Brief Report

Leydig Cell Loss and Spermatogenic Arrest in Platelet-derived Growth Factor (PDGF)-A–deficient Mice

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Abstract. Platelet-derived growth factor (PDGF)-A–deficient male mice were found to develop progressive reduction of testicular size, Leydig cells loss, and spermatogenic arrest. In normal mice, the PDGF-A and PDGF-Rα expression pattern showed positive cells in the seminiferous epithelium and in interstitial mesenchymal cells, respectively. The testicular defects seen in PDGF-A−/− mice, combined with the normal developmental expression of PDGF-A and PDGF-Rα, indicate that through an epithelial-mesenchymal signaling, the PDGF-A gene is essential for the development of the Leydig cell lineage. These findings suggest that PDGF-A may play a role in the cascade of genes involved in male gonad differentiation. The Leydig cell loss and the spermatogenic impairment in the mutant mice are reminiscent of cases of testicular failure in man.

Key words: PDGF-A • gene targeting • Leydig cell • spermatogenesis • testis

Introduction

The genes involved in the determination of the gonads comprise the Wilms’ tumor suppressor gene WT1, the orphan nuclear receptor SF-1 and the high mobility group family member SOX9 (Parker et al., 1999). After determination, the differentiation of the testis depends on the testis-determining factor SRY (Goodfellow and Lovell-Badge, 1993). SRY induces the Sertoli cells to differentiate and to secrete the Müllerian inhibiting substance, which suppresses the development of the female reproductive tract (Gustafson and Donahoe, 1994). Later, under the influence of Sertoli cells, the Leydig cells develop (Ge et al., 1996). The ontogenesis of Leydig cells involves two distinct cell generations (McLaren, 1998). The fetal Leydig cells originate prenatally and produce androgens required for the masculinization during fetal and neonatal life. They regress thereafter and are substituted during puberty by the adult Leydig cells, which supply the testosterone necessary for completion of spermatogenesis and maintenance of male reproductive function. Adult Leydig cells do not derive from preexisting fetal cells but from undifferentiated mesenchymal stem cells (McLaren, 1998). The factors that regulate the commitment and early differentiation of stem cells to adult Leydig cell lineage are unknown.

PDGF-A, a high-affinity ligand for the receptor tyrosine kinase PDGF-Rα, is required for embryonic and postnatal development (Betsholtz and Raines, 1997). PDGF-A–deficient animals develop lung emphysema secondary to the failure of alveolar septation (Boström et al., 1996), defective oligodendrogenesis (Calver et al., 1998; Fruttiger et al., 1999), and skin and hair defects (Karlsson et al., 1999). Previous studies have revealed that PDGF-A may play a role in testicular development (Gnessi et al., 1995) and that Leydig cells express PDGF-Rα (Gnessi et al., 1992; Loveland et al., 1995). Here we report the testicular phenotype of PDGF-A–deficient mouse generated by targeted gene disruption. From this study, we conclude that PDGF-A is an essential factor for adult Leydig cells development.

Materials and Methods

Animals

PDGF-A null mutant mice, heterozygous, and wild-type (WT)^1 litter-

^1 Abbreviations used in this paper: BrdU, bromodeoxyuridine; LH, luteinizing hormone; PAS, periodic acid-Schiff; PMC, peritubular myoid cells; RLF, relaxin-like factor; TUNEL, TdT-mediated dUTP biotin nick end labeling; WT, wild-type.
mates were obtained from heterozygous crosses all bred as 129Ola/C57 B16 hybrids (Böström et al., 1996). Transgenic mice carrying the reporter gene β-galactosidase (lacZ) under control of a 6 kb regulatory domain of the murine PDGF-Rα promoter were generated as previously described (Reinertsen et al., 1997).

In Situ Hybridization
Nonradioactive in situ hybridization using PDGF-A and PDGF-Rα probes was performed as described (Böström et al., 1996).

Histology, Immunohistochemistry, and lacZ Staining
Testes to be stained by periodic acid-Schiff (PAS)/haematoxylin, bromodeoxyuridine (BrdU), TdT-mediated dUTP biotin nick end labeling (TUNEL), PDGF-A, PDGF-Rα, relaxin-like actor (RLF), and GATA-1 immunohistochemistry were fixed in 4% buffered paraformaldehyde and paraffin embedded. The reactions were carried out on 5 μm sections. For routine histology, sections were stained by PAS/haematoxylin according to standard protocols. For BrdU labeling, BrdU (Sigma-Aldrich) was injected intraperitoneally (100 mg/g body mass). Injected animals were killed 2 h later and the fixed testes were processed as described (Karlsson et al., 1999). A apoptotic cells were detected by TUNEL using the ApopTag Plus in situ detection kit (Oncor) according to the instructions of the manufacturer. Sertoli cells were detected using a GATA-1–specific antibody (generously provided by Professor Richard Ivell, Institute of Hormone and Fertility Research, Hamburg, Germany); PDGF-A and PDGF-Rα–positive cells were detected using rabbit anti-PDGF-A (RDI Inc.) and rat anti-PDGF-Rα (PharMingen) antibodies. The antibodies (anti-GATA1, anti-PDGF-A, and anti-PDGF-Rα at 1:100 dilution; anti-RLF at 1:1,000 dilution) were incubated overnight at 4°C. The avidin-biotin immunoperoxidase system with 3-amino-9-ethylcarbazole as chromogen was used to visualize bound antibodies (Histostain-Plus kit; Zymed Laboratories). For lacZ staining, transgenic animals were fixed and stained with X-gal for lacZ activity, and 10-μm cryostat testicular sections were treated as described (Reinertsen et al., 1997).

