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Detection of platelet-derived growth factor-α (PDGF-A) protein in cells of Leydig lineage in the postnatal rat testis

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Summary. Platelet-derived growth factor-A (PDGF-A) is a locally produced growth factor in the rat testis secreted by both Sertoli cells and Leydig cells. It has been suggested that PDGF-A may be involved in modulation of testosterone production and may be essential to Leydig cell differentiation, however it is not known at what stage of differentiation PDGF-A begins to be expressed in the cells of Leydig lineage in the postnatal rat testis. Therefore, the objectives of this research were to determine at what postnatal age and in which cell type is PDGF-A first expressed in cells of the adult Leydig cell lineage, and does PDGF-A expression coincide with expression of 3ß-hydroxysteroid dehydrogenase (3ß-HSD), an indicator of steroid hormone synthesis. Male Sprague Dawley rats of postnatal day 1, 7, 9-14, 21, 28, 40, 60, and 90 were used (n=6). Animals were euthanized and their testicles removed, fixed in Bouin's solution, embedded in paraffin, and 5 µm sections were prepared. Immunolocalization of PDGF-A and 3ß-HSD was carried out using a peroxidase-streptavidin-biotin method. PDGF-A was first detected in cells of the Leydig cell lineage at postnatal day 10 in progenitor cells, which were surrounding the seminiferous tubules (peritubular). These cells were confirmed to be the progenitor cells and not the mesenchymal or any other spindle-shaped cells in the testis interstitium by immunolocalization of 3ß-HSD and PDGF-A in the cells in adjacent sections of testis tissue from rats of postnatal days 10-14. After postnatal day 10, PDGF-A was continued to be expressed in subsequent cells of the Leydig lineage through day 90 (adult), however, was not present in peritubular mesenchymal precursor cells of the Leydig cell lineage or any other spindle-shaped cells in the testis interstitium at any tested age. These results revealed that PDGF-A first appears in Leydig progenitor cells in the postnatal rat testis at the onset of mesenchymal cell differentiation into progenitor cells at postnatal day 10 and suggest that a functional role(s) of PDGF-A in postnatally differentiated Leydig cells in the rat testis is established at the time of the onset of postnatal Leydig stem cell differentiation. It is suggested that the significance of the first expression of PDGF-A in the Leydig progenitor cells may be associated with inducing cell proliferation and migration of this cell away from the peritubular region during Leydig cell differentiation.

Key words: PDGF-A, Testis, Leyding cell lineage, Rat, Immunocytochemistry

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Introduction

Platelet-derived growth factor (PDGF) is involved in a number of biological processes including embryogenesis and tumorigenesis (Bowen-Pope et al., 1991; Kadono et al., 2000), and is a mitogen for cells of mesenchymal origin (Heldin and Westermark, 1999). PDGFs exist as homo- or heterodimers of A-, B-, C-, or D-chains and bind to specific PDGF receptors (Betshotltz et al., 2001). Recently, the importance of PDGF in testicular function has been examined. The presence of PDGF and its receptors in the testis has been demonstrated in both humans (Basciani et al., 2002) and adult rats through mRNA (Gnessi et al., 1995; Loveland et al., 1995), immunolocalization (Gnessi et al., 1995), and binding studies (Gnessi et al., 1992). Both PDGF-A and –B and their receptors have been demonstrated in Sertoli and Leydig cells of healthy 20-week-old human fetal testis and in adult testis (Basciani et al., 2002). Interestingly, a more intense immunolocalization signal for PDGF was observed in neoplastic cells of Leydig

