pericytes are desmin positive but ASMA negative, whereas vSMC stain positive for both desmin and ASMA (Nehls and Drenckhahn, 1991; Nehls et al., 1992). In the adult mouse brain, we could confirm that desmin antibodies stained fine cytoplasmic processes running along but rarely surrounding the smallest diameter capillaries (Fig. 2B). Desmin also stained thicker processes encircling larger diameter capillaries/arterioles and the vSMC of some arteries (data not shown). ASMA staining generally overlapped that of desmin except for the finest diameter capillaries that were ASMA negative but desmin positive (Fig. 2A). Below, solitary desmin-positive mural cells in close proximity to capillaries will be referred to as pericytes, whereas ASMA-positive mural

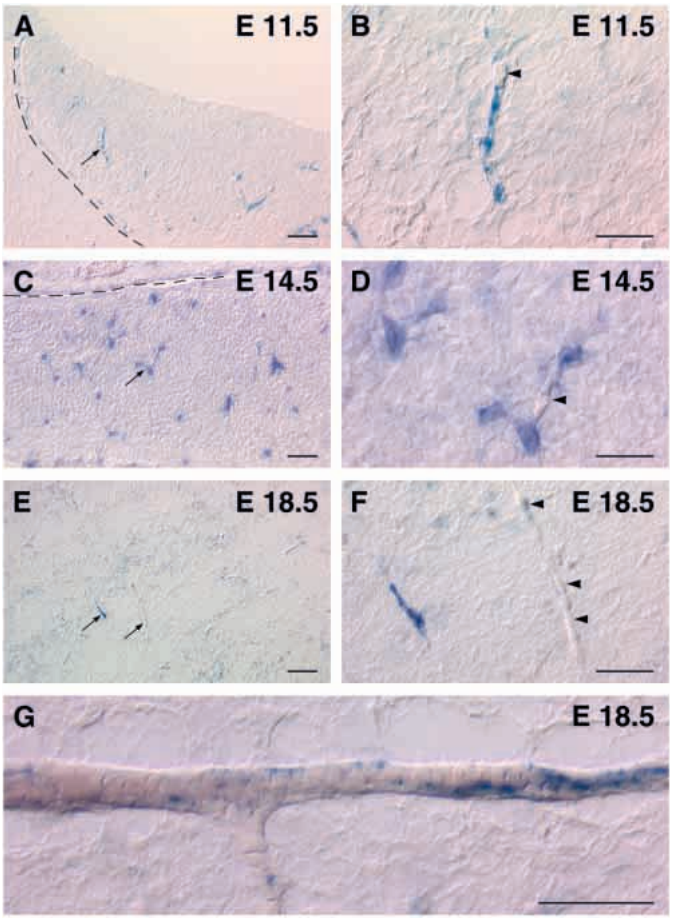

Fig. 1. *PDGF-B* expression by developing brain vascular endothelial cells. Non-radioactive in situ hybridization (blue labeling) on unstained sections viewed in interference contrast microscopy. The embryonic age is indicated. B,D and F represent high-power views of structures in A,C and E, respectively, indicated by arrows. Arrowheads in B,D and F point at erythrocytes. G shows a developing intracerebral artery. Dashed lines in A and C indicate the border between the brain and the perineural vascular plexus. Bars 100 μm in A,C,E; 50 μm in B,D,F,G.

Table 1. Presence of PDGF-Rβ- and desmin-positive pericytes

	PDGFR-β expression		Desmin staining			Microaneurysm
	wt	*PDGF-B*$^{-/-}$	wt	*PDGF-B*$^{-/-}$	*PDGFR-β*$^{-/-}$	*PDGF-B*$^{-/-}$; *PDGFR-β*$^{-/-}$
Brain capillaries	••••	•	••••	•	•	Yes
Heart	••••	•	n.a.	n.a.	n.a.	Yes
Brown adipose tissue	••••	•	••••	•	•	Yes
Lung	••••	•	••••	••	••	n.a.
GI villi	••••	•	••••	••	••	Yes
Skeletal muscle	••••	••	n.a.	n.a.	n.a.	Yes
Skin	••••	••	••••	••	••	Yes
Adrenal	••••	•••	••••	•••	•••	No
Perineural plexus	••••	••••	••••	•••	•••	No
Plexus choriodeus	••••	••••	••••	•••	•••	No
GI plexa	••••	••••	n.a.	n.a.	n.a.	No
Liver	–	–	••••	••••	••••	No

Differential loss of *PDGFR-β*- and desmin-positive pericytes, and its correlation to microaneurysum formation in *PDGF-B*$^{-/-}$ and *PDGFR-β*$^{-/-}$ embryos. The abundance of labeled cells was estimated by counting labeled cells on tissue sections.

