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ORIGINAL ARTICLE

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Ultracytochemistry of 3β-hydroxysteroid dehydrogenase in Leydig cell precursors and vascular endothelial cells of the postnatal rat testis

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Abstract In the biosynthesis of steroid hormones 3β-hydroxysteroid dehydrogenase (3β-HSD) is a key enzyme. The present report describes the subcellular localization of the enzyme in the fetal-type Leydig cells, the fibroblast-like precursors of adult-type Leydig cells and in endothelial cells of interstitial capillaries. Histochemical methods for light microscopy and ultracytochemical methods for electron microscopy were used on rat testes of postnatal day 15. 3β-HSD reactivity was located at subcellular levels by means of the ferricyanide method. A specific, distinct localization of reaction product in the form of copper ferrocyanide precipitates was observed on the membranes of the smooth endoplasmic reticulum not only in the fetal-type Leydig cells and the fibroblast-like precursors of adult-type Leydig cells, but also focally in the endothelial cells of interstitial blood capillaries. Topographically, the 3β-HSD-positive precursors were most often found in the outer layer of the boundary tissue and surrounding interstitial blood vessels. The capillaries with 3β-HSD-positive endothelial cells were usually located in the vicinity of 3β-HSD-positive Leydig cells. For the first time, 3β-HSD has been located at the subcellular level in precursors of adult-type Leydig cells and focally in capillary endothelial cells associated with them. Due to the close association between 3β-HSD-positive vascular endothelial cells and Leydig cells a paracrine relationship between the two cell types may be involved in the acute regulation of steroidogenesis by blood-borne luteinizing hormone.

Key words Adult-type Leydig cells · Endothelium · 3β-HSD · Ultrastructure · Differentiation

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Introduction

Two morphologically and functionally different populations of Leydig cells can be recognized in the rat testis during postnatal development: fetal-type Leydig cells (FTLC), and adult-type Leydig cells (ATLC; Roosen-Runge and Anderson 1959; Lording and de Kretser 1972; Tapanainen et al. 1984; Haider et al. 1986; Zirkin and Ewing 1987; Kuopio et al. 1989; Teerds 1989; Teerds et al. 1989; Dupont et al. 1993; for reviews see: Huhtaniemi et al. 1984; Tähkä 1986). FTLC are arranged in compact round to oval clusters in the interstitial spaces between the seminiferous tubules. They are enzyme histochemically positive for 3β-hydroxysteroid dehydrogenase (3β-HSD) from fetal day (fd) 16 onward with a peak concentration of the enzyme on fd 19. An immunohistochemical reaction for androgen receptors (Majdic et al. 1995) and for LH receptors (Haider et al. 1997) can be demonstrated in FTLC from fd 16 onward. FTLC can be clearly recognized at the level of the light microscope until postnatal day (pnd) 25. The ATLC appear for the first time on about pnd 13 as indicated by histochemical reactions for LH receptors and 3β-HSD. These cells are uniformly distributed around the seminiferous tubules and, unlike FTLC, are not found exclusively in clusters.

Hardy et al. (1989) subdivided the postnatal differentiation of rat Leydig cells into three stages: (1) progenitor stage – Leydig cells are mesenchyme-like progenitors (pnd 14–28); (2) immature stage – Leydig cells produce small amounts of testosterone around pnd 35; (3) mature stage – Leydig cells are fully functional in the sexually mature animal, at about pnd 90 (see also Hardy et al. 1990; Shan and Hardy 1992; Shan et al. 1995, 1997).

In continuation of our work on the origin of ATLC in the rat (Haider et al. 1995), we have attempted to functionally characterize the precursors of ATLC by employing ultracytochemistry for 3β-HSD. To date, this has not been reported in the literature. Up to pnd 12 the peritubular fibroblasts in the outer layer of the lamina propria (also known as boundary tissue) have the ultrastructural features typical for fibroblasts. However, from pnd 13 on-

