Immunolocalization of Steroid 5α-Reductase Type 2 in Human Prostate, Seminal Vesicle and Vas Deferens —Light and Electron Microscopic Immunohistochemistry—

Tadayuki Miyamoto, Yoshihiro Tsuruo*, Susumu Kagawa, Hiromichi Yokoi* and Kazunori Ishimura*

Department of Urology, School of Medicine, The University of Tokushima, Tokushima 770 and *Department of Anatomy, School of Medicine, The University of Tokushima, Tokushima 770

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Localization of steroid 5α-reductase type 2 in human prostate, seminal vesicle and vas deferens was investigated by light and electron microscopic immunohistochemistry using a rabbit polyclonal antiserum raised against a synthetic peptide fragment representing amino acids 225-240 of the human 5α-reductase type 2 enzyme. Under light microscope, the enzyme was localized in the cytoplasm of basal epithelial and stromal cells of the prostate. Secretory epithelial cells were negative. There was no immunoreaction in cell nuclei. Double immunostaining for 5α-reductase and muscle specific actin indicated that all smooth muscle cells had 5α-reductase type 2. However, a few type 2-positive cells did not react with a muscle specific actin antibody. Electron microscopic immunocytochemistry revealed that the cytoplasm of smooth muscle cells and of a few fibroblasts had immunoreaction products for this enzyme. In the seminal vesicle and vas deferens, basal and smooth muscle cells were positive for the type 2 enzyme. These findings indicate that the major sites of the DHT production are basal epithelial cells and stromal smooth muscle cells in human prostate, seminal vesicle and vas deferens. These cells may play a role in maintaining the functional activity of these male accessory sex organs through the production of DHT.

Key words: Human 5α-reductase type 2, Human prostate, Basal epithelial cell, Stromal cells, Immunocytochemistry

I. Introduction

The differentiation and development of the prostate as well as the male external genitalia are dependent on androgens, especially dihydrotestosterone (DHT) which is converted from testosterone by steroid 5α-reductase [30]. Therefore, steroid 5α-reductase is the key enzyme in the androgen-mediated effects on these male organs [21]. Individuals with a genetic deficiency of this enzyme, fail to develop a normal prostate [12, 30]. There are two distinct 5α-reductase isozymes, type 1 and 2, and mutations of the gene encoding the latter have been known to induce male pseudohermaphroditism [20].

The human prostate contains high levels of 5α-reductase activity. This activity is exclusively due to the type 2 enzyme [27]. Studies of the relationships between 5α-reductase activity and prostatic diseases have demonstrated that the enzyme activity is higher in hyperplastic prostate than in normal one [3, 10]. The development of benign prostatic hyperplasia (BPH) may depend on the action of DHT synthesized by 5α-reductase. Thus, 5α-reductase inhibitors have been used to treat BPH [9].

The localization of the type 2 enzyme in human urogenital tissues has been studied immunohistochemically using polyclonal antibodies against synthetic peptide fragments of this enzyme [6, 26]. Silver et al. [26] used a peptide fragment (amino acids 227–251) as an antigen and demonstrated that basal and stromal cells of the human prostate were positive, whereas the basal cells of the seminal vesicle were negative. However, Eicheler et al. [6] showed that the entire epithelial component was positive in both the prostate and the seminal vesicle, when an antiserum against a peptide fragment (amino acids 234–245) of the enzyme was applied. These findings are still con-
trouversial. Besides, the type of the 5α-reductase-positive cells in the stromal compartments has not yet been completely identified. To address these issues, we prepared an antibody against a synthetic peptide fragment (225-240) of human 5α-reductase type 2, and immunohistochemically studied the localization of the enzyme in the male genital organs including the prostate, seminal vesicles, vas deferens and ejaculatory ducts. Furthermore, we tried to identify the cell types of stromal cells expressing this enzyme in the prostate by double immunohistochemical staining as well as electron microscopic immunocytochemistry.

II. Materials and Methods

Tissues

Human prostate tissues were obtained during open prostatectomy (n=5, due to BPH, 63-74 years of age, mean 70.0 years old) and total cystoprostatectomy (n=5, due to bladder cancer, 59-71 years of age, mean 65.0 years old). Seminal vesicles and vas deferens in the surgical specimen of total cystoprostatectomy were employed for this study. The plasma testosterone levels of all patients were within normal range. None of the patients had undergone endocrine therapy.