•••• Wild-type abundance.

••• <70% of the wild-type abundance.

•• <30% of the wild-type abundance.

• <5% of the wild-type abundance.

There was no perisinusoidal *PDGFR-β* expression detectable in the liver, indicated '–'. Pericytic desmin staining was no applicable (n.a.) in tissues with parenchymal desmin expression, e.g. heart and skeletal muscle. Microaneurysm formation could not be evaluated in the embryonic lung. *PDGFR-β* staining by in situ hybridization was quantified in E14.5 and E16.5 embryos, desmin staining was quantified in E16.5 embryos and microaneurysms were scored in E17.5-18.5 embryos.

cells that cover vessels in single, or multiple concentric, layers will be referred to as vSMC.

Desmin-positive pericyte populations are differentially sensitive to the loss of PDGF-B or PDGFR-β

The abundance of desmin-positive pericytes was analyzed in E16.5 and E17.5 *PDGF-B*$^{-/-}$, *PDGFR-β*$^{-/-}$ embryos and littermate controls. In both types of mutants, there was a differential loss of pericytes depending on the tissue examined (Table 1). Both *PDGF-B*$^{-/-}$ and *PDGFR-β*$^{-/-}$ embryos completely lacked desmin-positive pericytes in the brain (Fig. 3A-E and data not shown). Most other tissues in the mutants exhibited severe but not complete loss of desmin-positive pericytes, e.g. the lung and gastrointestinal villi (Fig. 3J,K and data not shown). The perineural vascular plexus and the adrenal gland of both types of mutants showed a smaller but significant reduction in the abundance of desmin-positive pericytes (Fig. 3D-G, and data not shown). In agreement with lack of vascular *PDGF-B* and *PDGFR-β* expression in the liver sinusoids, there was no alteration in perisinusoidal desmin-positive cells in mutant embryos (Fig. 3H,I), suggesting that Ito cells (liver pericytes) develop independently of PDGF-B/PDGFR-β signaling. Loss of desmin-positive pericytes at E16.5 correlates spatially with subsequent microaneurysm formation at E17.5-18.5 in both *PDGF-B*$^{-/-}$ and *PDGFR-β*$^{-/-}$ embryos (Fig. 3C,E, and data not shown, summarized in Table 1). This supports the hypothesis that pericyte loss from the microvessel wall may trigger microaneurysm formation (Cogan et al., 1961; Buzney et al., 1977; Lindahl et al., 1997a,b).

In summary, the results obtained by using *PDGFR-β* or desmin as a marker for mural cells were similar: developing *PDGFR-β*- or desmin-positive mural cells were differentially sensitive to the loss of *PDGF-B* in a tissue-specific manner (Table 1). Moreover, the phenotypic analysis including desmin as a marker for developing pericytes shows that the phenotypes of *PDGF-B*$^{-/-}$ and *PDGFR-β*$^{-/-}$ embryos were indistinguishable regarding pericyte loss and microaneurysm formation.

Defective vSMC coverage of arteries in *PDGF-B*$^{-/-}$ and *PDGFR-β*$^{-/-}$ embryos

Large vessel dilation appears in the *PDGF-B* and *PDGFR-β* mutants at late gestation (Levéen et al., 1994; Soriano, 1994; Fig. 4I,J, and data not shown), but the cause of this phenotype has been unclear. The loss of pericytes in the mutants pointed to a possible defect in the vSMC coat of the larger vessels. Distal limb arteries and cardiac coronary arteries were chosen for analysis since corresponding vessels in mutant and wild-

