Immunocytochemical Localization of 17β -Hydroxysteroid Dehydrogenase (17β -HSD), and its Relation to the Ultrastructure of Steroidogenic Cells in Immature and Mature Rat Ovaries*

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Summary. Immunocytochemical localization of 17β -hydroxysteroid dehydrogenase (17β -HSD) and its relation to the ultrastructure of steroidogenic cells were examined in mature and immature rat ovaries.

In mature (8-10 weeks old) rat ovaries, the theca interna cells of secondary as well as Graafian follicles, and the interstitial gland cells were all strongly stained with anti- 17β -HSD antibody. However, granulosa cells, corpus luteum cells, oocytes and peritoneal epithelial cells were negative against this staining. In the ovaries of 1-week-old rats, all these cells were negative to immunostaining for 17β -HSD. In the ovaries of 2-week-old rats, the theca interna cells of secondary follicles and the interstitial gland cells showed a positive reaction for the 17β -HSD activity.

Electron microscopic examination demonstrated the presence of characteristic structures for steroid secretory cells such as many lipid droplets, well developed smooth endoplasmic reticulum, and oval mitochondria with tubular cristae in the theca interna cell of secondary as well as Graafian follicles and in the interstitial gland cell of mature rat ovaries. In the ovaries of 1-week-old rats, all the theca cells of the primary and secondary follicles were fibroblast-like in their shape and fine structure, and typical interstitial cells were not recognized. In the 2-week-old rats, some of the theca interna cells and interstitial cells were well differentiated in ultrastructure, showing characteristic features for steroid secretory cells.

These findings indicate that by 2 weeks after birth, theca interna cells and interstitial gland cells acquire the ability for testosterone production as seen in mature rat ovaries.

This paper is a part of our fine structural and immunocytochemical studies on the mechanism of hormone production in steroidogenic cells.

It is well known that ovarian estrogens are synthesized from androgens (for a review, see TAMAOKI, 1973). In order to clarify the site of estrogen biosynthesis in the ovary, we previously investigated in vivo localization of aromatase, the enzyme aromatizing androgens to estrogens, by means of immunocytochemistry using anti-

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aromatase II cytochrome P-450 antibody. We found that the enzyme is localized in the theca interna cells, the interstitial gland cells, and corpus luteum cells (MATSUDA et al., 1984).

The site of androgen biosynthesis in the ovary is also still controversial. Many studies have been carried out to localize 17β -hydroxysteroid dehydrogenase (17β -HSD), which is an enzyme converting androstenedione to testosterone. For example, IANNETTA and MELLODE OLIVEIRA (1979) showed 17β -HSD activity in the theca interna cells, the granulosa cells, the corpus luteum cells and the peritoneal epithelial cells, while AONO et al. (1981) reported that 17β -HSD activity is localized in the granulosa cell. To resolve these discrepancies, the immunocytochemical approach using anti- 17β -HSD antibody was applied for its conduciveness toward bringing more informative results concerning the *in vivo* location of androgen synthesis in the ovary, as in the case of aromatase.

In regard to the onset of the steroid production, it has been reported that neonatal rat ovaries already possess enzymes engaged in steroid hormone biosynthesis (QUATTROPANI and WEISZ, 1973; FUNKENSTEIN et al., 1980; SHAHA and GREENWALD, 1983; CARSON and SMITH, 1986). In addition, through their biochemical studies, CARSON and SMITH (1986) and CSERNUS (1986) drew attention to the significant increase in testosterone synthesis around 2 weeks after birth. Immunocytochemistry using anti- 17β -HSD antibody is necessary to clarify the onset of ovarian testosterone production from the morphological aspect.

Steroid secreting cells are known to have ultrastructural characteristics such as well-developed smooth endoplasmic reticulum (SER), round mitochondria with tubular or tubulovesicular cristae, and many lipid droplets. In fact, it has been reported that the theca interna cells acquire these characteristic structures when they are developed from the fibroblast-like cells (HIURA and FUJITA, 1977). Therefore, ultrastructural observation is needed to evaluate whether or not the cells show a steroidogenic activity.

