Abnormal kidney development and hematological disorders in PDGF β-receptor mutant mice

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Platelet-derived growth factor, a major mitogen and chemoattractant for a number of cell types, is implicated in the processes of wound healing, tumorigenesis, and differentiation and is recognized by two receptors, α and β. To begin understanding the role of these receptors in development, β-receptor-deficient mice were generated by gene targeting in ES cells. Mutant mice are hemorrhagic, thrombocytopenic, and severely anemic, exhibit a defect in kidney glomeruli because of a lack of mesangial cells, and die at or shortly before birth. However, many cell types and tissues that express the receptor, including major blood vessels and the heart, appear normal in the absence of the receptor. These results indicate that whereas the β receptor is essential in certain cell types during embryonic development, its broader role may be masked because of compensation by the α-subunit.

[Key Words: Kidney development; PDGF; mouse genetics; ES cells]

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Platelet derived growth factor (PDGF), one of the best-characterized mitogens for a number of cell types (Kohler and Lipton 1974; Ross et al. 1974), consists of a disulfide-bonded dimer of two polypeptides chains, A and B. It can be found either as a homodimer, AA or BB, or as a heterodimer, AB. Although PDGF was first identified in platelets, it is also known to stimulate other cell types than fibroblasts to divide, such as glial cells (Westermark and Wasteson 1976), and to be expressed in macrophages, smooth muscle cells, and endothelial cells (for review, see Ross et al. 1986). On the basis of its expression pattern and ectopic expression studies, PDGF is believed to be involved in wound healing, atherosclerosis, tumorogenesis, and differentiation.

The receptors for PDGF are protein tyrosine kinases, which consist of two chains, α (Matsui et al. 1989) and β (Yarden et al. 1986). The α receptor (αR) can bind both PDGF A and PDGF B, whereas the β receptor (βR) binds only PDGF B with high affinity (Hart et al. 1988; Heldin et al. 1988; Seifert et al. 1989). Ligand binding induces the receptors to dimerize as αα, ββ, or αβ units. Dimerization leads to a conformational change that activates the kinase domain and a downstream signaling cascade involving a number of molecules that interact with the intracellular domain of the receptors (for review, see Williams 1989; Kazlauskas 1994). According to the specificities of the two receptor chains, the αα receptor should bind all three PDGF forms, the αβ receptor should bind both PDGF AB and BB, and the ββ receptor should bind only PDGF BB (Seifert et al. 1989). However, it has been shown that PDGF AB can bind and signal through the ββ receptor with low affinity (Seifert et al. 1993; Abboud et al. 1994).

During mammalian development, PDGF and its receptors are expressed at the preimplantation stage (Rappolee et al. 1988), in a variety of mesenchymal cells in the mid-gestation embryo (Mercola et al. 1990; Orr-Urtreger and Lonai 1992; Orr-Urtreger et al. 1992; Schatteman et al. 1992), in the developing and adult central nervous system (Sasahara et al. 1991; Yeh et al. 1991, 1993) and in the kidney glomerulus (Alpers et al. 1992). Mice carrying mutations in either receptors or ligands might help shed light on the physiological role of PDGF and its receptors in vivo. To understand the role of the β receptor, I have introduced two mutations into the gene by homologous recombination in embryonic stem (ES) cells and derived mutant mice. Homozygotes do not survive past birth, are anemic and thrombocytopenic, and exhibit defects in the formation of kidney glomeruli.