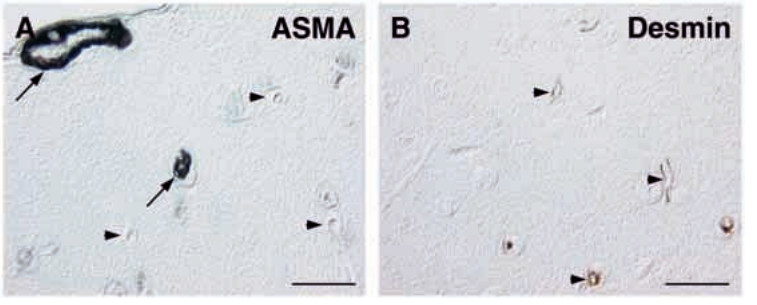

Fig. 2. ASMA and desmin expression by pericytes and vascular SMC. ASMA (A) and desmin (B) immunohistochemistry on unstained sections viewed by interference contrast microscopy. ASMA stains arteries and arterioles (arrows) but only few capillary-sized vessels (arrowheads). Desmin stains arteries and arterioles (not shown) and pericyte processes associated with capillaries (arrowheads). Bars 50 μm.

type embryos could easily be identified by spatial relationship to surrounding structures.

ASMA staining of E17.5 embryos indicated that the vSMC coat of mutant limb arteries was thinner than in wild-type arteries (Fig. 4A-D). In *PDGF-B*$^{-/-}$ and *PDGFR-β*$^{-/-}$ hearts, intramyocardial branches of the coronary arteries entirely lacked ASMA-positive vSMC (Fig. 4G,H). However, superficial pericardial coronaries were partially coated by vSMC (data not shown). A similar situation was found in the brain: No ASMA-positive developing arteries were found within the brain tissue of *PDGF-B*$^{-/-}$ or *PDGFR-β*$^{-/-}$ embryos, whereas vessels with partial vSMC cover could be found in the meningeal tissues surrounding the brain (data not shown). We also studied the vSMC coating of skin arteries. Although the ASMA-positive vSMC coat of deeper skin vessels was only slightly reduced (data not shown), the superficial vessels invariably exhibited a very poor ASMA-positive vSMC coat in *PDGF-B*$^{-/-}$ or *PDGFR-β*$^{-/-}$ embryos. This correlated with the extensive dilation of these vessels in the mutants (Fig. 4K,L).

Proliferation of *PDGFR-β*-positive vSMC/PC progenitors in the brain

Although PDGF-B clearly plays an important role in pericyte and vSMC recruitment to blood vessels, the cellular mechanism(s) controlled by PDGF-B in this process is unclear. Double-labeling experiments for *PDGFR-β* expression (in situ hybridization) and proliferation (BrdU immunohistochemistry) were performed at E11.5, E14.5 and E18.5, in wild-type embryos. Almost half of the *PDGFR-β*-positive vSMC/PC progenitors in the brain were BrdU labeled at E11.5 and E14.5 following a single BrdU injection (Fig. 5A). However, at E18.5, only few pericapillary *PDGFR-β*-positive cells became BrdU labeled (Fig. 5B). At the same age, *PDGFR-β*-positive cells surrounding developing brain arterioles showed a high labeling index (Fig. 5C).

These results show that the presence of cycling *PDGFR-β*-positive vSMC/PC progenitors in the brain correlate spatially and temporally with endothelial PDGF-B expression. As shown above, most brain capillary endothelial cells expressed *PDGF-B* at E11.5 and E14.5, at which time the *PDGFR-β*-positive cells cycled at a high rate. However, only few capillaries were *PDGF-B* positive at E18.5 and later (Fig. 1 and data not shown), at which time capillary *PDGFR-β*-positive cells cycled at a slow rate. At E18.5, *PDGF-B* expression persisted in intracerebral arteries/arterioles, which correlated spatially with cycling of *PDGFR-β*-positive cells. The correlation between endothelial *PDGF-B* expression and vSMC/PC progenitor proliferation is consistent with a role