For light microscopic immunohistochemistry, all tissue samples were fixed in 4% formaldehyde in 100 mM phosphate buffer solution at pH 7.4 for 24-36 hr, then dehydrated in ethanol and embedded in paraffin. Hematoxylin and eosin-stained sections were screened to exclude incidental foci of carcinoma. For immunoelectron microscopy, the tissues were fixed in periodate-lysine-paraformaldehyde solution (PLP; 2% paraformaldehyde) at 4°C for 4 hr. After fixation, the tissues were immersed in phosphate-buffered saline (PBS) containing ascending concentrations of sucrose (10-20%), embedded in OCT compound, and stored at −80°C.

Antibody preparation

To avoid homology with amino acid sequences of other known proteins, the GENETYX database (SDC, Japan) was checked before selecting the peptide sequence used for immunization. A peptide representing amino acids 225 to 240 of human 5α-reductase type 2 isozyme [1] with an additional cysteine residue at the carboxy-terminus was synthesized and coupled to keyhole limpet hemocyanin using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) as a coupling reagent [16]. Antisera against the peptide were raised in young female Japanese white rabbits. Antibodies were purified from serum using HiTrap NHS-activated Sepharose columns (Pharmacia Biotech, Japan) coupled with the original antigenic peptide. Antibodies were eluted with 100 mM glycine buffer, pH 2.5 and collected in 1/20 volume of 1M Tris buffer. The antibody fractions were checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blotting

The tissue samples for immunoblotting were homogenized with a Polytron in 4 volumes of 10 mM potassium phosphate, pH 7.0, 150 mM potassium chloride, 1 mM ethylenediaminetetraacetic acid and a broad spectrum of protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, USA), 2 µg/ml aprotinin (Boehringer Mannheim, Mannheim, Germany), 1 µg/ml leupeptin (Boehringer Mannheim) and 1 µg/ml pepstatin (Boehringer Mannheim). Tissue homogenates were centrifuged at 4°C for 30 min at 10,000 × g and the supernatants were analyzed for 5α-reductase type 2.

The samples (10 µg protein) were denatured before electrophoresis by boiling for 5 min in sample buffer. The denatured protein samples were electrophoresed on a 12.5% polyacrylamide gel containing 0.1% SDS (wt/vol), and transferred to Immobilon membranes by electroblotting at 100 V for 1 hr in transfer buffer (25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol, pH 8.4). The membranes were soaked in Block Ace (Yukijirushi, Sapporo), and incubated at 22°C for 1 hr with affinity-purified 5α-reductase type 2 antiserum diluted to a final concentration of 1.0 µg/ml in Tris-buffered saline (TBS) containing 0.05% Tween 20. Thereafter, the membranes were washed 3 times for 10 min each in TBS containing 0.05% Tween 20, then incubated for 1 hr at room temperature in a 1 : 2,500 dilution of an anti-rabbit IgG coupled to horseradish peroxidase (Promega, Madison, WI, USA) followed by 3 washes in the above buffer. Immune complexes were visualized by incubation with substrates for enhanced chemiluminescence detection (ECL; Amersham, Buckinghamshire, England) for 1 min. The membranes were exposed to X-ray film for 1 to 5 min.

Immunohistochemistry

For light microscopy, sections in 6 µm thickness were cut, mounted onto gelatin/chrome alum-coated glass slides and dried overnight at 37°C. After deparaffinization and rehydration, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water. We performed antigen retrieval experiments as originally described by Shi et al. [24]. The sections were placed in a plastic box containing 300 ml of 0.01 M citrate buffer, pH 6.0 and heated in a microwave oven (Panasonic NE-A33, Japan) seven times for 3 min at 500 W. The sections were cooled to room temperature. Following several rinses in PBS, the sections were immersed in avidin D and biotin ( Vectastain, Burlingame, CA., USA) to block endogenous avidin-binding activity, then incubated with affinity-purified 5α-reductase type 2 antiserum (1.0 µg/ml) overnight at 4°C. The sections were reacted for 1 hr with a biotinylated goat anti-rabbit antibody (1 : 100; Vector Laboratory) and stained with avidin-biotin-peroxidase complex (ABC) (Elite ABC, Vector Laboratory). The sections were incubated with 0.01% diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6, and then the DAB...
chromogen was intensified with silver-gold [17]. The sections were coverslipped and photographed using a Nikon light microscope.