Results

Derivation of mutant mice

Two different targeting vectors were used to introduce a mutation at the βR locus (Fig. 1A). One of the vectors includes a promoterless β-geo (β-gal/neo fusion; Friedrich and Soriano 1991) gene inserted in-frame between a SmaI site in the exon coding for the signal peptide and an EcoRV site in an exon coding for the second immunoglobulin domain. Because the βR is expressed in ES cells (data not shown), homologous recombination at
the βR locus should lead to β-geo expression, which can be used both to select for the recombinants and to monitor βR expression during development by X-gal staining. In the second vector, a neomycin phosphotransferase (neo) expression cassette (PGKneobpA; Soriano et al. 1991) was used to replace the 1.8 kb of βR genomic sequences between the Smal and EcoRV sites. Both vectors include 6 kb of genomic sequences 5' of the β-geo or neo genes, 3 kb on the 3' side, and a herpes simplex virus (HSV) thymidine kinase expression cassette for negative selection. Following electroporation of the targeting vectors into ABI ES cells and positive/negative selection with G418/FIAU, 1 of 6 [β-geo vector] and 10 of 445 [neo vector] colonies scored positive for targeting at the βR locus by a polymerase chain reaction (PCR) assay. Southern blot analysis of the clones using a flanking probe confirmed that the vectors had inserted by homologous recombination (Fig. 1B). Hybridization with a neo probe demonstrated the presence of a single neo copy at the locus, but several [two to three] copies of β-geo arranged in a concatemer [data not shown].

Germ-line chimeras were derived from the β-geo clone and two neo clones to give rise to strains βR1, βR2, and βR3, respectively. Mutant mouse stocks were maintained as inbred 129Sv mice or as 129Sv/C57BL6J hybrids. Heterozygotes did not display an overt phenotype, nor was there a difference in the homozygous mutant phenotype among the three lines in either genetic background.

To examine the expression pattern of the βR during embryogenesis, embryos derived from matings of heterozygous βR1 males to wild-type females were isolated at different stages of gestation and stained for β-geo activity (Fig. 2). High levels of expression were observed in embryonic day 8.5 (E8.5) embryos especially in the cephalic mesenchyme but not in the heart (Fig. 2A). At E9.5 and E10.5, expression became more restricted, particularly to
the heart, although β-geo-positive cells were observed in the somites and mesenchyme of the limb buds [Fig. 2B,C]. At E12.5, high levels of expression were observed in the heart, the choroid plexus, and developing vertebrae along the neural tube [Fig. 2D]. Sections revealed that the staining in the heart was broad, but strongest in the endocardium and the septum separating the two ventricles. Little staining was observed in the pericardium. In addition, β-geo expression was observed in the notochord and surrounding cells, and in mesenchymal cells surrounding the esophagus and the aorta [data not shown].

**βR mutants die at birth**

Crosses between heterozygous parents failed to produce homozygous mutant offspring at weaning, suggesting lethality at an earlier stage [Table 1]. Embryos were isolated at different stages of gestation and genotyped by blot analysis of yolk sac DNA. Mutant embryos were recovered in the expected proportion between E11 and E18. No overt phenotype could be detected in mutant embryos younger than E16. At this stage, however, about one-third of the mutant embryos [Table 1] exhibited purpura, an accumulation of blood under the surface of the dermis. These embryos were also edematous and some of them were dead. These phenotypes were more striking in E18.5 embryos [Fig. 3]. However, two-thirds of the mutant embryos at both time points appeared normal and alive in utero and did not show any signs of growth retardation relative to their wild-type or heterozygous littermates [Table 1]. On occasion, dead pups were observed in newborn litters, which were mostly found to be mutants. These observations suggest that the majority of the homozygous mutant embryos die perinatally.

To examine the fate of E18.5 embryos in more detail, 10 live mutant pups with no purpura were isolated by cesarean delivery. In contrast to wild-type or heterozygous embryos that started breathing and turned pink within a few minutes after delivery, mutant pups died within a matter of minutes either after becoming very pale or suddenly losing most of their blood through the cut in the umbilical cord. Although the mutants could react to external stimuli such as pricking, they could only gasp for breath a few times before dying. Postmortem inspection revealed some air in the lungs indicating that the defect was not in their ability to breathe.

To verify that the mutation creates a null allele, RNA and protein were isolated from homozygous embryos and subjected to blot analysis. Using a probe representing all of the coding sequence downstream of the signal peptide as well as the 3’-untranslated region, RNA blot hybridization demonstrated the absence of PDGFβR message in the βR1 strain [Fig. 4] and the presence of a shorter transcript in the βR2 and βR3 strains [data not shown], presumably because of exon skipping [Lowell et al. 1994]. Western blot analysis of total protein isolated from these embryos likewise failed to reveal the presence of immunoreactive PDGFβR, using a polyclonal antibody directed to the carboxy tail [data not shown]. As the phenotypes from all three strains appear to be iden-

![Figure 3. Phenotype of a mutant βR embryo at birth. This embryo was dead at birth and exhibits a bloated appearance, extensive edema, and purpura.](image)

![Figure 4. The mutation at the βR locus creates a null allele. Total RNA from wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−) E16 embryos was separated on a 1% agarose gel in the presence of formaldehyde and analyzed by blot hybridization using a βR cDNA probe from the Sinai site at nucleotide 226 to a KpnI site at nucleotide 4952 (Yarden et al. 1986).](image)
tical, these data indicate that both mutations at the PDGFβR locus generate null alleles.