In the present study using the ovaries of 1, 2, 8 and 10-week-old rats, the *in vivo* localization of 17β -HSD was immunocytochemically investigated. At the same time, the ultrastructural changes of the steroidogenic cells were observed.

MATERIALS AND METHODS

The ovaries of Wistar rats at 1, 2, 8 and 10 weeks of age were used for this study.

For immunocytochemistry the rats were perfused via the left heart ventricle with buffered 4% paraformaldehyde solution, and the bilateral ovaries were removed. The organs were cut and immersed in the same fixative for 24 hrs at 4°C. After fixation, the tissue samples were rinsed in 10%, 15%, and 20% sucrose/phosphate buffered saline (PBS) solutions for 6 hrs. Cryosections (6 μ m in thickness) were cut at -25°C using Ames-II cryostat (Miles) and mounted on albumin-coated glass slides. Antiporcine testicular 17β -HSD antibody was used as the first antibody. All of the procedures for the purification of the antigen (17β -HSD), the production of the antibody, and assay of the immunoactivity of the antibody to the antigen have been previously described (INANO et al., 1980). The sections were incubated in the rabbit antibody against 17β -HSD (1:500) overnight at 4°C. After rinsing in PBS, the sections were treated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Miles-Yeda) (1:40) for 2 hrs at room temperature. The other sections were treated with horseradish peroxidase

(HRP)-conjugated anti-rabbit IgG (Miles-Yeda) overnight at 4°C. After being rinsed in PBS, the sections were treated with a medium containing 0.005% 3-3' diaminobenzidine (DAB) and 0.03% H₂O₂ for 5-10 min at room temperature. The sections were examined with a Nikon fluorescein microscope or a Zeiss Photomicroscope. Control sections were treated with normal rabbit serum instead of the first antibody.

For electron microscopy, the ovaries were fixed in 3% glutaraldehyde solution buffered at pH 7.4 with 0.1 M Millonig's phosphate for 4 hrs at 4°C. Post-fixation was performed in 1% OsO₄ solution for 1 hr at 4°C. After dehydration in a graded series of alcohol, the tissue blocks were embedded in Epon epoxy resin. Thin sections were stained with uranyl acetate and lead citrate, and examined in a Hitachi H-500 electron microscope.

RESULTS

Immunocytochemistry

In the mature rat (8 and 10 weeks old) ovaries, corpora lutea and follicles at various

Fig. 1. Light microscopic immunocytochemistry of 17 β -HSD in the 8-week-old rat ovary. The theca interna cells (*T*) of secondary and Graafian follicles, and the interstitial gland cells (*I*) are immunostained. The granulosa cells (*G*) and the corpus luteum cells (*C*) are entirely negative. $\times 40$

Fig. 2. Light microscopic immunocytochemistry of 17 β -HSD in the 2-week-old rat ovary. Some theca interna cells (*T*) of small secondary follicles, and the interstitial gland cells (*I*) are positively stained. The granulosa cells (*G*) and oocytes (*O*) are entirely negative. No corpus luteum is present. $\times 40$

stages of development were seen. Well-developed interstitial glands occupying a large part of the interstitium in the ovary were also recognized. The theca interna cells of secondary and Graafian follicles, and the interstitial gland cells were always stained positively with anti- 17β -HSD antibody. The granulosa cells, corpus luteum cells, oocytes and peritoneal epithelial cells were negative for 17β -HSD (Fig. 1).

The ovaries of 1-week-old rats were characterized by many primordial follicles

Fig. 3. Electron micrograph of an 8-week-old rat ovary. A part of a secondary follicle with the theca interna (*T*) is seen. *O* oocyte, *G* granulosa cells, *I* interstitial gland. $\times 4,200$

and a few small secondary follicles, while no interstitial glands, and no corpora lutea were seen. All the cells in these young ovaries were immunocytochemically negative for 17 β -HSD.

In 2-week-old rats, the ovary showed many primary and a few secondary follicles, and several small interstitial glands. Positive reactions for 17 β -HSD were seen in some theca interna cells of the small secondary follicle consisting of two or three granulosa cell layers, and in the interstitial gland cells (Fig. 2). No corpus luteum was present in these immature ovaries. The granulosa cells, oocytes, peritoneal epithelial cells and other connective tissue cells were entirely negative to immunostaining for 17 β -HSD.