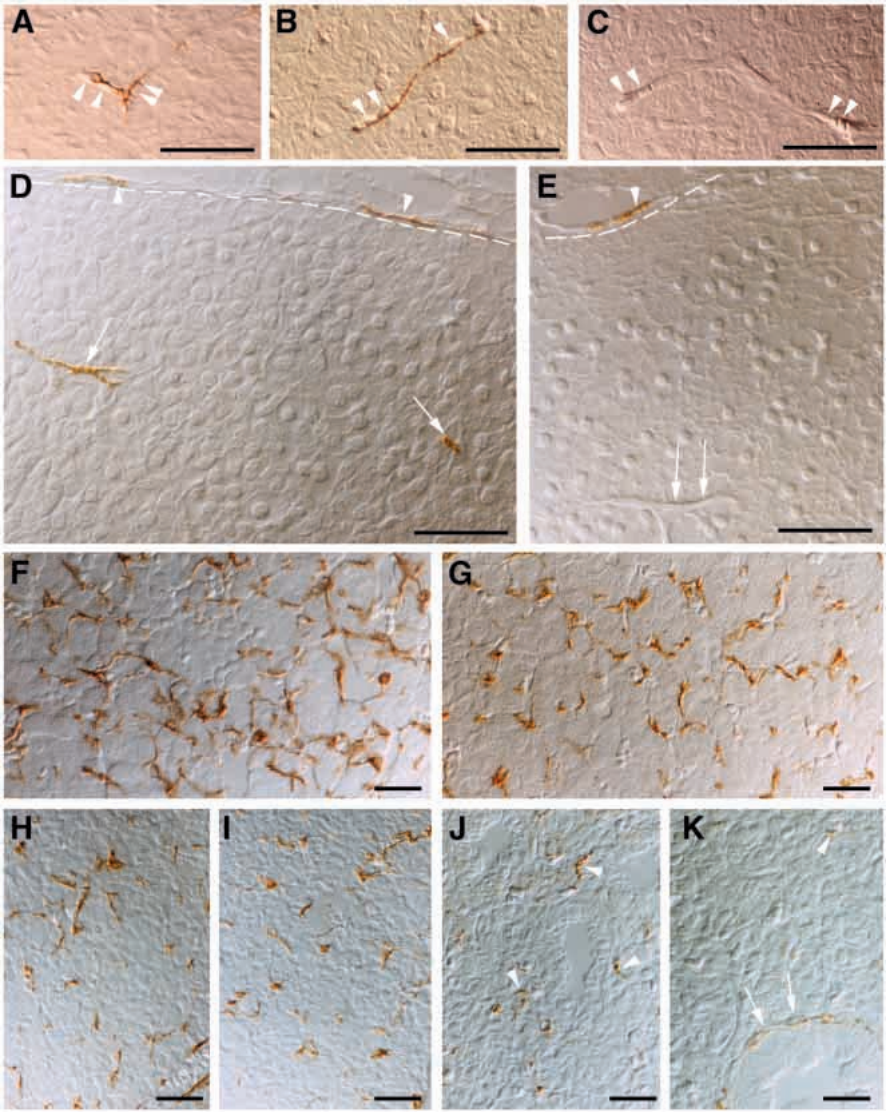

Fig. 3. Differential loss of desmin-positive pericytes in *PDGF-B*$^{-/-}$ tissues. Desmin immunohistochemistry on unstained sections viewed by interference contrast microscopy at E16.5. The tissues shown are brain (A-E), adrenal (F,G), liver (H,I) and lung (J,K). Thin desmin-positive pericyte processes were seen in association with wild-type brain capillaries (A,B,D; arrowheads point at erythrocytes that reveal the outline of the capillaries), but were not seen in *PDGF-B*$^{-/-}$ brain vessels (C,E). When pericyte cell bodies were seen in the sections, the stellate shape of the cells was confirmed (left arrow in D). Blood vessel-associated desmin staining was seen in the perineural vascular plexus of both wild-type (D; arrowheads) and *PDGF-B*$^{-/-}$ embryos (E; arrowhead). The capillaries in the *PDGF-B*$^{-/-}$ embryos exhibited microaneurysms (arrow in E). The wild-type adrenal showed abundant desmin-positive pericytes (F) whereas those cells were reduced in the *PDGF-B*$^{-/-}$ embryos (G). Desmin-positive perisinusoidal cells in the liver (Ito cells) were seen at the same abundance in wild-type (H) and *PDGF-B*$^{-/-}$ (I) embryos. In the lung (J,K), desmin stained developing vascular as well as bronchial smooth muscle. In general, there was a clear reduction in the abundance of solitary desmin-positive cells in the *PDGF-B*$^{-/-}$ lung (K; arrowhead) compared with the wild-type lung (J; arrowheads). As an internal control, the *PDGF-B*$^{-/-}$ lung showed normal desmin staining in association with developing bronchi (arrows in K). Dashed lines indicate border between brain and perineural vascular plexus. Bars 50 μm.