cell tumors indicating that PDGF may influence growth of this neoplasm (Basciani et al., 2002). In the fetus, PDGF expression corresponds with rapid proliferation of the Leydig cells (Basciani et al., 2002), and expression of PDGF and its receptors in both fetal and adult testis suggests that the components of the testis may either be targets for PDGF or a source of growth factor for neighboring cells. PDGFs have also been demonstrated in rat Sertoli cells in prenatal and early postnatal life and are thought to chemoattract PDGF receptor-expressing peritubular myoid cells (PMC) in close proximity of the seminiferous tubule (Gnessi et al., 1995). It is reported that in the adult rat, only Leydig cells are immunopositive for PDGF-A and -B and the PDGF receptors α and ß (Gnessi et al., 1995). In *in vitro* conditions, pre-exposure of adult rat Leydig cells to PDGF-BB was necessary to cause a significant stimulation for testosterone production when cells were stimulated by luteinizing hormone (LH; Risbridger, 1993). Research conducted *in vivo* in *Pdgf-/-* mice demonstrated that prenatal fetal populations of Leydig cells were present and functional, and testicular development was normal. However, postnatally the *Pdgf-/-* mice in this study underwent progressive reduction in testicular size, loss of Leydig cells, reduced circulating testosterone, and other deficiencies despite normal plasma levels of LH (Gnessi et al., 2000). Therefore, multiple functions have been proposed for PDGF in the testis, including mediating testicular cell-cell interactions, regulating Leydig cell proliferation, modulation of testosterone production and controlling Leydig cell differentiation (Gnessi et al., 2000).

Adult Leydig cells differentiate postnatally, and in the rat, and the onset is been reported to occur on postnatal day 10 (Mendis-Handagama et al., 1987; Ariyaratne et al., 2000a,b,c). Adult Leydig cells have been suggested to arise from peritubular mesenchymal cells (Mancini et al., 1963; Lording and de Kretser, 1972) and later this fact has been confirmed by using a marker for all steroid-secreting cells, 3ß-hydroxysteroid dehydrogenase (3ß-HSD), as well as cytochrome P450 side-chain cleavage and cytochrome P450 17α-hydroxylase (Ariyaratne et al., 2000a), which are also steroidogenic enzymes. Leydig progenitor cells further differentiate into newly formed adult Leydig cells, immature adult Leydig cells, and finally to mature adult Leydig cells (Mendis-Handagama and Ariyaratne, 2001). Studies have shown that LH is not required for mesenchymal cell differentiation into progenitors, however, it is required for adult Leydig cell proliferation and functional maturation (Mendis-Handagama and Ariyaratne, 2001). A study by Ariyaratne et al. (2000a) showed that when precursor cells differentiate into progenitor cells of the Leydig cell lineage, they simultaneously acquire steroidogenic enzymes, prior to gaining LH receptors. Furthermore, Baker et al. (2003) used gonadotrophin-deficient mice to demonstrate that steroidogenic enzyme markers expressed in adult Leydig cell lineage of the normal adult animal were also expressed in the gonadotrophin-deficient mice, suggesting that LH is not required for the first step in Leydig cell differentiation. Numerous other hormones and growth factors which includes PDGF have been suggested to be of importance at different times during the differentiation process of Leydig cells (review by Mendis-Handagama and Ariyaratne, 2001). However, it is not known at what stage in the Leydig cell lineage PDGF-A is expressed. This information is needed to help elucidate the role of PDGF in the process of Leydig cell differentiation and/or function. Therefore, the purpose of this study was to determine the timing and the stage in the Leydig cell lineage for the onset of PDGF-A expression in the postnatal rat testis.

Materials and methods

Animals

Male and female Sprague Dawley rats obtained from Harlan (Madison, WI), were paired (1:1) and housed in a single cage, under conditions of controlled lighting (14hours light:10 hours dark) and temperature (25°C) in the animal facility of The University of Tennessee College of Veterinary Medicine; food (Agway Prolab formula, Syracuse, NY) and water were provided ad libitum. Rats were observed daily for litters and the day the pups were born was considered Day 1 of birth. Male rats of age 1, 7, 9-14, 21, 28, 40, 60 and 90 days postnatal were used in this study.

Collection and preparation of testis tissue

Rats were euthanized by excess carbon dioxide and their testicles were removed, immersed in Bouin solution for 5-6 hours for fixation. Fixed testis tissues were washed with 70% ethanol for several days until the yellow color (picric acid in Bouin solution) disappeared from the ethanol. Tissues were processed through cycles of graded ethanol and xylene using an automated tissue processor (Tissue Tek, Miles Scientific, MA), infiltrated with, and then embedded in paraffin (Paraplast, Oxford Labware, St. Louis, MO). Blocks of paraffin-embedded tissues were cut into 5mm sections on a Leitz microtome followed by adhering the sections to ProbeOn Plus glass microscope slides (Fisher Scientific, Pittsburgh, PA). Serial sections were obtained from day 10 and 14 testes.