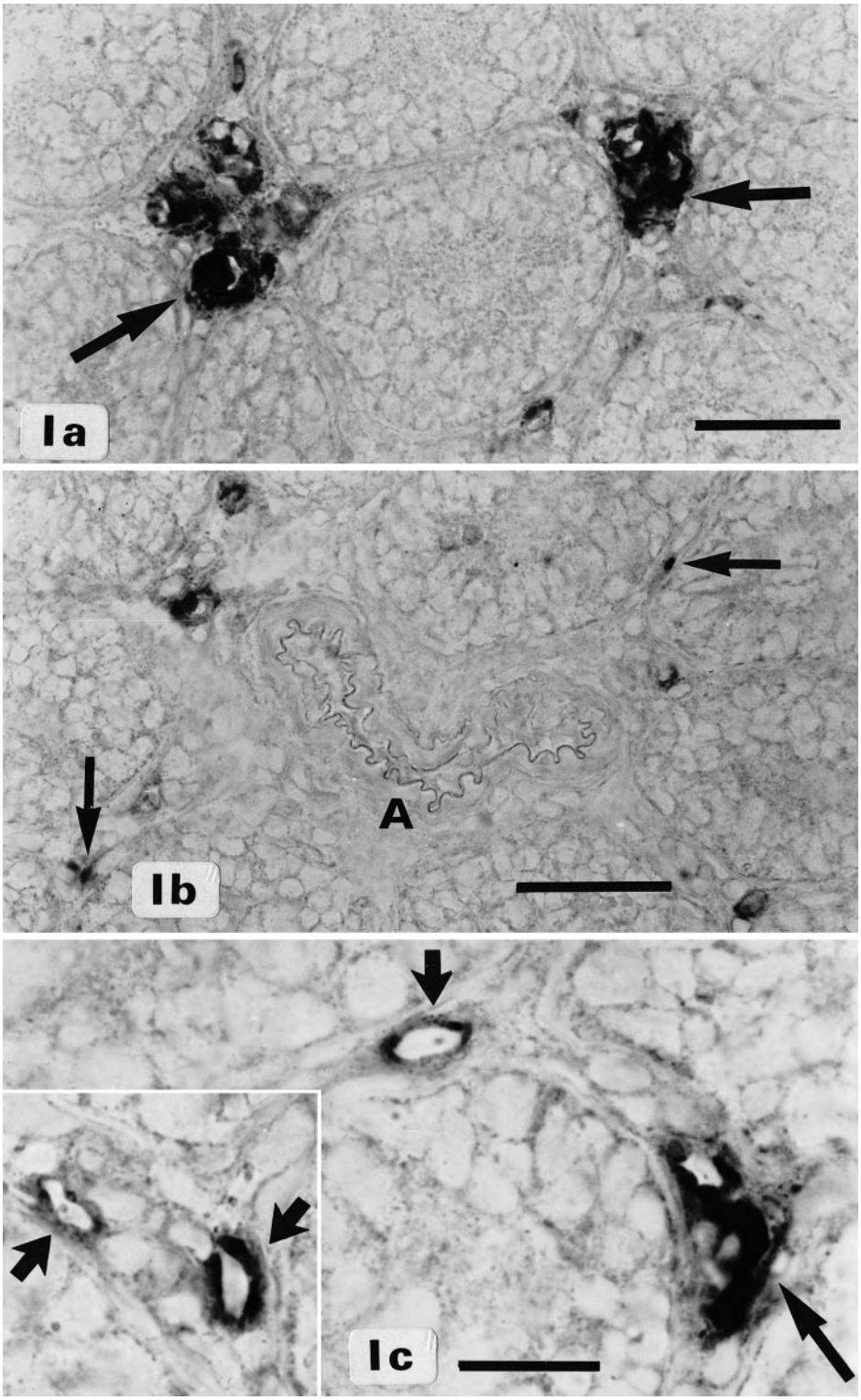

Fig. 1a–c Cryostat sections on pnd 15. **a** A strong enzymatic reaction in the clusters of fetal-type Leydig cells (*arrows*). **b** A moderate to strong reaction in the peritubular fibroblast-like precursors of ATLC (*arrows*). The wall of the interstitial artery (*A*), with a distinct internal elastic membrane, does not show the enzyme reaction. **c** A strong reaction in the cytoplasm of the FTLC (*arrow*); a moderate to strong reaction in the wall of the capillary (*small thick arrow*). *Inset* Moderate to strong reaction for 3β-HSD in the walls of two capillaries (*small thick arrows*); the shadows of erythrocytes are weakly visible in the lumen of these capillaries. *Bars* **a**, **b** 40 µm; **c** (including inset) 15 µm

ward these cells contain in addition organelles typical for steroid-synthesizing cells, i.e., smooth endoplasmic reticulum, mitochondria with tubular cristae and lipid droplets. Employing H3-thymidine, these fibroblast-like cells had the highest labeling index among interstitial cells and a duration of DNA synthesis of 10 h (Haider et al. 1995). Such data indeed suggest that the peritubular fibroblast-like cells lying in the outer layer of the boundary tissue ultimately differentiate – after a phase of transition – into ATLC. In order to collect further information on the functional characterization of these precursors, the present study describes the ultracytochemical distribution patterns of 3β-HSD in the rat testis on pnd 15, an age at which both FTLC and precursors of ATLC are present.