**Hemolytic defects in βR mutants**

The pallor and blood loss of embryos delivered by cesarean section might reflect an inability to oxygenate or hemorrhaging attributable to blood cell disorders. To examine this possibility in more detail, microhematocrits were performed on mutant embryos. This analysis revealed that among five mutants examined, all were significantly anemic (18–21% with one at 30%) compared with their wild-type or heterozygous littermates (37–50%). Blood cells were analyzed further by Giemsa-Wright smears (Fig. 5). The most noticeable feature was a significantly elevated number of nucleated erythrocytes, which was also readily detected in paraffin sections. Nuclear fragments, present as Howell-Jolly bodies, were also observed frequently. The analysis revealed significant polychromasia, consistent with the increase in erythropoiesis. In addition, mature erythrocytes were often shaped irregularly and of varying sizes, indicating anisocytosis, and there were numerous debris and schistocytes. Mutants were significantly thrombocytopenic, as platelet counts were on the order of 400,000/μl, compared with 900,000/μl for heterozygous or wild-type littermates. The low platelet count did not, however, prevent the blood from clotting, suggesting the absence of an inherent defect in clotting factors. These findings might indicate an inherent defect in maturation of erythroid and thrombocytic series cell maturation. Alternatively, the mutants might suffer from microangiopathic hemolytic anemia, perhaps because of a generalized defect in the microvasculature. The combined effects of the hematological disorders and the hemorrhages likely play a major part in the perinatal lethality.

**Anatomical defects in mutant embryos**

Homozygous embryos were isolated at various stages of gestation and examined anatomically and by histology. Gross dissection of E18 embryos revealed an abnormal phenotype only in the kidneys, which showed specks of blood. Further examination by histology showed the defect to be at the level of the glomeruli [Fig. 6]. This phenotype was fully penetrant and was observed in mature glomeruli of five E16 and eight E18 embryos. The capillary tuft normally consisting of podocytes and mesangial cells was missing in every glomerulus examined and was replaced with blood cells filling up the capsule space [Fig. 6C,D]. These abnormal glomeruli were present, however, in normal numbers. Immature glomeruli identified as U- or S-shaped vesicles (arrows) did not exhibit an abnormal phenotype. These observations suggest that the defect lies in glomerulus maturation rather than in glomerulus formation.

Further examination by electron microscopy confirmed the abnormalities in the mutant glomeruli [Fig. 7]. In contrast to the intricate capillary network found in wild-type glomeruli, few capillaries were observed in the mutants. These were delineated by well-formed basement membrane, endothelial cells, and podocytes with their characteristic pedicels extending around the basement membrane [Fig. 7B]. Because capillaries were so scarce, mesangial cells could not be identified positively within the loops.

Mutant embryos exhibited a dilation of veinules and accumulation of red blood cells under the surface of the skin, consistent with the purpura observed in some embryos [data not shown]. In contrast, major veins and arteries appeared normal, indicating that the defects in the circulatory system were not generalized. The heart, which based on the β-galactosidase staining of βR1 embryos expresses relatively high levels of the βR, did not exhibit an overt phenotype either.