Immunolocalization of PDGF-A and 3ß-HSD in rat testes

Testis tissue sections were de-waxed with xylene and rehydrated with decreasing concentrations of ethanol, then brought to deionized water. They were washed in phosphate bufferd saline (PBS, pH 7.3) for 5 minutes and then incubated in 3% hydrogen peroxide for 20 minutes. After incubation, sections were washed in PBS and normal goat serum was added to tissues overnight (4°C) to bind nonspecific proteins. Rabbit polyclonal anti-PDGF-A (Santa Cruz Biotechnology,

CA) was used at a dilution of 1:200 in streptavidin-peroxidase diluent (BioGenex, San Ramon, CA) and incubated on tissue sections overnight at 4°C. Testis tissue sections used as negative controls were incubated with normal rabbit serum. PDGF-A was detected using a commercially available biotin-streptavidin kit (BioGenex, San Ramon, CA) with 3,3'-diaminobenzidine as the chromagen, according to the manufacturer's instructions. Sections were counterstained with Harris' hematoxylin, dehydrated with increasing concentrations of ethanol then brought to xylene and cover-slipped using Permount. To immunolocalize PDGF-A and 3ß-HSD in adjacent testis tissue sections from rats of postnatal day 10-14, anti-3ß-HSD was used at a dilution of 1:2000. The polyclonal antibody against 3ßHSD was a rabbit IgG antibody against purified human placental 3ß-HSD (Lorence et al., 1990) and has previously been used for immunolocalization of 3ß-HSD antigen in rat testis in many studies including Leydig cell differentiation studies in rats (Majdic et al., 1996, 1998; Ariyaratne and Mendis-Handagama, 2000; Ariyaratne et al., 2000a-c).

Results

Immunohistochemistry

Fetal type Leydig cells in the postnatal rat testis showed positive cytoplasmic labeling for PDGF-A (Fig. 1A). In the Leydig cell lineage of the adult population, PDGF-A was first detected at day 10 in elongated, spindle-shaped cells in the peritubular region (Fig. 1B). Positive immunolabeling for PDGF-A was continued to be expressed in subsequent cells of the Leydig lineage (Fig. 1C,D,F) through day 90, but was absent in peritubular mesenchymal precursor cells and any elongated spindle-shaped cells in the testis interstitium at any age studied (Fig. 1 C,D).

These elongated spindle-shaped cells that were positive for PDGF-A were confirmed to be Leydig progenitor cells by immunolabeling of the adjacent tissue sections for 3ß-HSD (Fig. 2A,C) and PDGF-A (Fig. 2B,D). Furthermore, Sertoli cells and blood vessels (smooth muscle cells and endothelial cells) showed positive immunolabeling for PDGF-A (Fig. 2B,D), from postnatal day one. Negative control sections showed no immunolabeling for PDGF-A or 3ß-HSD (Fig. 1F).

Discussion

Our study showed that PDGF-A is first expressed in the cells of postnatal Leydig lineage in the rat testis at day 10 and the first cell type is the Leydig progenitor cell in the peritubular region. Thereafter, PDGF-A continued to be expressed in subsequent cells of the Leydig cell lineage through day 90. In the peritubular region of the testis interstitium, there are several cell types which are elongated and spindle-shaped, namely, mesenchymal cells, lymphatic endothelial cells, myoid cells and Leydig progenitor cells. The unequivocal identification of these elongated spindle-shaped cells in the peritubular region at postnatal day 10 that first showed positive immunolabeling for PDGF-A was possible in the present study as immunocytochemistry was performed to localize the steroidogenic enzyme 3ß-HSD and PDGF-A in these cells in adjacent tissue sections. These findings confirmed that the elongated spindle-shaped cells in the peritubular region that were positive for PDGF-A on postnatal day 10 were indeed the Leydig progenitor cells, and not the peritubular myoid or mesenchymal cells. Leydig progenitor cells but not mesenchymal or myoid cells possess steroidogenic capacity and therefore are positive for 3ß-HSD. It is important to note that not only at postnatal day 10, but at all other postnatal ages tested, PDGF-A was absent in mesenchymal precursor cells and all other spindle-shaped cells in the testis interstitium. However, it is reported that mesenchymal cells in testes of mouse embryos have receptors for PDGF-ß (Gnessi et al., 2000), although it is not known yet whether mesenchymal cells in the postnatal rat testes express PDGF-B receptors.