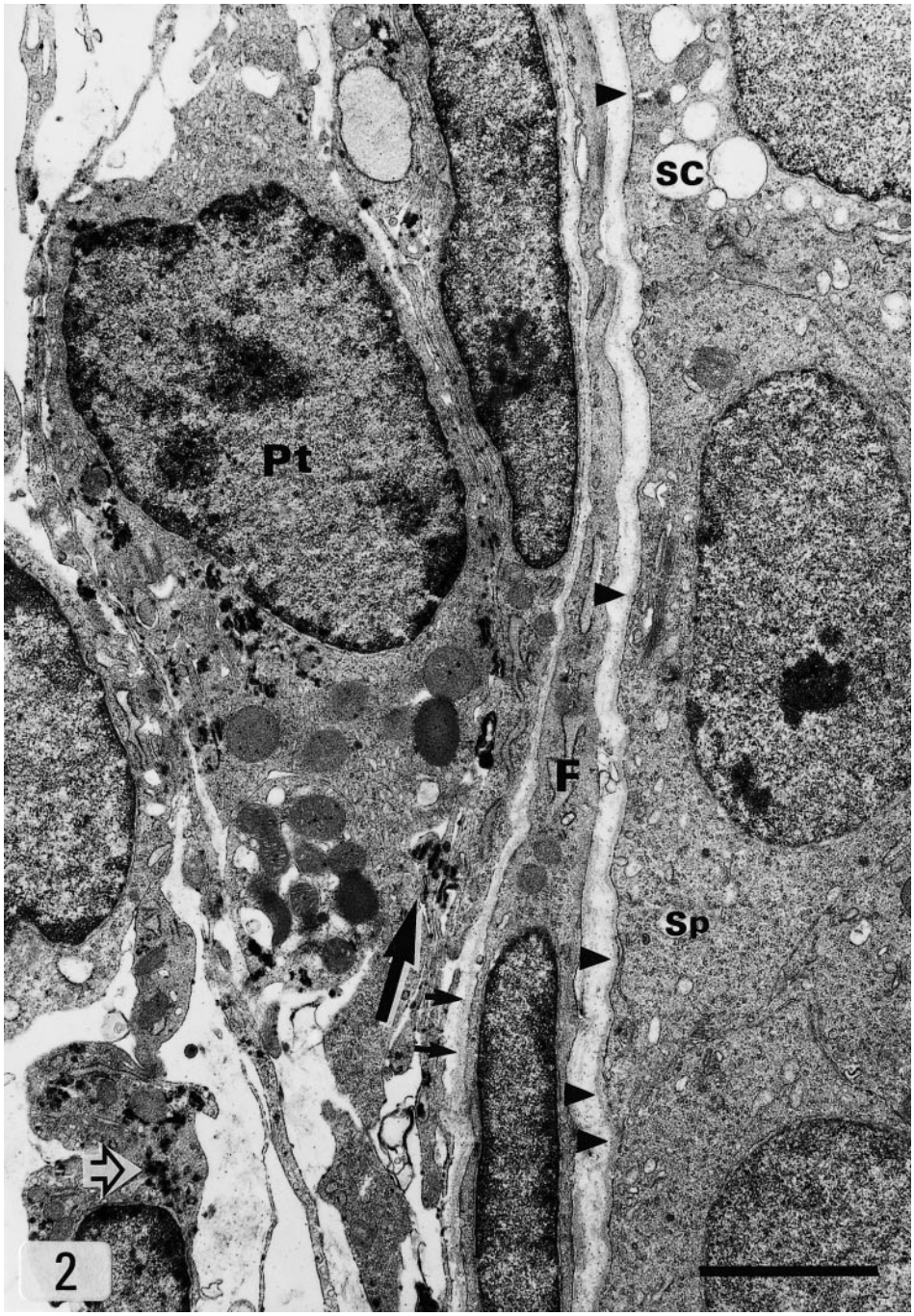

Fig. 2 Ultracytochemistry for 3β-HSD on pnd 15. A distinct reaction product is localized in the form of electron dense granules on the membranes of smooth endoplasmic reticulum (*arrow*) in the peritubular fibroblast-like cell (*Pt*) and in the one next to it (below left with a *small hollow arrow*). Sertoli cell (*SC*) and spermatogonia (*sp*), at the basal lamina of the tubule (*arrowheads*), are without enzyme reaction. The fibroblast (*F*) in the inner layer of the boundary tissue, separated from the interstitium by a basal lamina (*small black arrows*), also lacks the enzyme reaction. *Bar* 2 μm

Materials and methods

Wistar white rats were obtained from the Animal House, Heinrich Heine University Düsseldorf. The criteria of the German Law for Animal Protection were adhered to and the study was approved by the university authorities. The animals were killed by cervical dislocation under deep ether anesthesia. All rats were 15 days old.

Histochemistry of 3β-HSD

The testes of six rats were removed and quickly frozen in liquid nitrogen at –190°C, and then preserved in CO_2 snow. 10 μm thick cryostat sections were prepared at –23°C and prefixed in acetone at 4°C for 3 min. The incubation medium for the enzyme reaction for 3β-HSD contained: 2 mM dehydroepiandrosterone as substrate dissolved in dimethylformamide, NAD as a co-factor, tetranitroblue tetrazolium as an H^+ acceptor, 0.1 M potassium cyanide, 0.05 M magnesium chloride, 20% polyvinyl alcohol, and 0.1 M phosphate buffer at pH 7.5. The sections were incubated for 90 min at 37°C in a humid chamber in darkness. Parallel to each reaction, a few sections were incubated without substrate as negative controls in order to study the non-specific dehydrogenase effect.

Ultracytochemistry of 3β-HSD

The testes and 0.5-cm-long sections of ileum were quickly removed from seven further rats. The intestinal tissue served as a control organ. After removing the tunica albuginea the testes were cut into thin square slices (3–4 mm each side and ca. 2 mm thick).