For electron microscopic examination, frozen sections of 8 μm thickness were immunostained as described above without antigen retrieval. The positive cells were intensified with silver-gold and postfixed in 1% osmium tetroxide for 1 hr at room temperature. They were dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were cut on a Reichert Ultracut E, mounted on copper grids and examined using a Hitachi H-500 electron microscope.

For controls, some sections were incubated with preimmune serum or first antiserum that had been incubated with an excess of the corresponding antigens (100 μg/ml).

**Double immunolabeling**

Some paraffin embedded sections were double-immunostained for 5α-reductase type 2 and muscle-specific actin. The sections were incubated with muscle-specific actin monoclonal antibody (HHF-35) (1:100; DAKO, CA., USA) at 4°C overnight. This antibody does not react with fibroblasts, endothelial cells, or macrophages [31]. After rinsing with PBS, the sections were incubated with a fluorescent isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:100; Vector Laboratory, Burlingame, CA., USA) for 1 hr at 32°C, rinsed with PBS, then incubated with affinity-purified 5α-reductase type 2 antiserum (5 μg/ml) at 4°C overnight. After washing with PBS, a biotinylated goat anti-rabbit IgG (1:100; Vector Laboratory) was applied to the sections for 1 hr. Thereafter, rhodamine-avidin D (1:500; Vector Laboratory) was applied for 1 hr. Following several rinses the stained sections were coverslipped with 80% glycerol in PBS containing 0.1% p-phenylene-diamine dihydrochloride. Immunofluorescent labeling for muscle-specific actin (FITC) and 5α-reductase type 2 (rhodamine) in the same area were photographed in turn by changing the appropriate filters.

### III. Results

**Western blotting**

When the protein extracts from the prostate, seminal vesicle and vas deferens were immunoblotted using affinity-purified 5α-reductase type 2 antiserum, two bands were detected with an apparent molecular mass of 29 and 50 kilodaltons (kDa) (Fig. 1, left panel). When the antiserum preabsorbed by an excessive antigen peptide (100 μg/ml) was applied, the band at 29 kDa disappeared, whereas that at 50 kDa remained (Fig. 1, right panel). Hence, the band of 29 kDa protein was thought to specifically react with the antiserum.

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**Peptide(-)**   **Peptide(+)**

Fig. 1. Steroid 5α-reductase type 2 expression in the prostate, seminal vesicle and vas deferens. **Left panel:** Aliquots of the tissue extracts were immunoblotted using affinity-purified 5α-reductase type 2 antiserum. Protein molecular mass standards are indicated on the left. **Tissues:** lane 1, prostate; lane 2, seminal vesicle; lane 3, vas deferens. Two bands were detected with an apparent molecular mass of 29 and 50 kDa. **Right panel:** The band at 29 kDa in each lane is abolished in the presence of excess antigen peptide (arrow).

**Localization of 5α-reductase type 2 in the prostate**

**Light microscopic immunohistochemistry**

Histological observation revealed that hyperplastic changes proceeded with age in both samples of BPH and total cystoprostatectomy. We chose areas of normal prostatic architecture and examined them immunohistochemically. Areas of inflammation, cystic atrophy, basal cell hyperplasia and premalignant status were excluded. Since no distinct differences in the immunohistochemical staining between these two groups were observed, we described the results together.

Positive immunoreaction for 5α-reductase type 2 was found in the cytoplasm of stromal and basal epithelial cells of the prostate (Fig. 2a). There was no immunoreactivity in the secretory epithelial cells, or in the nuclei. The stained basal cells were continuously observed on the basement membrane. There was no immunoreaction when the anti-5α-reductase type 2 serum was preabsorbed with the antigen peptide (Fig. 2b). There was no significant difference in the staining intensity between the central (close to the urethra) and peripheral (underneath the capsule) region in the prostate (Fig. 2c, d). The basal cells of the periurethral ducts were stained (Fig. 2c), but no apparent positive staining was present in the epithelial cells of the prostatic utricle (Fig. 2f). The staining intensity did not vary with the degree of the stromal proliferation. Positively stained cells were also seen in the capsule (Fig. 2d), and in the wall of blood vessels (Fig. 2e).