All the control sections were negative.

Electron microscopy

Mature rats

The ovaries of mature (8 and 10 weeks old) rats had numerous follicles in various stages of development, corpora lutea, and well developed interstitial glands (Fig. 3).

Fig. 4. Electron micrograph of parts of theca interna cells of the secondary follicle from a 10-week-old rat ovary. Elements of SER, oval or elongated mitochondria with tubular cristae, and several lipid droplets are seen. $\times 37,000$

The cells surrounding the primary follicle were flat and fibroblast-like in shape. They contained moderately developed rough endoplasmic reticulum (RER), elongated mitochondria with lamellar cristae, and few lipid droplets. The small secondary follicles with two to three layers of granulosa cells were surrounded by theca interna layers. Though all the cells in this layer were fibroblast-like in shape, a few lipid droplets, and oval mitochondria with tubular cristae were noticed in some theca cells. In more developed follicles, many typical theca gland cells showing characteristic structures for the steroid-secreting cell, such as many lipid droplets, well developed smooth endoplasmic reticulum (SER), and oval mitochondria with tubular cristae appeared, though they were flat and fibroblast-like in shape. In addition, typical fibroblasts, whose cytoorganelles do not show any characteristic structures for steroid secreting cells, were also present in the theca interna layer. The cells located in the inner part of the theca interna showed a not-so-characteristic ultrastructure for the steroid-secretory

Fig. 5. Electron micrograph of parts of the interstitial gland cells from a 10-week-old rat ovary. Well developed SER, oval mitochondria with tubular cristae, and many lipid droplets are seen. $\times 38,000$

cell, while other cells located in the outer part of the theca interna showed a more typical ultrastructure for the steroid-secreting cell. They possessed numerous lipid droplets, well developed SER, and large oval or round mitochondria with tubular cristae (Fig. 4).

Interstitial gland cells, polygonal in shape, showed a more characteristic appearance for the steroid-secreting cell as compared with the theca interna cell (Fig. 5).

The follicle epithelial cells of the primary follicle were flat in shape and had numerous free ribosomes, poorly developed RER, and a few elongated mitochondria with lamellar cristae. In secondary and Graafian follicles, the granulosa cells derived from the follicle epithelial cells had abundant free ribosomes, many—but small—elements of RER, an increased number of elongated mitochondria with tubular cristae, and prominent Golgi apparatus. Lipid droplets were often observed in the granulosa cells located near the basal lamina of the Graafian follicle.

Fig. 6. Electron micrograph of a part of the secondary follicle from a 2-week-old rat ovary. Some cells (*F*) of the theca interna (*T*) are fibroblast-like, while others (*S*) show characteristic structures of the steroid-secreting cell. $\times 5,000$

Immature rats

The ovaries of 1-week-old rats contain numerous primordial or primary follicles and a few small secondary follicles. The secondary follicle has two or three layers of granulosa cells showing an almost identical ultrastructure to those of the mature rats.

Fig. 7. Electron micrograph of a part of the interstitial gland of a 2-week-old rat ovary. The interstitial gland cells are polygonal in shape, and have many lipid droplets and oval mitochondria. $\times 4,000$

Fig. 8. Higher magnification of parts of the interstitial gland cells in a 2-week-old rat ovary. Many lipid droplets (*L*), well-developed SER and oval mitochondria (*M*) with tubulovesicular cristae are seen. $\times 35,000$

Most cells surrounding the secondary follicle were fibroblast-like in shape and had elongated mitochondria with lamellar cristae, small elements of RER, and many free ribosomes. Though small lipid droplets were seldom present, no characteristic features for the steroid secretory cell were recognized in this cell. Interstitial glands were scarcely observed in this stage.

The ovaries from 2-week-old rats had several antral follicles and interstitial glands in addition to the primary and secondary follicles (Fig. 6). The ultrastructure of the granulosa cell in the antral and secondary follicles was generally similar to that in mature rats. Certain fibroblast-like cells surrounding these larger secondary follicles as well as all the interstitial gland cells showed the typical appearance of the steroid-secretory cell such as lipid droplets, oval mitochondria with tubular cristae, and many profiles of SER (Fig. 7, 8).