**Up-regulation of αR mRNA**

PDGF B can associate both with the βR and the αR. To examine whether the PDGFβR is up-regulated in response to the βR mutation, total RNA was isolated from E16 embryos, and Northern blots were quantified using a PhosphorImager [Fig. 8]. No significant difference was observed in the amount of αR mRNA in heterozygous embryos relative to wild type. However, a 1.6-fold increase was observed in homozygous embryos relative to heterozygous embryos, when the amount of RNA in the different lanes was normalized for the amount of rRNA. This small increase was observed in four mutants among three litters in three separate experiments. Similar observations were made using RNA from E18 embryos [data not shown]. This variation in the level of mRNA was not detected at the protein level, nor were there

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**Figure 5. Blood disorders in mutant embryos.** A blood smear from a mutant E18 pup was stained using Giemsa-Wright. Note nucleated [thin arrows] and fragmented [thick arrows] red blood cells, and irregular shapes indicating anisocytosis.
differences in the levels of PDGF A or PDGF B mRNA in the mutant embryos (data not shown).

Discussion

Several lines of evidence using a variety of tissue culture cell lines indicate that the βR only binds PDGF B with high affinity, whereas the αR binds both PDGF A and PDGF B (Seifert et al. 1989). A simplistic model in which the downstream pathways and final targets of both receptors are the same, after binding of PDGF AA, AB, and BB, would predict that disruption of either of the receptors or the ligands should lead to phenotypes with varying degrees of severity: In a βR mutant background, however, all three PDGF dimers [AA, AB, BB] should still bind to the αR (Fig. 9). The analysis of the βR mutant phenotype presented here indicates that the βR has an essential role in some tissues or cell types. These may either not express the αR, or the signaling downstream of the αR receptor might be different from that of the αβ or ββ receptors. If the downstream targets of all three receptor dimers are the same, expression of the αR at sites normally expressing the βR should be able to rescue the mutant phenotype.

Examination of the β-galactosidase staining in βR1 heterozygous embryos revealed βR expression in the heart and in a variety of mesenchymal tissues. These results are similar to a recent in situ hybridization study (Shinbrot et al. 1994). Some of these locations also show expression of the αR, but the general patterns of expression differ significantly. For instance, the heart is a predominant site of expression of the βR but not of the αR (Orr-Urtreger et al. 1992, Schatteman et al. 1992). In addition, the strongest β-geo staining was observed in the endocardium and the septum separating the two ventricles, whereas αR expression is restricted to the pericardium (Orr-Urtreger et al. 1992). These data indicate that the two receptors are often expressed at distinct sites during embryogenesis.

βR mutants died perinatally and exhibited defects in the blood and in the kidney glomerulus. Cesarean-delivered pups were anemic and exhibited high numbers of nucleated erythrocytes and polychromasia. This increase in erythropoiesis is presumably in response to the loss of blood cells. Blood cells were, however, of varying shape and numerous debris, and schistocytes were observed and platelet counts were low. There are two possible explanations for the hematological disorders. First, loss of the βR might lead to an inherent deficiency in erythropoiesis. It might be possible to test this hypothesis by transferring mutant fetal liver cells into irradiated wild-type newborn pups. Second, the defects might be attributed to microangiopathic hemolytic anemia. This type of anemia is usually associated with narrowing or obstructions in the capillary microvasculature and is observed both in disseminated intravascular coagulation (DIC) and in thrombotic thrombocytopenic purpura (TTP). Whereas some of the mutant pups exhibited purpura, further analysis will be required to classify the defects more precisely.

Capillaries are normally composed of a single layer of endothelial cells surrounded by pericytes. In contrast, veins and arteries are composed of endothelial cells surrounded by layers of smooth muscle cells and collagen and elastic fibers. It has been shown that the βR and PDGF B are expressed during angiogenesis in the syncytiotrophoblasts of the developing human placenta, which subserve endothelial function in capillaries, whereas endothelial cells from the larger blood vessels express PDGF B but not the βR (Holmgren et al. 1991). PDGF may thus serve in an autocrine mode to stimulate growth of capillary endothelial cells but have a paracrine role to stimulate the growth of βR-positive mesenchymal cells surrounding the larger blood vessels. The βR,
PDGF-β-receptor mutant mice

Figure 8. PDGFαR expression is up-regulated in the absence of PDGFβR. Total RNA from wild-type (+/+), heterozygous (+/-), and homozygous mutant (-/-) E16 embryos was separated on a 1% agarose gel in the presence of formaldehyde and analyzed by blot hybridization using a full-length αR cDNA probe.