Hormone Assays
Serum testosterone and luteinizing hormone (LH) levels were determined by radioimmunoassays using DiaSorin and Amersham assay systems according to the manufacturers’ instructions.

Results and Discussion
Newborn PDGF-A−/− mice are outwardly normal but fail to thrive after birth. Most die within a few days, but some individuals survive for as long as 6 wk, eventually dying of pulmonary failure. Due to the lethal phenotype of the homozygous mutants, our analysis was restricted to male mice recovered between postnatal day 10 (P10) and P42, killed before the first signs of respiratory failure were apparent. The heterozygous mice were phenotypically in-distinguishable from WT. Surviving null mice weighed less than WT littersmates and their growth curve was slower. PDGF-A−/− males had no defects in the external genitalia and testicular descent appeared normal. The testes were reduced in size (Fig. 1 A), however, the testicular size reduction in the mutants was more pronounced than the average reduction of the body size, and this feature increased dramatically with age (Fig. 1 B). Testis histology revealed profound differences between PDGF-A null mice and WT (Fig. 1, C−J). At P10, −/− animals showed absence of tubular lumen whose formation was starting in the controls; no significant difference was observed in tubular diameter and germ cells number. Sertoli cells, interstitial cells, peritubular myoid cells (PM C) and extracellular matrix layers appeared intact as well (Fig. 1, C and D).
At P18, the mutant testes had reduced seminiferous tubule diameter and poor lumen formation but, likewise control littermates, spermatogenesis had proceeded up to pachytenic spermatocytes; Sertoli cells and PMC were normal. However, the null mice showed an initial reduction in number of morphologically recognizable Leydig cells, scattered in the interstitium among small undifferentiated spindle-shaped mesenchymal cells of fibroblastic appearance (Fig. 1, E and F). At P25, round spermatids were frequently found in the tubules of +/- animals and the interstitium was filled with Leydig cells; no spermatids and a further reduction of the interstitial cells population were observed in the mutants (data not shown). A t P32 and P42 all stages of the spermatogenic cycle and abundant Leydig cells were seen in control testis (Fig. 1, G, and I). In +/- P32 testis, the tubular diameter was greatly reduced and a spermatocytic arrest with further loss of Leydig cells was observed (Fig. 1 H). P42/-/- testis displayed a dramatic decrease of spermatocytes, adluminal multinucleated giant cells, and an almost complete absence of Leydig cells (Fig. 1 J). Immunostaining of P10-P42 +/- testicular sections with an antibody against the Sertoli cell–specific gene GATA-1 (Yomogida et al., 1994) confirmed the presence of Sertoli cells in the null testis, indicating that the lack of mature forms of germ cells in PDGF-A -/- mice did not result from a loss of the supporting cells (data not shown). We also performed an immunohistochemical analysis of RLF, which is a specific marker for the mature forms of fetal as well as adult Leydig cells (Balvers et al., 1998). Anti-RLF staining identified mature fetal Leydig cells in both mutant and WT P10 testis (Fig. 2, A and B). A t P18, concomitantly with the involution of fetal Leydig cells and with the first appearance of the adult Leydig cells progenitors, no RLF was expressed in +/- testis while sporadic punctate staining was seen in the interstitium of +/- testis (Fig. 2, C and D). This trend increased at P25. Here there was no staining in the few interstitial cells of the KO testis, while the majority of the interstitial cells of the WT testis exhibited RLF-specific staining (Fig. 2, E and F). By day 32 and onward, a more homogeneous cytoplasmic distribution of RLF positivity within the interstitial cells of the WT animals was observed while, concomitantly with the progressive depletion of the interstitium, RLF staining in the KO testis was absent (data not shown). This RLF staining pattern indicates that the mutant prepubertal testis contains mature fetal Leydig cells and confirms the absence of the adult Leydig cell population in older +/- testis.