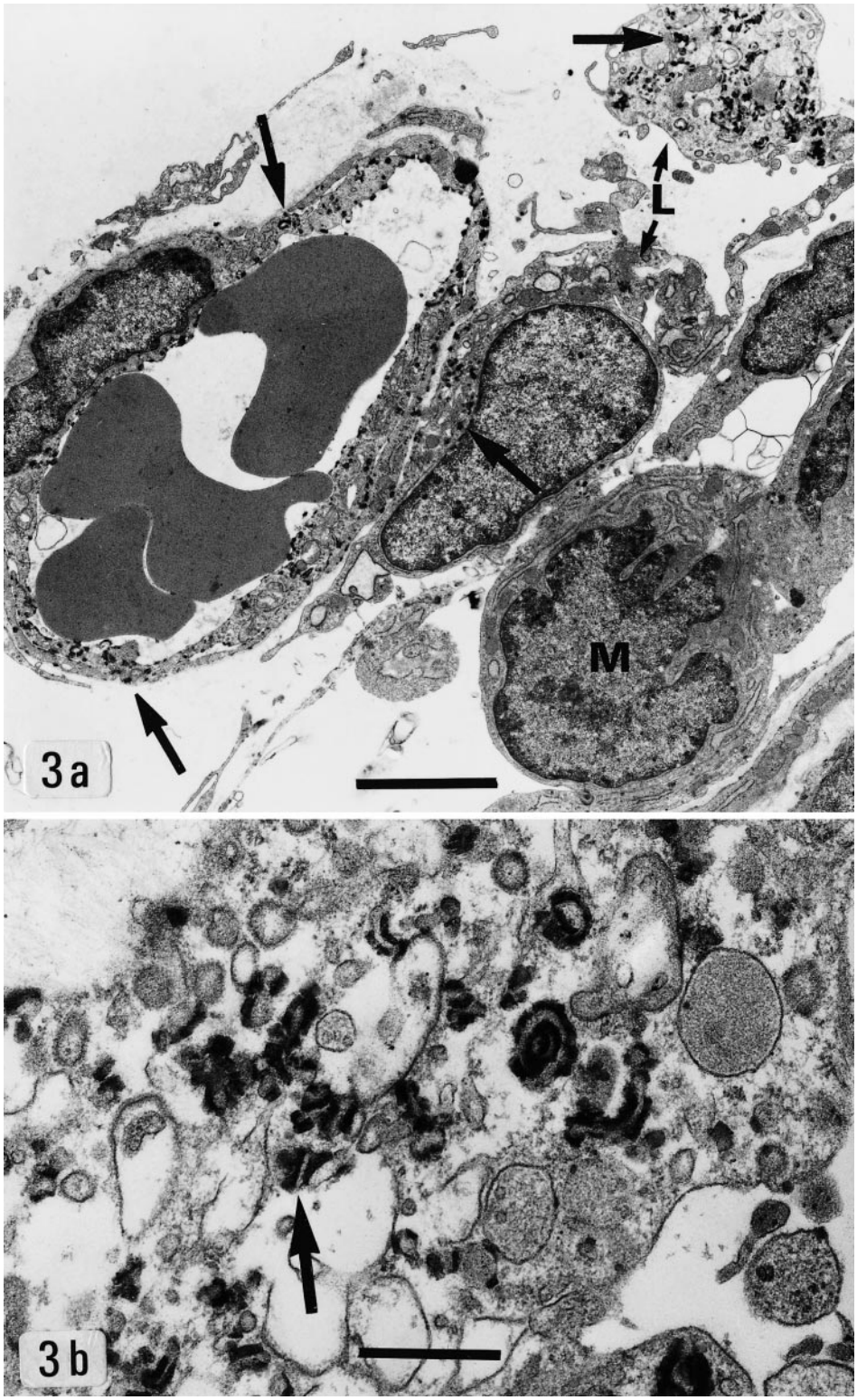

Fig. 3 a Rat testis on pnd 15. Ultracytochemical localization of 3β-HSD reaction products, visible as small dark spots (*arrows*) in the endothelial cell of an interstitial blood capillary with erythrocytes in the lumen (left half of the figure), as well as in the Leydig cells (*L*, with two *small arrows*); the *upper arrow* indicates a portion of a FTLC and the *lower arrow* a perivascular fibroblast-like precursor of ATLC. The macrophage (*M*) has no reaction product. **b** Precipitates distinctly located on the membranes of smooth endoplasmic reticulum (*arrow*) of a FTLC.
Bars **a** 2.5 µm; **b** 0.5 µm

The slices and samples of ileum were prefixed in a solution consisting of 1% paraformaldehyde and 0.25% glutaraldehyde in phosphate buffer at pH 7.4, for 20 min at 0°C. The ferricyanide method for the ultracytochemical demonstration of 3β-HSD was adapted according to the basic technique of Benkoel et al. (1976), which was modified by Hiura et al. (1981). Two additional changes were made: PMS (phenazine methosulfate, as an electron carrier) was added to the medium prior to incubation, as described by Berchtold (1977) and PVA (polyvinyl alcohol) was added to minimize the loss of intracellular enzyme by diffusion through the cell membrane as recommended by Henderson et al. (1978). The substrate was purchased from Sigma (Munich, Germany). After prefixation the slices were washed and kept in 0.1 M phosphate buffer overnight, at 4°C and subsequently incubated in the following medium in a humid chamber at 37°C in darkness for 90 min: substrate – 3 mM dehydroepiandrosterone (1.2 mg dissolved in 1.5 ml dimethylformamide); 0.1 M phosphate buffer, pH 7.4 (12.5 ml); 0.1 M sodium citrate (2 ml); 15 mM potassium hexacyanoferrate