**Immunofluorescence microscopy**

To determine whether the type 2 enzyme was expressed in smooth muscle cells in stroma of the prostate, double immunofluorescence labeling was carried out using 5α-reductase antiserum and actin monoclonal antibody HHF-
Fig. 2. Light microscopic immunohistochemistry of 5α-reductase type 2 in human prostate. a: Positive reaction for 5α-reductase type 2 is localized in the cytoplasm of basal epithelial and stromal cells. Secretory epithelial cells are negative. b: Immunoreactivity was not demonstrated in the section exposed to the antisera preabsorbed by an excess amount of the antigen peptide. c–e: There are positive reactions in the basal cells of periurethral ducts (c) and of prostatic gland near the capsule (d). Smooth muscle cells of the capsule (d) and the vascular wall (e) are also positive. f: The immunoreaction is absent in the utriculus. a $\times$ 380, b $\times$ 190, c–e $\times$ 380, d–f $\times$ 95.
As shown in Fig. 3, most of the type 2-positive cells (Fig. 3a) were stained by actin antibody (Fig. 3b). But, some type 2-positive cells were negative for actin (Fig. 3a, arrow). These cells were spindle-shaped, and smaller than stromal smooth muscle cells. These results indicated that the type 2 enzyme was mainly present in the smooth muscle cells.

**Electron microscopic immunocytochemistry**

An electron microscopic observation was performed to elucidate the cell types expressing 5α-reductase type 2 and the subcellular distribution of the enzyme. Smooth muscle cells in the prostatic stroma had immunoreaction products for 5α-reductase. Electron-dense grains indicating the presence of this enzyme were scattered throughout the cytoplasm of the smooth muscle cell (Fig. 4-a, b) but they were not found in the nucleus (Fig. 4b). We could not determine precise subcellular localization of the grains in the cytoplasm (Fig. 4-a, b).

While the majority of fibroblasts were unlabeled or weakly labeled (Fig. 4a), some were positively stained for the type 2 enzyme (Fig. 4-c, d).

**Localization of 5α-reductase type 2 in seminal vesicle and vas deferens**

Positive immunoreaction for 5α-reductase type 2 was seen in the cytoplasm of basal epithelial (Fig. 5-a, c) and smooth muscle cells (Fig. 5-b, d) of the seminal vesicle and vas deferens. There was no immunoreactivity in the cell nuclei. The staining intensity was similar to that in the prostate. However, there was no immunoreactivity in the epithelial cells of the ejaculatory duct (Fig. 5e).

**IV. Discussion**

**Specificity of the antiserum**

In the present study, we chose peptide fragment (225-240) as an antigen in order to avoid the possibility that the antiserum crossreacted with type 1 enzyme.

Western blotting using affinity-purified antiserum recognized two immunoreactive bands with apparent molecular masses of 29 and 50 kDa. The band at 29 kDa disappeared in the presence of excessive antigen peptide. Therefore, the antiserum recognized the protein at 29 kDa. The size of the protein corresponded to the molecular weight calculated from the cDNA sequence of human 5α-reductase type 2 [1]. The 50 kDa band was considered nonspecific, since it was present when the antiserum was incubated with the antigenic peptide. This was confirmed by the results of the immunohistochemistry in which preabsorbed antiserum did not positively immunostain the prostate and other tissues. These findings indicated that our antiserum specifically reacts with human 5α-reductase type 2.

**Localization of 5α-reductase type 2 in the prostate, seminal vesicle and vas deferens**

The present results demonstrated the presence of 5α-reductase type 2 in basal epithelial and stromal cells of the human prostate. These findings coincided well with those of Silver et al. [26], but not those of Eicheler et al. [6], who showed that this enzyme was present in secretory cells in addition to the basal and stromal cells. Since preparation procedures of materials were almost the same among three groups, these discrepancies may be due to the difference in the specificity of the antibodies. Recently, we immunohistochemically demonstrated that the type 1 enzyme was present only in secretory cells of rat prostate [19]. Therefore, it is possible that the two isozymes of 5α-reductase may exist in different cell types in the prostate, and it is interesting to examine whether different components express their own isozyme.