To our knowledge, the present study is the first to report the expression of PDGF-A protein in rat Leydig cells before adulthood. Loveland et al. (1995) have demonstrated expression of mRNAs encoding PDGF-A, -B and the PDGF receptor subunits PDGFR-α and PDGFR, in Leydig cells and Sertoli cells in the rat testis. This paper (Loveland et al., 1995) states that rat Leydig cells contain relatively low levels of PDGF-A mRNA and are likely to synthesize predominantly PDGF-B. The present study revealed that PDGF-A is also synthesized to some abundance by the rat Leydig cells as well as other cell types in the adult Leydig cell lineage except the mesenchymal cells. However, it remains to be determined whether PDGF-B is synthesized more than PDGF-A by the Leydig cells in the adult rat testis.

Gnessi et al. (1992) performed immuno-histochemical and receptor binding studies and reported that Leydig cells from 50-55 day old rats produce PDGF and possess PDGF binding sites. In other studies, Gnessi et al. (1995) have reported immunolabeling of PDGF-A and B in Sertoli cells of 1 week-old postnatal rats but not in Leydig cells. mRNA analysis has revealed that adult Leydig cells express PDGF-A and B-chains, however, early pubertal Leydig cells did not show measurable amounts of these PDGF chains (Gnessi et al., 1995). This information does not dismiss the possibility of PDGF being expressed in prepubertal rat Leydig cells since specificity of PDGF-A antibodies used in others' studies and our study may be different. Furthermore, although expression of PDGF-A in early cells of Leydig lineage in the postnatal rat has not been reported previously, the presence of PDGF receptors (PDGFR) in mesenchymal cell of the testis interstitium has been shown previously in species such as mice (Gnessi et al., 2000) and humans (Basciani et al., 2002). Additionally, mRNAs for PDGF-A, PDGF-B and PDGFR-α and