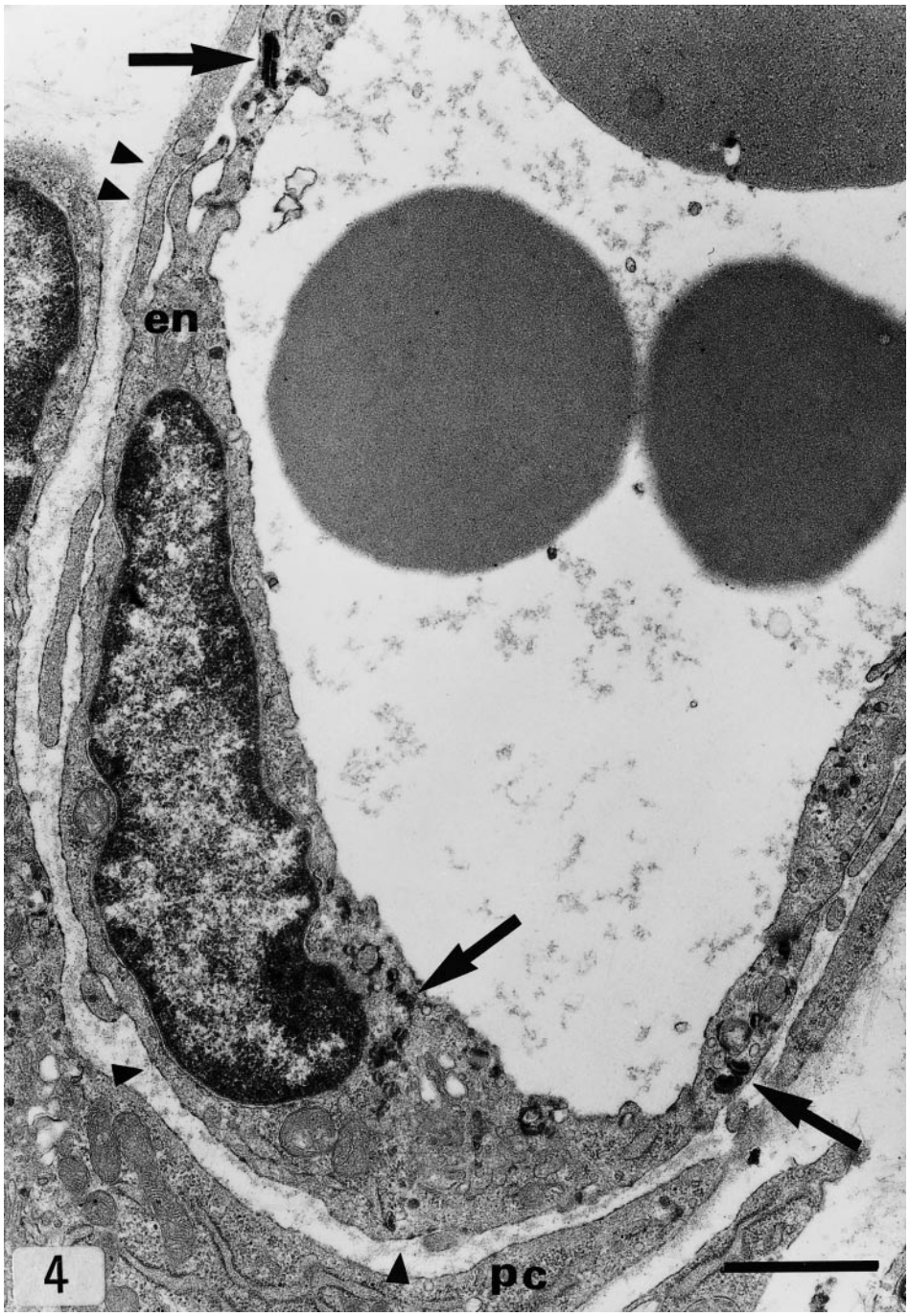

Fig. 4 Rat testis on pnd 15. The ultracytochemical localization of 3β-HSD (*arrows*) in sER of an endothelial cell (*en*), but not in mitochondria in an interstitial blood capillary. The endothelial cell is surrounded by a basal lamina (*arrowheads*). No reaction products were observed in the erythrocytes or in the pericyte (*pc*). *Bar* 1 μm

III (2 ml); 90 mM copper sulfate (2 ml); sucrose (2 g); NAD (7.2 mg); 20% PVA (2 ml); 1 mM PMS – added just before incubation (2 ml). The substrate was omitted in the incubation medium for the negative control sections.

After incubation the tissue slices were washed in phosphate buffer (2×10 min) and postfixed in 1% OsO_4 (in 0.1 M phosphate buffer) at room temperature for 30 min. After brief rinsing in phosphate buffer (2×5 min) the tissue was dehydrated (10 min each in 80%, 90%, 100% ethanol, 100% isopropanol and finally 2×5 min in toluol). The samples were then embedded in Epon 812 (2×1 h at 40°C), and polymerized in gelatin capsules at 60°C for 48 h. Ultrathin sections were prepared with an Reichert-Jung ultramicrotome, stained with saturated uranylacetate and lead citrate, then observed and photographed with an electron microscope (HITACHI H-600). Semithin sections were stained with 1% toluidine blue solution.

Results

Histochemistry for 3β-HSD

The gray-brown precipitates of the reaction product were observed in the cytoplasm of FTLC and in the slender, peritubular fibroblast-like precursors of ATLC. The intensity of the reaction was moderate to strong (Fig. 1). The nuclei were devoid of precipitate and always appeared as colorless round or oval structures around which the cytoplasmic enzyme reaction product was located. The FTLC were often arranged in clusters in the interstitial areas. In