The functional significance of the basal cells in the human prostate has not been elucidated. Ultrastructural

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**Fig. 3.** Double immunostaining of 5α-reductase type 2 (a) and muscle-specific actin (b). Most of the 5α-reductase-containing stromal cells are also positive for HHF-35, but some are not (arrow). Type 2-positive basal cells (a, arrowhead) are devoid of muscle specific actin (b, arrowhead). a, b × 625.
Fig. 4. Electron microscopic immunocytochemistry of 5α-reductase type 2 in the prostate. a, b: Electron dense grains indicating the presence of 5α-reductase type 2 are scattered throughout the cytoplasm of smooth muscle cells (M). A type 2 immunonegative fibroblast (F) is shown. a × 7,200, b × 18,000. c, d: Dense grains are present in the fibroblast. c × 14,000, d × 18,000.
studies in the early days suggested that the basal cells were undifferentiated stem cells which gave rise to secretory epithelial cells, because these cells were characterized by poorly developed cytoplasmic organelles and abundant free ribosomes [5, 18, 28]. However, the present results suggested that the basal cells were functionally rather active in steroid metabolism. Other studies have also revealed that the basal cells differ from secretory epithelial cells...
in terms of proliferative activity and hormonal regulation [7, 8]. Androgen deprivation induces atrophy or loss of the secretory epithelial cells, whereas the basal cells are maintained [7]. Issacs and Coffey [13] reported that the basal cells persisted even in long-term castrated animals. Since both basal and secretory cells contain androgen receptors [2, 14], the difference in the persistence may depend on the absence or presence of the enzyme which convert the steroids. The basal cells probably produce sufficient DHT to maintain themselves even in the presence of low testosterone levels.

In the stroma, HHF-35-positive cells also retained 5α-reductase type 2. In addition, electron microscopic immunochemistry confirmed that this enzyme was expressed in the cytoplasm of the smooth muscle cells. These cells contain androgen receptors [14] and shrink following exposure to 5α-reductase inhibitor [22]. These findings indicate that DHT synthesized by this enzyme in smooth muscle cells acts in an autocrine fashion to direct cell growth.

Biochemical studies of androgen metabolism in the human prostate have shown that 5α-reductase activity was detected in stroma as well as in epithelium and the Vmax values for the enzyme were larger in the stroma of BPH than in that of the normal prostate [3, 15]. Our study showed that all smooth muscle cells retained this enzyme and that there was no significant difference in the staining profiles between BPH and total cystoprostatectomy specimens.

This study also showed that a small number of fibroblasts in the stroma contained 5α-reductase type 2. However, the majority of the fibroblasts were negative for the enzyme. This coincides well with the previous study showing that the 5α-reductase activity was very low in prostatic fibroblasts [4]. Therefore, it seems unlikely that fibroblasts play a major role in DHT production in the prostate.

In contrast to the prostatic glands and stroma, the region of the utriculus was devoid of 5α-reductase type 2. Although Wernert et al. [29] reported that the function of the utriculus did not differ from that of the prostate, our results indicate that the ability to metabolize steroids is different in these two portions. This may be related to their origin. The utriculus is derived from the Müllerian ducts and is more sensitive to estrogens than the rest of the gland [23].

In seminal vesicle, the stromal cells and basal epithelial cells were positive for 5α-reductase. This result is the same with that of Eicheler et al. [6]. However, Silver et al. [26] reported that only stromal cells are positive for 5α-reductase. The growth and postnatal epithelial development have been known to depend on DHT [11, 25]. Based on these findings, Shima et al. [25] suggested a possibility that testosterone is converted to DHT in epithelial compartment of seminal vesicle. Our result of the type 2 enzyme expression in basal epithelial cells supports this speculation.

The localization of 5α-reductase in human vas deferens was first demonstrated in the present study. The enzyme in this organ may play a role similar to that in seminal vesicle, though the physiological significance of 5α-reductase remains unknown. This issue should be clarified.

In conclusion, the major sites of the DHT production are basal epithelial and stromal smooth muscle cells in the human prostate, seminal vesicle and vas deferens. These cells may play a role in the maintenance of functional activity of these male accessory sex organs through production of DHT.

V. References


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