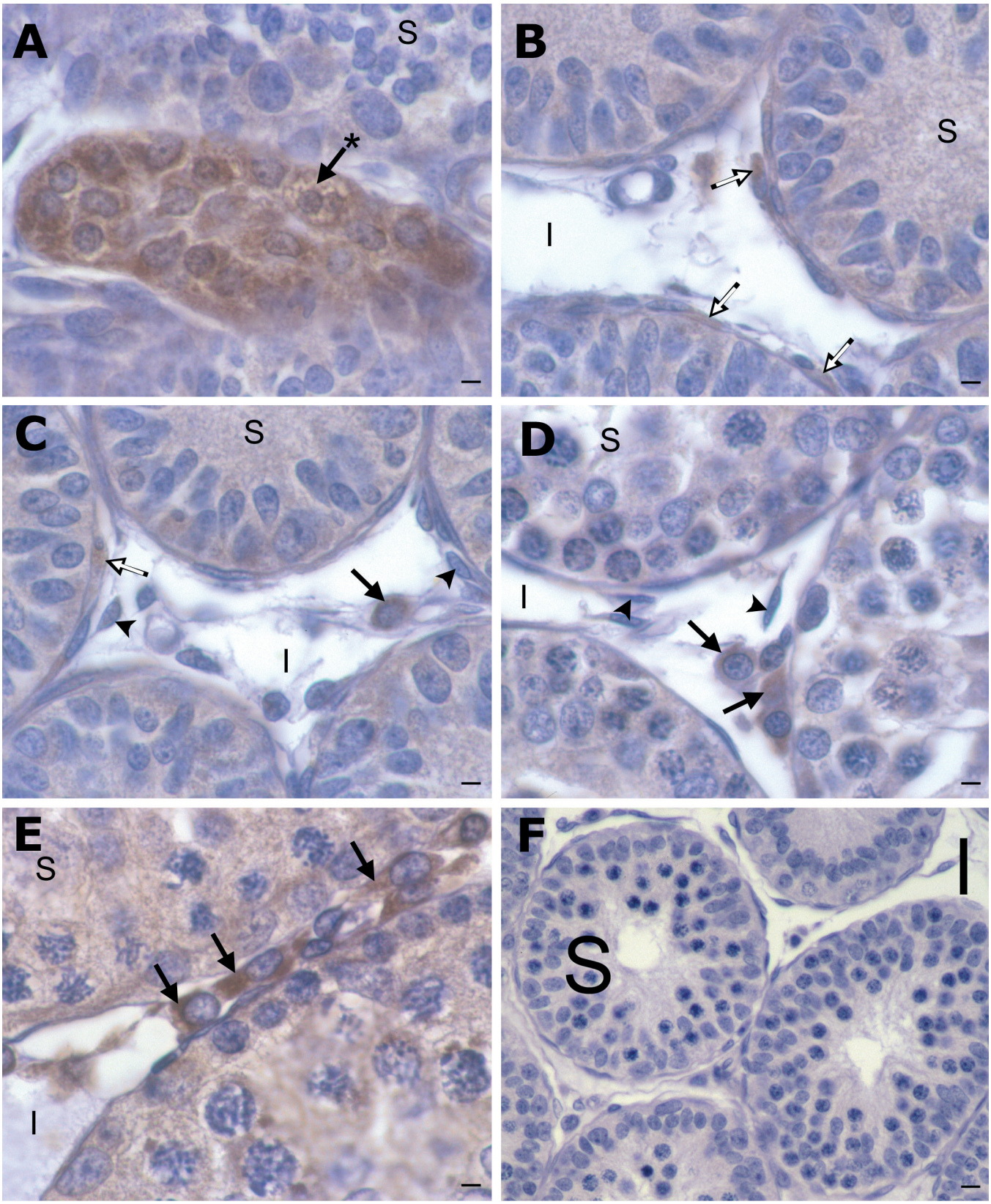

Fig. 1. A-C. Light micrograph of postnatal day 10 testis showing a fetal Leydig cell cluster (arrow*) **(A)**, peritubular Leydig cell progenitors (open arrows) **(B)** and immunolabeled for PDGF. **C.** A newly formed adult Leydig cell (arrow) and peritubular progenitor cell (open arrow) immunolabeled for PDGF-A. **D.** Light micrograph of postnatal day 21 testis showing newly formed adult Leydig cells (arrow) immunolabeled for PDGF. **E.** Light micrograph of postnatal day 40 testis showing newly formed adult Leydig cells (arrow) immunolabeled for PDGF. **F.** Representative light micrograph of negative control testis with no PDGF or 3ß-HSD immunolabeling. Platelet-derived growth factor was not present in mesenchymal cells (arrow head) of the Leydig cell lineage at any of the ages studied. I: interstitium, S: seminiferous tubules. Bars: A-E, 2.5 µm; D, 4.5 µm.