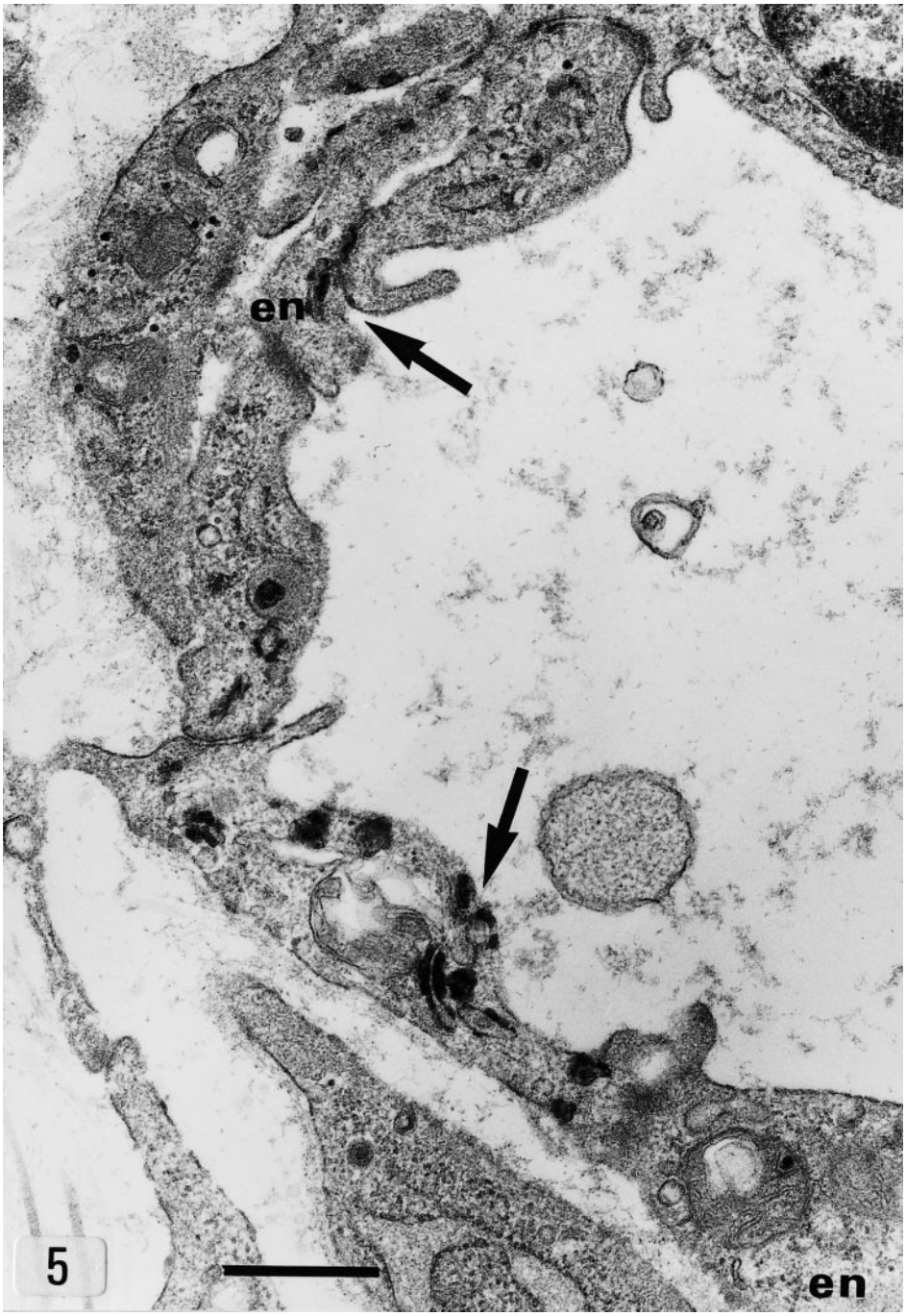

Fig. 5 Rat testis on pnd 15. A higher magnification of endothelial cells (*en*) showing a distinct localization of reaction products of 3β-HSD on the membranes of smooth endoplasmic reticulum (*arrows*). *Bar* 0.5 µm

addition, there was a moderate to large amount of 3β-HSD reaction product distinctly located in the walls of interstitial blood capillaries (Fig. 1c). These 3β-HSD-positive capillaries were found almost exclusively in the vicinity of 3β-HSD-positive Leydig cells. The counting of interstitial fields in cryostat sections yielded the following scores for the enzymatic reaction in randomly selected areas using an ocular (×10) grid and an objective ×40 (data expressed here in mean percentage of total no. of counted fields per section): Leydig cells as well as associated endothelial cells negative =56%, both cell types associated and positive =12%, only positive Leydig cells without any clear topographic association with endothelial cells =31%, positive Leydig cells and negative associated endothelial cells =0%, positive endothelial cells with negative associated Leydig cells =1%. No enzyme reactivity was seen in the walls of interstitial arteries, arterioles, venules or veins. This also holds true for the blood vessels lying within the tunica albuginea.

Ultracytochemistry for 3β-HSD

Reaction products of copper ferrocyanide for the oxidative activity of the enzyme 3β-HSD were found mainly on the membranes of the smooth endoplasmic reticulum

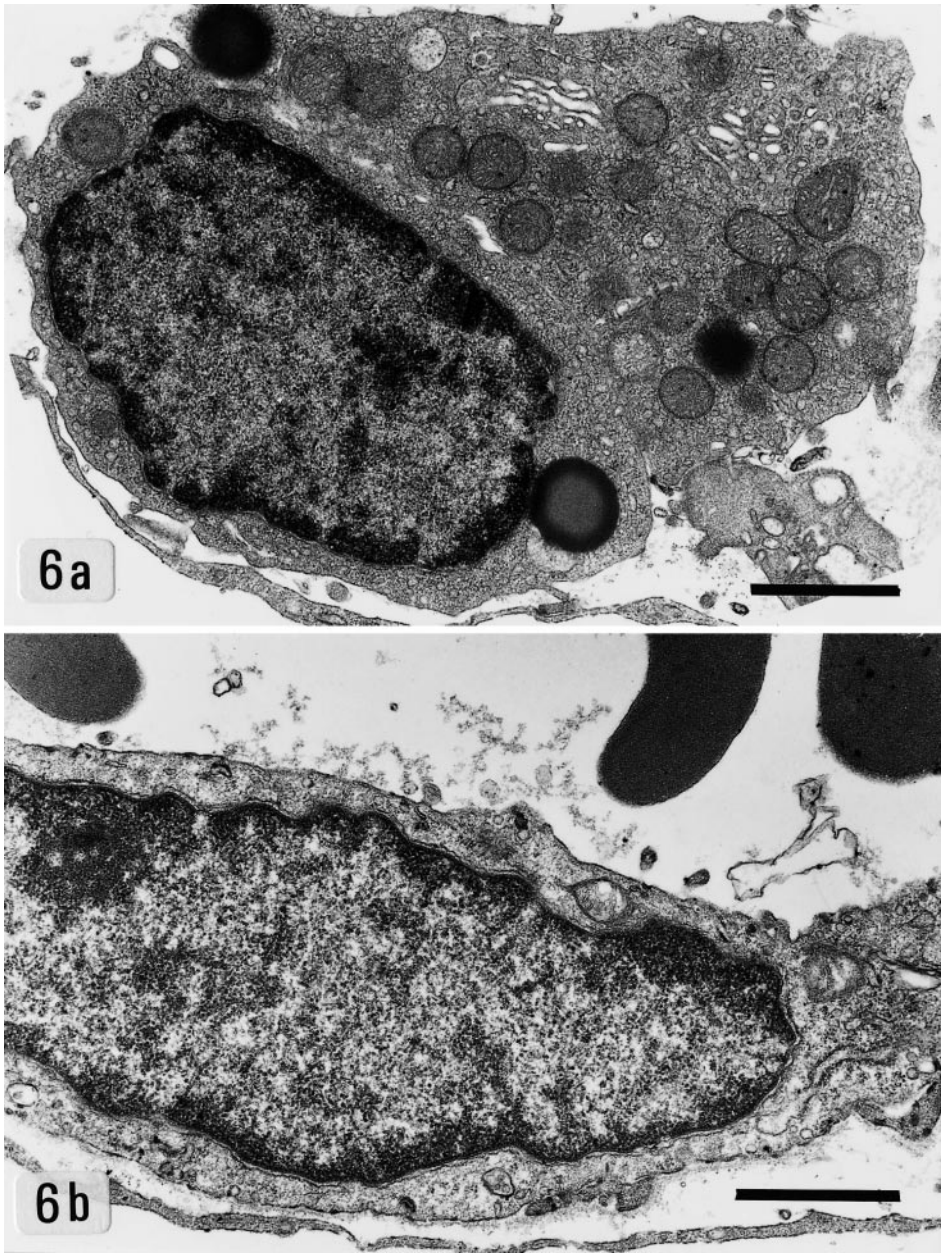

Fig. 6a, b Rat testis on pnd 15. Negative control sections, incubated without substrate for 3β-HSD. **a** A FTLC without reaction products. **b** No reaction product in an endothelial cell in close vicinity of Leydig cells (3 dark areas are erythrocytes). *Bar* 1 μm