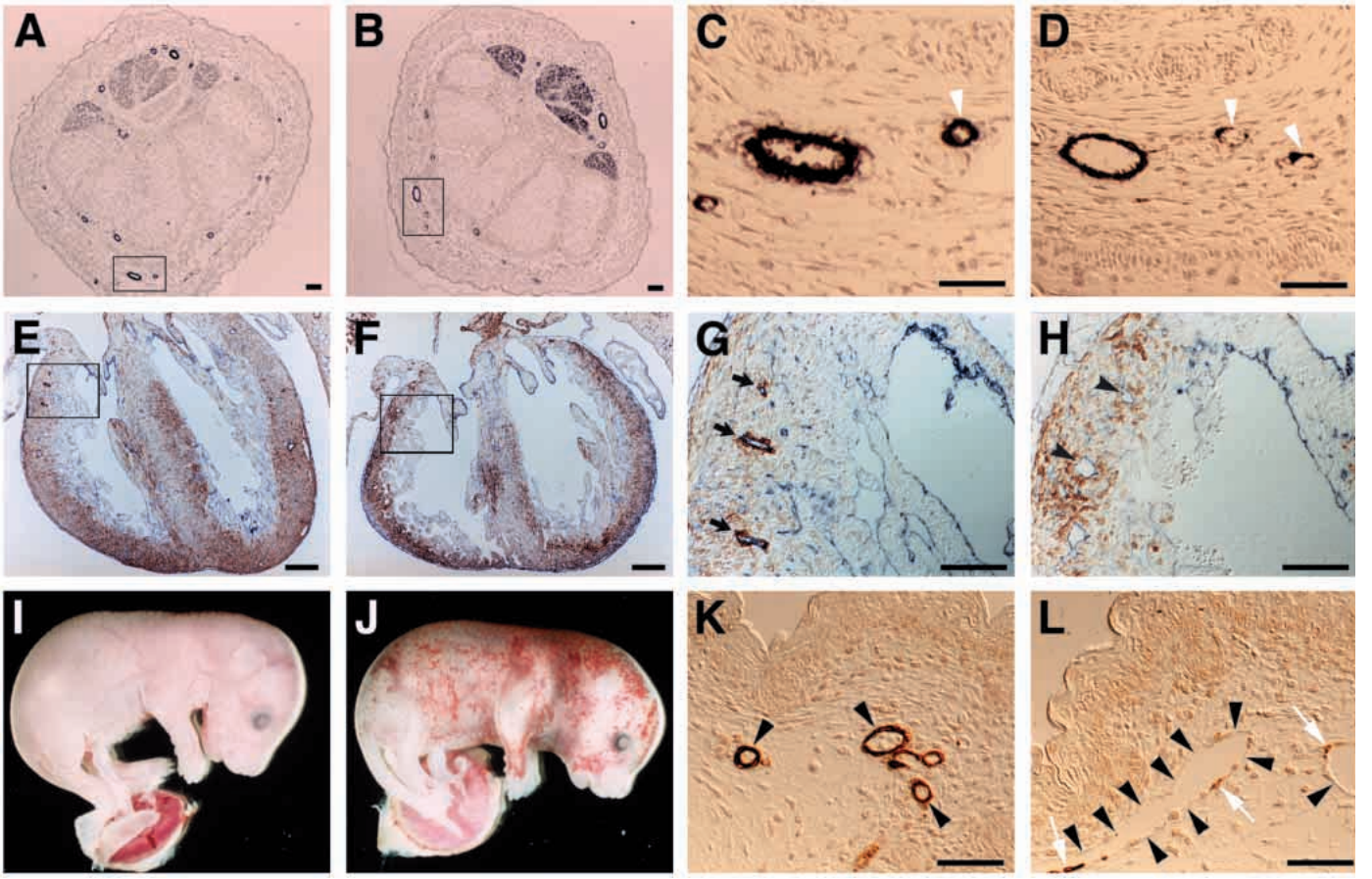

Fig. 4. Failure of recruitment of vSMC to small arteries in limb, heart and skin of *PDGF-B*$^{-/-}$ and *PDGFR-β*$^{-/-}$ embryos. ASMA staining of sections from the distal limb (A-D), heart (E-H) and skin (K,L) of wild-type (A,C,E,G,K), *PDGFR-β*$^{-/-}$ (B,D,F,H) or *PDGF-B*$^{-/-}$ (L) embryos. (C,D) Regions in wild-type and *PDGFR-β*$^{-/-}$ limbs as indicated in the overview sections (A,B). ASMA staining revealed that the distal limb arteries had a reduced ASMA-positive vSMC coat (compare D with C). (E-H) ASMA staining (brown) was combined with B4 lectin staining (blue) that labels endothelium and endocardium. (G,H) show indicated regions in (E,F) at higher magnification. The *PDGFR-β*$^{-/-}$ intramyocardial coronaries completely lacked ASMA-positive vSMC coat (compare G and H). (I,J). External appearance of E18.5 wild-type and *PDGF-B*$^{-/-}$ embryos. Note the abundance of dilated blood vessels in the *PDGF-B*$^{-/-}$ embryo. In the wild-type skin, small arteries and arterioles were readily identified by ASMA staining (K, arrowheads). In the *PDGF-B*$^{-/-}$ skin, the dilated blood vessels (L, arrowheads outline the vessels) had very few associated ASMA-positive cells (white arrows). Bars 50 μm.