To analyze whether the reduced size of the PDGF-A mutant testis and its histologic appearance could be due to decreased cell proliferation and/or increased apoptosis, BrdU and TUNEL labeling were performed. At P18, P25, P32, and P42 there was no difference in BrdU incorporation of the actively proliferating germ cells between WT and mutant testes (Fig. 3, A-D; data not shown). During the third week of postnatal life, the stem cell precursors of the adult Leydig cell proliferate (Ge et al., 1996). Accordingly, BrdU staining was observed in some interstitial cells of P18 WT animals (Fig. 3 A); on the contrary, no BrdU labeling was seen in P18 PDGF-A -/- littermates (Fig. 3 B). Regarding TUNEL, few apoptotic cells were seen in the tubules of both WT and +/- siblings between P10 and P32 (data not shown). However, at P42 a large number of spermatocytes undergoing apoptosis was observed in null mice compared with control (Fig. 3, E and F). No evidence for apoptosis-mediated death of other testicular cell types was seen. Thus, an arrest of differentiation followed by increased apoptosis seem to be responsible for the seminiferous tubules aspect in the PDGF-A +/- testis. The lack of BrdU incorporation and the absence of TUNEL labeling in the interstitium of +/- testis, indicate that the paucity of Leydig cells might be due to a proliferative arrest. Thus, we speculate that in the PDGF-A +/- testis the normal pubertal replacement of the fetal Leydig cells with the adult population of cells is deranged because of a deficiency in the commitment of the adult Leydig cell precursors to proliferate and perhaps to differentiate.

Such a model requires the knowledge of the localization of PDGF-A and its receptor in normal testis. In situ hybridization for PDGF-A and PDGF-Rα in embryonic-day-17.5 (E17.5)-P30 WT testis showed the mRNA expression in seminiferous epithelium and interstitial mesenchymal cells, respectively (Fig. 4, A-H). At E17.5 the PDGF-A and PDGF-Rα labeling was rather homoge-
aceous. Later, some PDGF-A–positive tubules and distinct populations of PDGF-Rα–positive interstitial cells could be seen. PDGF-Rα expression was moreover localized using transgenic mice carrying the reporter gene β-galactosidase (lacZ) under control of the murine PDGF-Rα promoter. According to the in situ data, lacZ staining was found in the interstitium (Fig. 4, I and J). The immunohistochemistry for PDGF-A confirmed the intratubular expression of the growth factor which, in agreement with previous results (Gnessi et al., 1992, 1995; Loveland et al., 1995), was mainly localized in the cytoplasm of the Sertoli cells (Fig. 5). A t P18, although with different intensities, all the tubules were stained (Fig. 5 A). A t P42, in line with the in situ findings, only the Sertoli cells in some tubules, corresponding to spermatogenic stages IX–X (Russell et al., 1990), were positive (Fig. 5 B). A t P42, a weak signal was also found in some interstitial cells (Fig. 5 B), which is consistent with the reported production of PDGF-A by adult Leydig cells in vitro (Gnessi et al., 1992, 1995; Loveland et al., 1995). Concerning PDGF-Rα, in prepubertal animals the immunostaining was localized in the interstitial cells of both +/- and +/-/− testes, although in the latter the interstitial cells were less in number (Fig. 5, C and D). In older animals, the WT testis continued to show PDGF-Rα–positive cells between the tubules (Fig. 5 E), while the null testis showed a steadily decreasing number of positive cells, culminating in their complete absence at P42 (Fig. 5 F). The comparison between the testicular defects found in +/-/− mice and the normal expression pattern of PDGF-A and its receptor, indicates that paracrine PDGF-A/PDGF-Rα signaling constitutes a critical part of the epithelial-mesenchymal interaction, essential for adult Leydig cells development. PDGF-A does not influence the fetal generation of Leydig cells, as suggested by both the normal adrogenization and RLF-positive interstitial immunohistochemical staining of prepubertal +/-/− animals. Due to the lack of specific markers, we could not produce direct evidence that among the interstitial PDGF-Rα–positive cells are indeed the precursors of the adult Leydig cells, but the spatiotemporal distribution of PDGF-Rα-interstitial cells in normal mice seems to suggest this possibility. It is also reasonable to predict that a cell type specifically lost in PDGF-A/−/− mice should carry the receptor for PDGF-A, and should locate close to the source of the ligand, as is the case for the PDGF-Rα–positive interstitial mesenchymal cells.
described. However, given the chemotactic activity of PDGF-A (Heldin and Westermark, 1999), we cannot exclude that, similarly to what is described for other PDGF-dependent developmental processes (Boström et al., 1996; Lindahl et al., 1997a,b; Calver et al., 1998), PDGF-A might influence the long-range migration of Leydig stem cells, thus preventing their arrival in the gonads from primordial germ layer source besides proliferation and differentiation. Regardless of whether PDGF-A influences the migration of the Leydig cells precursors or their proliferation/differentiation, the main consequence of the lack of replacement of the fetal Leydig cells with adult cells would
be a progressive reduction of testosterone, followed by spermatogenic arrest and germ cells degeneration. The testicular phenotype of the null animals strongly indicates that this is the case. In \(-/\)- testis the initiation of spermatogenesis, which depends mainly on follicle-stimulating hormone, occurs normally, while its testosterone-dependent subsequent completion and maintenance (Sharpe, 1994) are lacking. Indeed, serum testosterone levels were similar in prepubertal/early pubertal (P10-P18) WT (1.79 ± 0.7 ng/ml; n = 5) and mutant mice (2.0 ± 0.89 ng/ml; n = 3), while in \(-/\)- older animals, circulating testosterone was not detectable, confirming that the spermatogenic arrest and germ line apoptotic regression in PDGF-A \(-/-\) animals are mediated by androgen deficiency.