(sER) of peritubular fibroblast-like precursors of ATLC (Fig. 2). These cells were located in the outer layer of the boundary tissue, i.e. lamina propria of seminiferous tubules and contained an oval nucleus, a moderately well-developed Golgi apparatus, mitochondria with tubular or lamellar cristae and a few lipid droplets. The FTLC were also well marked with electron dense reaction products on the membranes, predominantly the outer ones, of the sER (Fig. 3a, b). The inner layer of the lamina propria contained spindled shaped fibroblasts with elongated nuclei, only few mitochondria with tubular or lamellar cristae, rough endoplasmic reticulum, and long thin cytoplasmic processes (Fig. 2). These cells did not show the enzyme reaction product. This also holds true for the cell organelles of spermatogonia (Fig. 2), Sertoli cells (Fig. 2), and interstitial macrophages (Fig. 3a).

Apart from the two types of Leydig cells, the reaction product for the enzyme was also observed in endothelial cells. Certain interstitial blood capillaries displayed distinct precipitates on the membranes of the sER (Figs. 3a, 4, 5). The slender endothelial cells contained elongated, slightly indented nuclei, mitochondria with lamellar cristae, a moderate amount of sER, but no lipid droplets. Pericytes did not contain any reaction product; nor did the erythrocytes in the lumen of the capillaries (Figs. 3a and 4). Enzyme-positive capillaries were detected almost exclusively in the neighborhood of Leydig cells which were also enzyme positive as shown in Fig. 3a. Capillaries, whose endothelial cells were devoid of the reaction product, however, were located either far from Leydig cells or in the vicinity of Leydig cells that had at most traces of reaction products. Leydig cells devoid of pre-

cipitate were almost invariably accompanied by capillaries also lacking the enzyme.

The intestinal (ileum) tissue sections did not show any specific copper ferrocyanide precipitates, either in the endothelial cells or in any other cells. The negative control sections of the testis, i.e., the sections incubated without substrate, were completely devoid of the specific enzyme reaction product as shown in Fig. 6a (FTLC) and in Fig. 6b (an interstitial capillary). The semithin sections of testicular tissue, treated for 3β-HSD, were unsuitable for evaluating the enzymatic reaction because it was not clearly visible.

Discussion

This report presents two completely new observations: (1) the subcellular localization of 3β-HSD reaction products in FTLC and in the precursors of ATLC; (2) the focal presence of 3β-HSD in endothelial cells of interstitial blood capillaries.

The enzyme 3β-HSD has been studied at the ultrastructural level by several authors who employed either the tetrazolium salt method (Bara and Anderson 1973; Bara 1979; Frederiks et al. 1989), the ferricyanide method (Benkoel et al. 1976; Berchtold 1977; Hiura et al. 1981), or immunocytochemistry (Ishimura et al. 1988; Spek et al. 1996). Some of these reports localized the enzyme at the subcellular level, but none of them deals with developmental stages of Leydig cells. The results of the present study were obtained with the ferricyanide method. In preliminary experiments we also tested the tetrazolium salt method, but it was unsatisfactory. The precipitates were not clearly bound to structures and the results were inconsistent. The ferricyanide method of Benkoel et al. (1976), as modified by Berchtold (1977) and Hiura et al. (1981) led to a reproducible distinct localization of enzyme reaction product for 3β-HSD in the form of copper ferrocyanide precipitates on the membranes of the sER. The enzyme reaction was specific, since the negative control sections, incubated without substrate, were devoid of precipitates.

Earlier reports have localized 3β-HSD not only on the membranes of the sER but also on mitochondrial cristae (Bara and Anderson 1973; Benkoel et al. 1976; Berchtold 1977; Bara 1979; Hiura et al. 1981). However, the authors who used immunocytochemistry (Ishimura et al. 1988; Spek et al. 1996) localized the enzyme only on the membranes of the sER. Our findings are in complete agreement with these latter reports although our approach was cytochemical: the specific, distinct reaction product for 3β-HSD was located only on the membranes of the sER.