Figure 7. Electron microscopy of wild-type and mutant glomeruli. (A) Wild type, (B) mutant. (P), podocyte; (pe) pedicel processes; (bl) basal lamina; (M) mesangial cell; (ce) capsular epithelium; (E) endothelial cell. Mutant glomeruli accumulate red blood cells but mesangial cells could not be identified positively. Magnification, 800× (A); 1200× (B).

but not the αR, has been shown to be expressed in rat brain capillary endothelial cells as well (Smits et al. 1989). In the absence of the βR, endothelial cells from capillaries might be affected, resulting in microangiopathic hemolytic anemia. Preliminary observation indicates that capillaries in skeletal muscle are more narrow and of irregular shape relative to control littermates.

In the kidney of mutant pups, the capillary tuft was considerably reduced or even lacking, resulting in a reduced glomerular filtration area. Electron microscopy observation revealed that the basement membrane surrounding the capillaries was present and that endothelial cells were present. The most conspicuous phenotype, however, was the absence, or significant paucity, of mesangial cells. Although several mutations in the mouse affect early kidney development, including that in another receptor tyrosine kinase, c-Ret (Schuchardt et al. 1994), this and the PDGF B mutation described in the accompanying paper (Levén et al., this issue) are the first mutations that lead specifically to this type of glomerular defect. The phenotype suggests that mesangial cells only develop when in presence of a capillary net-

Figure 9. Receptor–ligand interactions in wild-type and mutant embryos. Binding of ligands to their receptors is indicated by black lines. According to this model, an αR knockout leaves only PDGF BB binding to the βR, whereas in a βR knockout, all three ligand forms can still bind to the αR. In PDGF B mutants, only PDGF AA can bind the αR, and in PDGF A mutants, all three receptor forms are still present but can bind only PDGF BB.
ney has been examined for a number of years. It is known work or that they have a structural role to support the growth of the capillary network.

The role of PDGF and its receptor system in the kidney has been examined for a number of years. It is known that mesangial cells respond mitogenically to administration of PDGF (Shultz et al. 1988; Floege et al. 1993). Mesangial cells express the βR, but not the αR, and βR levels are increased in mesangial proliferative nephritis (Iida et al. 1991). Conversely, mesangial cell proliferation in glomerulonephritis is inhibited by antibodies to PDGF. Expression of the βR and of PDGF B has been examined during glomerulogenesis in human embryos (Alpers et al. 1992). In the early stages of glomerulogenesis, βR is expressed in the undifferentiated metanephric blastema and becomes restricted primarily to mesangial cells at later stages. It is thus believed that PDGF may serve as a paracrine growth factor and chemoattractant to recruit mesangial progenitor cells to the glomerulus and that at the later stages it serves a more autocrine role. Both of these functions might be compromised in βR mutant mice.

In many tissues of the mutant mice known to express the βR, no defect was observed. In particular, no defect was observed in the heart, where β-galactosidase staining indicates high levels of βR expression. Likewise, in mesenchymal cells such as smooth muscle surrounding the major blood vessels, expression of both αR and βR subunits has been detected, suggesting that the αR may be able to compensate for the loss of the β-subunit. Alternatively, the observed αR mRNA up-regulation in the βR mutants may lead to changes at the protein level in some tissues, albeit not in general, and thus to an attenuation of the mutant phenotype. Finally, no defects were observed in the developing central nervous system of the βR mutants, despite the documented expression of both PDGF chains and the αR in neural tissues (Sasahara et al. 1991; Yeh et al. 1991, 1993).