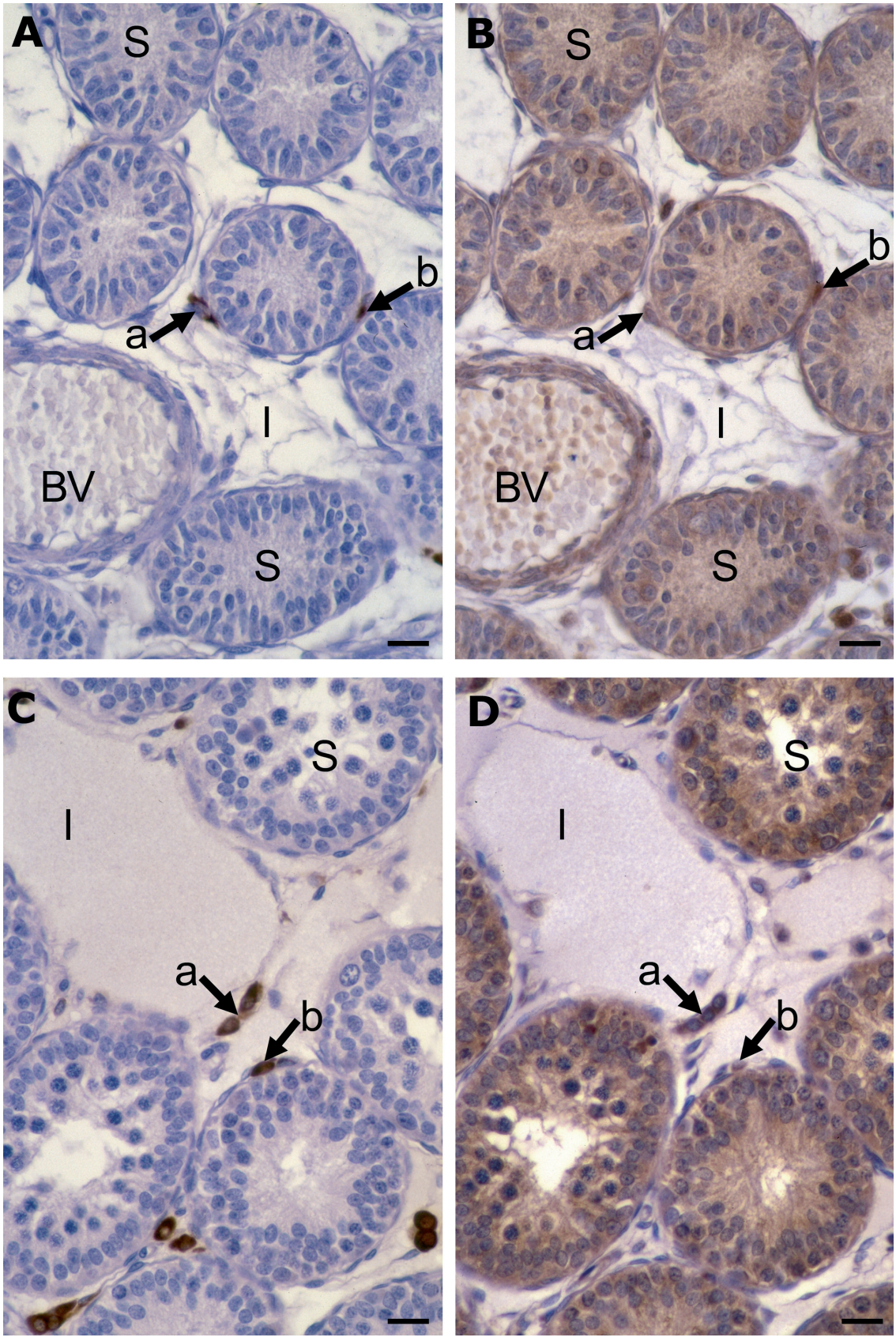

Fig. 2.A.B. Light micrographs of adjacent sections of postnatal day 10 testis showing simultaneous immunolabeling for 3ß-HSD and PDGF-A in the cells of Leydig lineage (alb arrows). **C and D.** Light micrographs of adjacent sections of postnatal day 14 testis showing simultaneous immunolabeling for 3ß-HSD and PDGF-A in the cells of Leydig lineage (**a, b** arrows). Immunolabeling in blood vessels (BV) for PDGF-A is seen in the endothelium and the surrounding smooth muscles (**B**). PDGF-A immunolabeling in the seminiferous tubules (S) is observed in cytoplasm of Sertoli cells (brown color in seminiferous tubules). I: interstitium, Bar: 12 µm.

PDGF-ß have all been localized in Leydig cells. We also verified that the same cells expressing PDGF-A also expressed 3ß-HSD, to confirm that Leydig progenitor cells express PDGF-A protein. This has not been shown in any of the previously published studies.

The exact role that PDGF-A may play in Leydig cell differentiation is still not known and warrants further investigations. Studies in PDGF-A -deficient mice (PDGF-A -/-) have shown reduced Leydig cell numbers in their testes at postnatal day 18 and an absence of the adult Leydig cell population in older PDGF-A -/- mice (Gnessi et al., 2000). The latter observation is also in agreement with the undetectable levels of circulating testosterone, arrest of spermatogenesis and degeneration of germ cells observed in adult PDGF-A -/- mice reported by the same authors. It is clear that in PDGF-A -/- mice, the initiation of the spermatogenic process, which depends mainly on follicular stimulating hormone, occurs normally. However, the subsequent completion of the process, which depends on testosterone (Sharpe, 1994) is lacking, due to testosterone deficiency resulting from paucity of Leydig cells.