Up to pnd 12 only FTLC are the main source of testosterone in the rat testis (Tapanainen et al. 1984; Haider et al. 1986; Kuopio et al. 1989). The FTLC are completely surrounded by a basal lamina that separates them from the collagen fibers and slender fibroblasts in the interstitial tissue; the plasmalemm bears short microvilli and often thin membrane foldings. The cytoplasm contains sER, mitochondria and large lipid droplets. The FTLC possess 3β-HSD, LH receptors, androgen receptors and are able to synthesize testosterone (Kuopio et al. 1989; Haider et al. 1986, 1995, 1997; Majdic et al. 1995). Up to pnd 12 the lamina propria surrounding the seminiferous tubules consists of two thin layers – an inner and an outer layer – of peritubular fibroblasts. From pnd 13 onward the fusiform peritubular fibroblasts in the outer layer, which borders the interstitium, also contain sER as well as mitochondria with tubular cristae. At this age such fibroblasts are already histochemically positive for 3β-HSD, express receptors for androgen and LH, and are thus the most probable precursors for ATLC (Haider et al. 1986, 1995, 1997; Teerds et al. 1989). They are henceforth termed peritubular fibroblast-like precursors of ATLC. The ATLC most probably originate from these fibroblast-like cells, also known as mesenchyme-like progenitors (Hardy et al. 1989, 1990; Teerds et al. 1989; Chemes et al. 1994; Haider et al. 1995; Shan et al. 1997). We have localized 3β-HSD at the subcellular level in both types of Leydig cells which are simultaneously present on pnd 15. The ultracytochemical evidence of 3β-HSD activity on the sER in the precursors of ATLC strongly suggest that these cells have the capacity for testosterone biosynthesis and agree with the observations of Hardy et al. (1990) that they produce androgens. These cells are most likely morphogenetically and functionally identical to the cells that have been designated as mesenchyme-like progenitors of Leydig cells by Hardy et al. (1989, 1990) and Shan et al. (1997). The fibroblast-like precursors of ATLC possess androgen receptors and are sensitive to testosterone produced by the FTLC (Tapanainen et al. 1984; Kuopio et al. 1989; Teerds 1989; Shan et al. 1997). It can be assumed that LH from pituitary gland and testosterone from the FTLC play an important role in the functional differentiation and development of ATLC from peritubular fibroblast-like precursors. The precursors are probably a transitory cell form and ultimately differentiate into ATLC, as also shown by the morphological findings of Russel et al. (1995). The ATLC, in contrast to the FTLC, do not possess a basal lamina, the plasmalemm has only a few slender membrane foldings and the cytoplasm contains sER, mitochondria, only few small lipid droplets, and a large well-developed Golgi apparatus (Haider et al. 1995). The present results on the differentiation of ATLC are supported by the in vitro studies of Chemes et al. (1992, 1994) on the cytodifferentiation of Leydig cells in human and in rat testis. The studies in rat (Chemes et al. 1994) revealed that the cells isolated 30 days after ethylene dimethyl sulfonate treatment contain a mixed cell population with a predominance of mesenchymal elements showing an intermediate stage of differentiation with various degrees of 3β-HSD activity. These cells responded to hCG stimulation with a 3–4-fold increase in androgen secretion and were considered to be the precursors of Leydig cells. In our sections the cell organelles of the interstitial macrophages did not show the ultracytochemical reaction for 3β-HSD. Gaytan et al. (1994) have suggested that the secretion of essential growth factors by macrophages may

be needed for the differentiation of Leydig cells from mesenchymal precursors, as well as for the proliferative activity of the newly formed Leydig cells after treatment with ethylene dimethane sulfonate. The mode of differentiation of ATLC from the peritubular fibroblasts in the testis, as discussed here, and the mode of differentiation of theca cells from perifollicular fibroblasts in the ovary (Hiura et al. 1981) seem to be analogous; the perifollicular fibroblasts attain the ultrastructural features of a steroid-producing cell and contain 3β-HSD at the subcellular level, and such "transitory cells" ultimately develop into the cells of the theca interna (thecal gland), which produces steroids (Familiari et al. 1991).

The present work also revealed the ultracytochemical reaction of 3β-HSD on the membranes of the sER in the endothelial cells of interstitial blood capillaries, lying in the vicinity of 3β-HSD-positive Leydig cells. This is, to our knowledge, a novel observation. The focal localization of 3β-HSD reaction in the endothelial cells seems to be specific for the testis, since the endothelial cells of the blood capillaries in the wall of the ileum, which served as a negative control organ from the same animals, did not exhibit any specific reaction product. Ghinea et al. (1994) have described a receptor-mediated transendothelial transport of LH in rat testis; they found the LH/hCG receptors not only in Leydig cells but also in endothelial cells. Misrahi et al. (1996) examined the transport of hCG in rat testicular microvasculature and reported that LH/hCG receptors are present in endothelial cells and are involved in hormone transcytosis through these cells (for reviews, see Ghinea and Milgrom 1995). The possibility that LH within endothelial cells stimulates the biosynthesis of 3β-HSD should be further explored.

Some authors have recently shown the presence of androgen receptors in the smooth muscle cells of the tunica media of arteries and arterioles in the testis (Bergh and Damber 1992; Ergün and Ungefroren 1995). There is strong evidence showing that testosterone modulates vasomotion (rhythmical variations in capillary blood flow) and permeability of testicular vessels (Damber et al. 1989; Damber and Bergh 1992; Setchell 1994; Ergün et al. 1996). Leydig cells and endothelial cells seem to influence the contractility of smooth muscle cells and pericytes of the vessel walls by nitric oxide (Davidoff et al. 1995). Lissbrant et al. (1997) demonstrated the presence of NADPH diaphorase in the endothelial cells of rat testicular blood vessels, indicating the presence of nitric oxide synthase activity.

The cited reports on androgen receptors, testosterone and nitric oxide or NADPH in connection with blood vessels did not describe any specific distribution patterns. In our sections, however, the capillaries marked by precipitates for 3β-HSD were almost invariably found only next to Leydig cells, which were themselves marked for the enzyme. Those lacking precipitates were seen at greater distances from marked Leydig cells or next to unmarked ones (data not shown). The rare cases of a marked endothelial cell not accompanied by a marked Leydig cell may be an effect of sectioning; such Leydig cells might have been found in adjacent, unsectioned tissue. The possibility that the enzyme 3β-HSD is passed from Leydig cells to the blood and is only transiently present in the endothelium is highly unlikely because the precipitates were clearly associated with the sER within the endothelial cells. More information on the mechanisms that induce 3β-HSD gene expression in the various types of testicular cells would help to better understand the source, synthesis and significance of the enzyme in endothelial cells (Labrie et al. 1996).

Past reports have described a paracrine relationship between Leydig cells and the cells of the seminiferous tubules as well as autocrine and paracrine effects of androgens on Leydig cells (Berg 1983, 1985; Sharpe et al. 1990; Skinner 1991). The present observations show that there is a correlation between the functional status of Leydig cells and the presence of 3β-HSD in the endothelium of capillaries next to them. Further research on the interrelationship between Leydig cells and the other elements of the testicular interstitial tissue may reveal whether our findings are an aspect of regulation of testosterone biosynthesis or whether they belong to the paracrine aspect of testicular physiology.

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