Both the glomerular phenotype observed in the kidney and the hemolytic defects observed in βR mutants appear very similar to those observed in mice lacking PDGF B and described in the accompanying paper (Levén et al., this issue). These phenotypes therefore are most likely attributable to the lack of signaling of PDGF BB to PDGFβB. Some of the more severe defects observed in PDGF B mutants that do not appear in βR mutants, such as hypertrabeculation of the heart and dilation of the arteries, may be attributable to loss of signaling of PDGF BB or AB through the αR receptor. The predictions of the model presented in Figure 9 are consistent with the analysis of the two mutant phenotypes, as all of the defects observed in the βR mutants were observed in the PDGF B mutants as well. However, different phenotypes could have been predicted according to differential expression of ligands and receptors in different cell types or up-regulation of a receptor in a mutant background (only mRNA up-regulation has been observed here). Activation of the αR or the βR also has been shown to lead to different signaling events (Eriksson et al. 1992; Salhany et al. 1992; Heidaran et al. 1993). Finally, ligands exhibit differential affinities for their receptors. Although Scatchard analysis indicates that PDGF B binds the αR with a five- to eightfold lower affinity than PDGF A (Claesson-Welsh et al. 1989; Kelly et al. 1991), it does so still within the physiological range consistent with the similarities in the βR and PDGF B mutant phenotypes. The issue of differential affinity has been difficult to address without cell lines expressing only one of the receptors. Using embryonic fibroblasts derived from mutant Patch (Ph) mice, which only express the βR receptor, it was shown that PDGF AB can bind βR receptors with low affinity (Seifert et al. 1993). It is therefore also possible that AA dimers bind to αR or βR receptors. Cell lines derived from βR mutant mice might also be of use to elucidate differential affinities between the ligands and the αR.

Ultimately, the genetic analysis of both receptors and both ligands will be required to understand the role of PDGF and its receptors in development. It has been shown that Patch (Ph) mice carry a deletion of the PDGFAβR (Smith et al. 1991; Stephenson et al. 1991). These mice show dominant pigmentation defects and recessive embryonic lethality (Grüneberg and Truslove 1960) with defects in a number of mesenchymal lineages and in neural crest derivatives (Morrison-Graham et al. 1992; Orr-Uriet and Schatteman 1992). Because Ph represents a large deletion, it is uncertain whether the phenotype is attributable to the absence of the αR alone or of several genes in addition to the αR. Mice carrying a targeted mutation in the αR have now been generated in this laboratory. This and the availability of PDGF A mutant mice (C. Betsholtz, pers. comm.) should ultimately allow a side-by-side comparison of the phenotypes of mice lacking both receptors or both ligands and help elucidate the interactions and physiological roles of PDGF and its receptors.

Materials and methods

Derivation of mutant mice

A bgeo gene followed by a polyadenylation sequence (β-neoBAP, Friedrich and Soriano 1991), or a neo expression cassette (PGK-neoBAP, Soriano et al. 1991) were used to replace a 1.8-κb genomic Smal-EcoRV fragment spanning sequences coding for the signal peptide to the second immunoglobulin domain of the PDGFB (Yarden et al. 1986). The constructs were flank ed 5′ and 3′, respectively, by 6 and 3 κb of genomic sequences from the βR locus cloned from a 129Sv mouse library. A thymidine kinase gene expression cassette under the control of the phos phohgycerate kinase I (PGK) promoter was included 3′ of the shorter homologous arm. The constructs were linearized with XhoI and electroporated into AB1 ES cells, and colonies were selected with G418 and FIAU. Homologous recombination events were screened by PCR as described (Soriano et al. 1991) using an oligonucleotide from the neo gene (5′-TGGC-TACCCGTGATATTGCT-3′) and βR genomic sequences downstream of the short arm of homologous sequences (5′-GCCACATTCTPTGTGCCCGTGCTC-3′) for 40 cycles [93°C for 30 sec; 55°C for 30 sec; 65°C for 3 min]. Positive clones were analyzed further using an Accl cDNA fragment of 243 bases hybridizing to the sequences 3′ of the targeting vector. Tissue culture and blastocyst injections were carried out as described previously (Soriano et al. 1991).
Histological analysis

Embryos were isolated at different stages of gestation and fixed in Bouin’s at 4°C for 20–24 hr, processed for paraffin sectioning, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff according to standard procedures. Embryos were stained for β-galactosidase activity as described previously [Sanes et al. 1986]

Electron microscopy

E18 kidneys were fixed in half-strength Karnovsky’s fixative, rinsed in buffer, and postfixed in 2% collidine-buffered osmium tetroxide. They were dehydrated in graded ethanolos and propyleneoxide and embedded in Polybed 812. Sections were cut at 80–90 nm, stained with saturated aqueous uranyl acetate and lead citrate and photographed with a JEOL 100-SX electron microscope operating at 80 kV.

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