Platelet derived growth factors in general have been observed as mitogens for specific mesenchymal cells and glial cell types (Betsholtz et al., 2004). Specifically, PDGF-A has been cited as a potent inducer of glial cell mitosis within the central nervous system (CNS) and mesenchymal cells outside the CNS (Betsholtz et al., 2004). PDGF-A null mutants display thin dermis and disrupted hair cycles (Karlson et al., 1999) as evidence for arrest in cell mitoses in this region. It is also reported that PDGF-A expression in tissues such as retinal ganglion cells influences the final numbers of retinal astrocytes (Gerhardt et al., 2003). Over expression of PDGF-A in cells of the pulmonary epithelium (Hoyle et al., 1999; Li and Hoyle, 2001; Li et al., 2002) lens epithelium of the eye (Reneker and Overbeek, 1996a), and the retinal astrocytes of the eye (Frutigger et al., 1996; Reneker and Overbeek, 1996b; Betsholtz et al., 2004) results in over proliferation of cells in these regions. It is also interesting to note that PDGF-A is specifically recognized as a mitogen for progenitors of oligodendrocytes (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988; Levine, 1989).

In addition to it's mitogenic properties, PDGF-A has properties of controlling cell motility and migration (Ataliotis and Mercola, 1977; Heldin and Westermark, 1999; Betsholzis et al., 2001; Nagel et al., 2004). During vertebrate gastrulation, PDGF-A and its receptor (PDGFR·) are required for the directional migration of mesodermal cells (Harisson et al., 1993; Nagel et al., 2004). It is also reported that male-specific cell migration into the developing gonad is a conserved process involving PDGF-A/PDGFR· signalling (Smith et al., 2005). However, blocking PDGF-A/PDGFR· function in mesodermal cells does not inhibit cell migration per se, but results in randomized instead of a directional migration of these cells (Nagel et al., 2004).

At the onset of mesenchymal cell differentiation into Leydig progenitor cells at the peritubular region, progenitors become positive for PDGF-A. This is an intriguing observation because, the next step in the process of Leydig cell differentiation is rounding up of the progenitor cells and moving away from their peritubular location into the central interstitium. We have shown previously that Leydig progenitor cells undergo mitosis immediately upon their differentiation (Ariyaratna et al., 2000b,c). However, to date, the mechanism of proliferation and migration of these Leydig progenitor cells away from the peritubular region towards the central interstitium, immediately upon their differentiation is not fully understood. In the present study, we demonstrate that PDGF-A, which is an important factor that controls mesodermal cell proliferation and migration, is expressed in Leydig progenitor cells, simultaneously with their differentiation and immediately prior to moving away from the seminiferous tubules. This observation suggests that PDGF-A could be of significance in migration of Leydig progenitor cells away from the peritubular region towards the central interstitium. Whether this movement uses chemotaxis through PDGFR· signalling like many other situations of cell migration (Hosang et al., 1989; Ferns et al., 1990; Rosenkranz et al., 1999; Yu et al., 2001) is also an important issue to be resolved in future studies.

The presence of PDGF-A in Leydig progenitor cells and subsequent cells of the Leydig cell lineage supports that PDGF-A may be a crucial factor in cell proliferation and/or differentiation of cells in the postnatal testis. Additionally, the absence of PDGF-A in peritubular mesenchymal cells that are not migratory and the presence of PDGF-A in Leydig progenitor cells, which are migratory, suggest that PDGF-A may be an important factor in Leydig progenitor cell migration away from their peritubular location towards the central interstitium during Leydig cell differentiation. In conclusion, the exact cellular function(s) controlled by PDGF-A in cells of Leydig lineage has not been determined yet, it is reasonable to suggest that as in many other cell types, PDGF-A in cells of Leydig lineage may be associated with cell proliferation and migration which are essential events in the process of postnatal Leydig cell differentiation.

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