for PDGF-B in controlling cell proliferation during the recruitment of both pericytes and vSMC to brain blood vessels.

Reduced vSMC proliferation in *PDGF-B*$^{-/-}$ digital arteries

To further address if PDGF-B signaling is involved in vSMC proliferation, we compared ASMA/BrdU double-labeling index in E17.5 wild-type and *PDGF-B*$^{-/-}$ digital arteries. These distal, supposedly newly assembled, arteries were anatomically distinct and therefore allowed careful side-by-side comparisons between mutant and wild-type embryos. The anatomical level analyzed is illustrated in Fig. 6A, and the type of analysis – double labeling for propidium iodide (PI)/ASMA, and BrdU/ASMA – is illustrated in Fig. 6B,C. The PI/ASMA and BrdU/ASMA double stains were done to visualize total and proliferating vSMC, respectively. Data are summarized in Table 2 and show a 2-fold reduction in the BrdU-labeling index in *PDGF-B*$^{-/-}$ vessels compared with littermate control wild-type vessels. Similar data were obtained in a PDGFR-β mutant (data not shown). We conclude that lack of PDGF-B/PDGFR-β signaling in developing digital arteries leads to a reduced rate of cycling of ASMA-positive vSMC.

Table 2. vSMC proliferation in digital arteries in wild type and *PDGF-B* null mice

Genotype	No BrdU-positive vSMC/No. vessel cross sections	No. vSMC/no. vessel cross section	BrdU labeling index*
1. +/+	43/25	184/26	0,24
2. +/+	62/101	309/95	0,19
3. –/–	15/36	131/28	0,09
4. –/–	17/26	132/20	0,10

Animal no. 1 and 4; 2 and 3 are littermates.
*BrdU-labeling index is the proportion of BrdU-positive vSMC in the digital arteries. The value was calculated from the quotient of BrdU-positive vSMC/vessel cross section divided by the quotient of vSMC/vessel cross section.