DISCUSSION

It is well known that 17β -HSD is an enzyme involved in the synthesis of testosterone from androstenedione (for a review, see TAMAOKI, 1973). Testosterone, the main androgenic steroid in males, is an important precursor of estradiol- 17β which is one of the major sex steroids produced in the female gonad. As to the site of testosterone biosynthesis in the ovary, there has been some disagreement. IANNETTA and MELLO DE OLIVEIRA (1979) reported histochemically that 17β -HSD activity is localized in the theca interna cell, interstitial gland cell, granulosa cell and peritoneal epithelial cell of the rat ovary. On the other hand, AONO et al. (1981) attributed the 17β -HSD activity in the rat ovary to the granulosa cell, inferring from the biochemical data that the enzyme activity was lost when the granulosa cell was removed by X-ray irradiation. BOGOVICH and RICHARDS (1984) further suggested that the theca and granulosa cells from the small antral and preovulatory follicles might possess 17β -HSD activity, from evidence that testosterone was accumulated and increased in this tissue when these cells were incubated with androstenedione.

As to the site of testosterone biosynthesis in the testis, two controversial theories have been put forward: some investigators have reported that the 17β -HSD activity is demonstrated histochemically only in the Leydig cell of the interstitial tissue (BAILLIE et al., 1966; VAN STRAATEN and WENSING, 1978), while others have the opinion that this enzyme is localized in both the Sertoli cells of the seminiferous tubule and Leydig cells, using the same method (SAIDAPUR, 1976; LAZARD, 1979; BILASPURI and GURAYA, 1984). In order to clarify this problem, we previously investigated the localization of 17β -HSD in a porcine testis by means of immunocytochemistry using anti- 17β -HSD antibody, and found that only the Leydig cell was positively immunostained (KUROSUMI et al., 1986). It is believed that the immunocytochemical approach using an antibody against enzyme proteins involved in the steroid hormone biosynthesis is a more appropriate means than a histochemical one in order to detect the precise localization of the steroidogenesis.

The present immunocytochemical study clearly demonstrated that, in mature rat ovaries, theca interna cells of secondary as well as Graafian follicles and interstitial gland cells were positively stained with 17β -HSD antibody. This fact means that these cells synthesize testosterone in the rat ovary. In our laboratory, MATSUDA et al. (1984) immunocytochemically demonstrated aromatase, an enzyme converting testosterone to estradiol- 17β , in theca interna cells of secondary and Graafian follicles, in the interstitial gland cells and corpus luteum cells of ovaries of various mammalian species including the rat. From these findings, it is believed that in the rat ovary, the theca interna cell and the interstitial gland cell have the ability for *de novo* synthesis of estrogen, while the corpus luteum cell does not have such an ability because it lacks 17β -HSD. Electron microscopic observation demonstrated that cytoorganelles of both theca interna cells and interstitial gland cells show the ultrastructures characteristic of the steroid secretory cell. These ultrastructural findings obtained in young and adult animals coincide well with the immunocytochemical data on these cells.

It is well known that the ovary of the immature rat can produce progesterone, testosterone and estradiol (DÖHLER and WUTTKE, 1975; MEIJS-ROELOFS et al., 1975; CARSON and SMITH, 1986; CSERNUS, 1986). It has been thought that even neonatal rat ovaries possess the enzymes involved in steroid hormone biosynthesis (QUATTROPANI and WEISZ, 1973; FUNKENSTEIN et al., 1980; SHAHA and GREENWALD, 1983; CARSON and

SMITH, 1986), but the production of testosterone is known to be increased significantly in ovaries of 12 to 14-day-old rats (CARSON and SMITH, 1986; CSERNUS, 1986). In the present study, the theca interna cells of secondary follicle as well as interstitial gland cells of 2-week-old rat ovaries were positively immunostained against the 17 β -HSD antibody. In addition, ultrastructural observations showed that these cells of 2-week-old rats already obtained the characteristic structure for steroid-secreting cell. These findings indicate that the theca interna cell and the interstitial gland cell functionally differentiate and acquire the ability to convert to testosterone by 2 weeks of age. However, more research is needed to clarify the physiological significance of the steroid producing activity in the immature rat ovary.

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