The plasma levels of luteinizing hormone (LH) were also measured. They were similar in \(-/\-) (1.6 ± 0.4 ng/ml; n = 4) and \(+/+\) (1.4 ± 0.5 ng/ml; n = 6) animals between P10 and P25. In agreement with the testosterone reduction, in a null P42 animal the levels of LH (7.5 ng/ml) were higher than in control littermates (3.8 ± 0.3 ng/ml; n = 4). These data indicate that in PDGF-A \(-/-\) null animals, the fate of the Leydig cells cannot be ascribed to an LH deficiency.

In conclusion, understanding of Leydig cell development is still incomplete. Although LH, androgens and IGF-1 are recognized important elements required for the completion of Leydig cell maturation, they cannot be the factors that regulate the commitment of stem cells to adult Leydig cell lineage (Benton et al., 1995). In fact, initial proliferation of stem cells can occur when LH is absent (Teerds et al., 1989), and animals with androgen insensitivity (Murphy et al., 1994) or IGF-1 gene deletion (Baker et al., 1996) develop Leydig cells, suggesting that a separate factor regulates the earliest stage of adult Leydig cell evolution. Our findings indicate that PDGF-A may be this factor, and suggest that adult Leydig cells arise from PDGF-R\(\alpha\) progenitors.

The pivotal role played by PDGF-A in adult Leydig cells development suggests that PDGF-A may be a potential target for the master genes involved in testicular organogenesis. In this respect, it is worth mentioning that WT1, whose spatio-temporal Sertoli cells expression profile (Pelletier et al., 1991; Mundlos et al., 1993; Rackley et al., 1993; Del Rio-Tsonis et al., 1996) is virtually superimposable on that of PDGF-A, can either repress or activate the PDGF-A gene (Wang et al., 1992, 1993a, 1993b; Gashler et al., 1992), and has been involved in posttranscriptional processing within the Sertoli cells (Larsson et al., 1995). The dramatic testicular phenotype of the PDGF-A \(-/-\) animals is also of interest because recent studies have reported detection of PDGF-A and PDGF-R\(\alpha\) in the

Figure 5. Immunohistochemical localization of PDGF-A in \(+/+\) (A and B) testis and comparison of the localization of PDGF-R\(\alpha\) in \(+/+\) (C and E) and \(-/-\) (D and F) testis. PDGF-A staining is localized in the seminiferous epithelium of all the testicular tubules at P18 (A) and in a subset of tubules (spermatogenic stages IX-X) and some interstitial cells at P42 (B). At P18, PDGF-R\(\alpha\) expression is seen in the interstitial cells of \(+/+\) (C) and \(-/-\) (D) testis. At P42, PDGF-R\(\alpha\) is still localized in the interstitial cells of the WT testis (E). Note the lack of positive staining in the P42 \(-/-\) testis which is in line with the Leydig cells loss (F). Bar: (A, C, D, E, and F) 50 \(\mu\)m; (B) 100 \(\mu\)m.
human testis (Basciani, S., L. Gnessi, M. A. rizzi, N. Rucci, S. Mariani, S. Ullisse, E.A. J. Jannini, G. Spera. 1999. Proceedings of the 81st A nnual Meeting of the Endocrine Society, San Diego, CA. A bst. P2–P81). These considerations, coupled with our findings, may furnish a new approach for the understanding of the WT1-mediated mechanisms involved in testicular development and perhaps of WT1-dependent urogenital abnormalities and tumorigenesis (H astie, 1994; R eddy and Licht, 1996; M enke et al., 1998). Moreover, a local impairment of the PDGF-A/PDGFR-α system may provide a new conceptual framework for the comprehension of some forms of Leydig cell hypoplasia, with high LH, low testosterone, and no mutations of the LH receptor gene (Zenteno et al., 1999), and of cases of spermatogenetic failure, which are probably due to intratesticular testosterone deficiency in man (Sharpe, 1994).

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