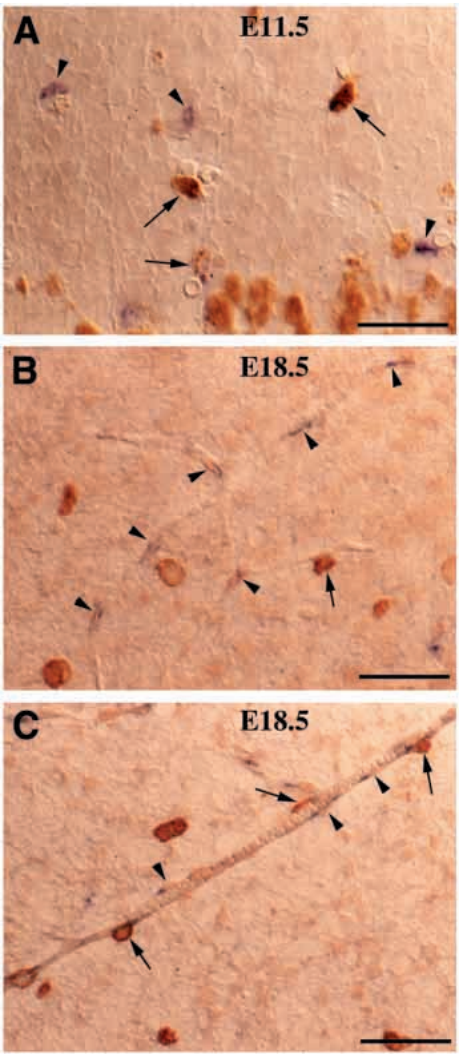

Fig. 5. Vascular SMC/PC progenitor cell proliferation in brain blood vessel wall formation. Wild-type brain sections from E11.5 and E18.5 embryos were double stained for BrdU (brown) and *PDGFR-β* (light blue). At E11.5 (A), double-labeled (arrows) cells were abundant in association with developing blood vessels. Non-BrdU-labeled *PDGFR-β*-positive cells are indicated with arrowheads. At E18.5 (B), mature capillaries can be seen using interference contrast microscopy through their content of erythrocytes. These vessels had associated *PDGFR-β*-positive cells which were predominantly non-BrdU-labeled (arrowheads in B). The arrow in B indicates a BrdU-labeled *PDGFR-β*-negative cell. Larger vessels (C) were associated with abundant double-labeled cells (arrows); arrowheads indicate *PDGFR-β*-positive non-BrdU-labeled cells. Bars 50 μm.

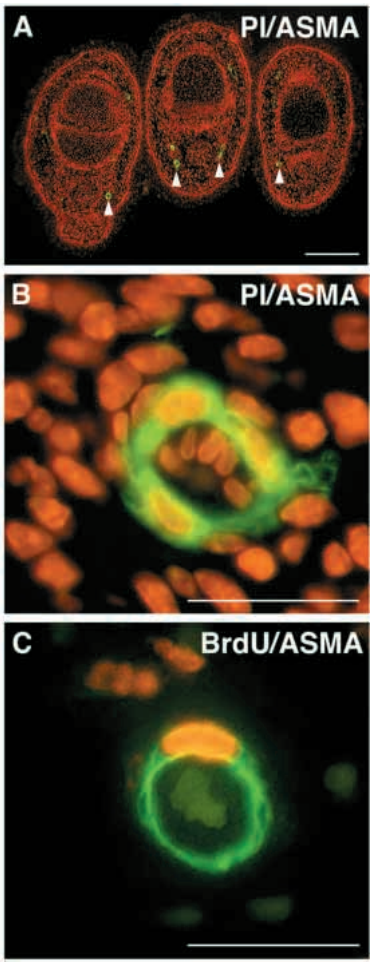

Fig. 6. vSMC proliferation in digital arteries. The figure shows examples of sections used to calculate the BrdU-labeling indices in the vSMC of digital arteries (Table 2). (A) Overview of propidium iodide (PI) and ASMA double staining. PI (red) stains all cell nuclei and ASMA (green) stains vSMC cytoplasm. Three cross-sectioned digits are seen. Arrowheads indicate the vessels selected for analysis. (B) A digital artery at high magnification shows that the vessel is surrounded by three vSMC sectioned through the nuclei. (C) A vessel section in which one vSMC has a BrdU-labeled nucleus (red). Bars 200 μm (A); 50 μm (B,C).

DISCUSSION

We have previously shown that PDGF-B/R-β signaling is required for pericyte co-migration along angiogenic sprouts in the brain (Lindahl et al., 1997a), but our data presented here establish that the dependence on PDGF-B/R-β-mediated pericyte and vSMC recruitment is variable in a tissue-specific manner. That some *PDGFR-β*-positive vSMC/PC progenitors remain in the *PDGF-B* mutants was expected, since vSMC/PC progenitors must express *PDGFR-β* before they can respond to PDGF-B secreted by the endothelium. Such *PDGFR-β*-positive vSMC/PC progenitors are likely formed by EC-mediated induction of perivascular mesenchymal cells. This hypothesis is supported by a recent demonstration that EC release a transforming growth factor-β (TGF-β)-like factor that promotes vSMC differentiation of 10T1/2 cells in vitro (Hirschi et al., 1998). Angiogenic sprouting into the developing limb may illustrate a situation in which PDGF-B-independent induction of SMC/PC progenitors is followed by PDGF-B-dependent expansion of the induced pool of vSMC. This scenario would explain why some vSMC remain in the mutant limb lacking PDGF-B or PDGFR-β. As a noticeable exception, liver perisinusiodal pericytes did not express *PDGFR-β*. In addition, liver capillary cells do not express *PDGF-B*. As anticipated, the abundance of liver pericytes was not reduced in *PDGF-B* or *PDGFR-β* mutants.

Once induced, the *PDGFR-β*-positive cells can be targeted by PDGF-B from the endothelium and be co-recruited along

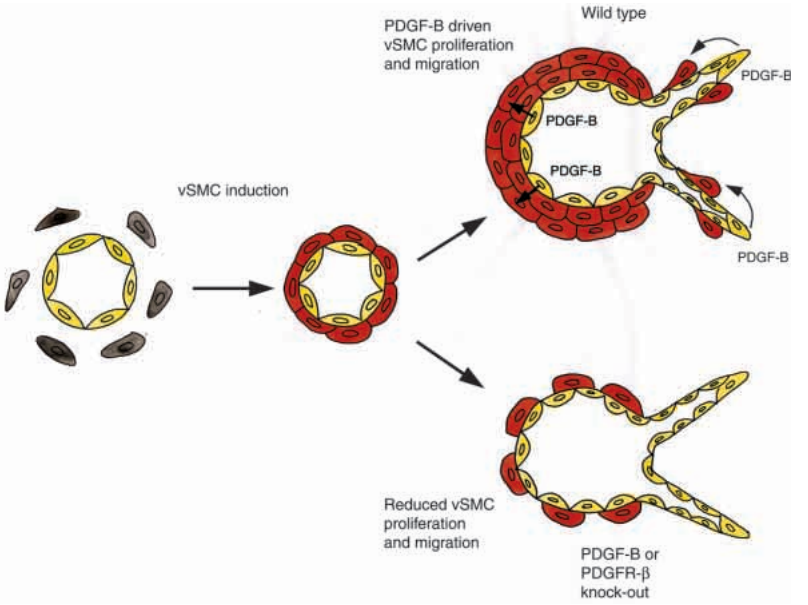

Fig. 7. Role of PDGF-B in vSMC/pericyte development. Undifferentiated mesenchymal cells (gray) surrounding the newly assembled endothelial tube (yellow) undergo induction to a vSMC/PC fate and assemble into a vascular wall (red). In subsequent vessel enlargement and angiogenic sprouting PDGF-B released by the endothelium drives vSMC proliferation and migration. Lack of PDGF-B or PDGFR-β leads to reduced vSMC/PC proliferation and migration, and vSMC hypoplasia of larger vessels and PC deficiency in capillaries.

sprouting capillaries. This is typically seen in the central nervous system (CNS). The initially avascular CNS would be presumed to lack cells (i.e. undifferentiated mesenchymal cells) that are competent to be induced to a vSMC/PC fate. Hence, the CNS would be completely dependent on vSMC/PC co-recruitment to acquire vascular mural cells to its vessels. PDGF-B-driven vSMC/PC recruitment to brain vessels likely involves both cell migration and proliferation. Pericytes are abundant in the *PDGF-B*$^{-/-}$ perineural vascular plexus but fail to enter the CNS along with the angiogenic sprouts. This suggests that PDGF-B normally promotes co-migration of pericytes along these vessels (Lindahl et al., 1997a). PDGF-B is indeed known to promote directed migration of mesenchymal cells in culture (Grotendorst et al., 1981 Nistér et al., 1988; Siegbahn et al., 1990; Vassbotn et al., 1992). A role for PDGF-B in promoting cell proliferation is suggested by the temporal and spatial correlation between endothelial *PDGF-B* expression and vSMC/PC progenitor cell BrdU incorporation in normal embryos, and the reduced proliferation rate of vSMC in *PDGF-B* mutants.

Available data are consistent with the model for PDGF-B driven vSMC/PC development illustrated in Fig. 7. The newly formed vessel releases a signal that leads to induction of *PDGFR-β*-positive vSMC/PC progenitors from surrounding undifferentiated mesenchyme. The model implies that the expression of PDGFR-β is an important part of the induced vSMC/PC progenitor cell phenotype. The requirement of PDGF-B for vSMC/PC proliferation and migration varies between different vessels. Thus, the differential vSMC/PC loss in *PDGF-B*$^{-/-}$ and *PDGFR-β*$^{-/-}$ embryos would reflect the proportion of induced versus PDGF-B/PDGFR-β-dependent SMC/PC formation.

The model is supported by a recent study in which the relative contribution of *PDGFR-β*$^{-/-}$ cells in mouse chimeras was found to be reduced in the smooth muscle compartment of all blood vessels (Crosby et al., 1998). Our data demonstrate a role for PDGF-B in many blood vessels but, for example, not in veins. However, veins have only rudimentary SMC coating before birth, when *PDGF-B*$^{-/-}$ and *PDGFR-β*$^{-/-}$ mice are available for analysis. The suggested model for PDGF-B function in arterial wall development before birth may well apply to venous wall development after birth.

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