Immunohistochemical Studies on the Localization of Aromatase and 17 α -Hydroxylase/C₁₇₋₂₀ Lyase (17 α -Lyase) in Estrous Cycling and Pregnant Hamster Ovaries

Hideaki TSURI¹, Tomoko YOSHINAGA-HIRABAYASHI¹, Kazunori ISHIMURA¹, Yoshio OSAWA², Shiro KOMINAMI³, Shigeki TAKEMORI³ and Hisao FUJITA¹

Department of Anatomy¹, Osaka University Medical School, Suita, Japan; Endocrine Biochemistry Department², Medical Foundation of Buffalo, Buffalo, U.S.A.; and Faculty of Integrated Arts and Sciences³, Hiroshima University, Hiroshima, Japan

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Summary. In order to clarify periodic changes in the localization of enzymes engaged in estrogen biosynthesis during the estrous cycle, immunohistochemical and fine structural studies were performed using estrous cycling and pregnant hamster ovaries. Results showed that ovulation takes place at midnight between Day 4 and Day 1 in the regular 4 day-cycle hamster. Immunoreactivity for aromatase is localized in the granulosa cells of the secondary follicle and granulosa lutein cells during the morning (10:00 am) of Day 1 to the evening (5:00 pm) of Day 4; in the night (9:00 pm) of Day 4, only the granulosa cells of the Graafian follicle showed a strong immunoreaction.

As for 17 α -lyase, theca interna cells of the secondary follicle are immunopositive throughout Day 2 to the morning (10:00 am) of Day 4. Only a few cells in the theca interna of the Graafian follicles are immunopositive in the evening (5:00 pm) of Day 4. No positive cells for this enzyme were detected in the night (9:00 pm) of Day 4 or morning (10:00 am) of Day 1. The rapid decrease of estrogen biosynthesis occurring just before ovulation is considered to be due to the disappearance of 17 α -lyase in the theca interna cells of the ovary.

On Day 10 of pregnancy, the granulosa cells of the secondary follicles and both the granulosa and theca lutein cells of the corpora lutea are immunostained with the aromatase antibody, while the theca interna cells of the secondary follicles reacted positively to the 17 α -lyase antibody.

Only the granulosa and theca interna cells from the large preovulatory Graafian follicle of Day 4 (proestrus) which are positively stained for aromatase as well as 17 α -lyase show ultrastructural features typical of steroid secretory cells.

Estrogen biosynthesis activity in the ovary changes regularly throughout the estrous cycle, under the control of hormones from the anterior pituitary. In our laboratory, the estrogen biosynthesis mechanism in the ovary has been studied by immunohistochemistry for enzymes necessary for estrogen synthesis. We previously reported that aromatase, the enzyme catalyzing the reaction from testosterone to estradiol, is localized in granulosa cells of the Graafian follicles around ovulation, and rapidly disappears from the cells after ovulation (ISHIMURA et al., 1989; ZHONG et al., 1989; YOSHINAGA-HIRABAYASHI et al., 1990).

We also reported that 17 α -hydroxylase/C₁₇₋₂₀ lyase (17 α -lyase, the enzyme catalyzing the reaction from progesterone to androstenedione, is localized in theca interna cells of the secondary follicles and interstitial gland cells, and rapidly disappears several hours before ovulation (ISHIMURA et al., 1990).

ZIRKIN et al. (1980) reported that an apparent increase in smooth endoplasmic reticulum in Leydig cells is related to the elevation of plasma testosterone levels.

In the present study, we planned to clarify the localizations of aromatase and 17 α -lyase immunohistochemically in the normal hamster ovary and the cyclic changes of their reactions throughout the estrous cycle and pregnancy. In addition, the relationship between the ultrastructural aspects and immunohistochemical reactivities of the ovary throughout the estrous cycle was examined.

MATERIALS AND METHODS

Among the numerous mature adult and pregnant hamsters maintained under 12-h light (8:00 am–8:00 pm) and 12-h dark (8:00 pm–8:00 am) conditions, only the animals showing 4-day regular estrous cycles by the vaginal smear test were used for this study. Here, Day 1 is estrus, Days 2 and 3 are diestrus, and Day 4 is proestrus; ovulation can be expected at the midnight between Day 4 and Day 1. About 40 ovaries were collected at 10:00 am on each estrous Day, 5:00 pm and 9:00 pm on Day 4, and Day 10 of gestation.

All the animals were anesthetized and perfused *via* the left ventricle with buffered 4% paraformaldehyde solution, and bilateral ovaries were removed. The ovaries were cut into halves and immersed in the same fixatives for 24 h at 4°C. After fixation, the tissue samples were rinsed in a phosphate buffered saline (PBS) solution containing 10%, 15% and 20% sucrose for 5 h, respectively. Cryosections (6 μ m in thickness) were cut at –25°C in Leitz cryostat, and mounted on albumin-coated glass slides.

Some sections were incubated with rabbit anti-aromatase cytochrome P-450 antisera (1:500) (from Drs. Y. OSAWA and J. KITAWAKI) for 24 h at 4°C, and other sections were incubated with a rabbit antiserum against cytochrome P-450 of 17 α -hydroxylase/C₁₇₋₂₀ lyase (17 α -lyase) (1:1000) (from Drs. S. KOMINAMI and S. TAKEMORI) for 24 h at 4°C. After rinsing in PBS, the sections were treated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:100) for 3 h at room temperature, and examined with a Nikon fluorescein microscope.

All the detailed procedures for purification of antigens and preparation of the antibodies have been previously described (KOMINAMI et al., 1982; OSAWA et al., 1987a,b; SHINZAWA et al., 1988). For the examination of the staining pattern of basal lamina, some sections were treated with an antiserum against laminin, and FITC-conjugated second antibody. As a

control, normal rabbit serum was used instead of the first antibody.

For electron microscopic examination, ovaries from Day 2 and 5:00 pm on Day 4 were cut into small pieces and immersed in 2.5% glutaraldehyde solution buffered at pH 7.4 with Millonig's phosphate for 2 h at 4°C. After dehydration in graded concentrations of ethanol, the tissues were embedded in Epon epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a JEOL 1200 EX electron microscope.

RESULTS

Immunohistochemistry

1. Normal cycle hamsters

At any estrous stage, many primary and small secondary follicles, interstitial glands, and corpora lutea were seen in the ovaries.

Day 2 (the first day of diestrus): Many antral follicles and some small atretic follicles were observed. The immunoreaction for aromatase was strongly positive in the granulosa cells of the secondary and antral follicles, and also in the granulosa lutein cells.

Theca interna cells of the secondary follicle were positively stained with the 17 α -lyase antibody, but all the cells around the corpus luteum were devoid of the immunostaining for 17 α -lyase.

Day 3 (the second day of diestrus) and 10:00 am on Day 4 (proestrus) (Fig. 1): Some large antral follicles and a small number of Graafian follicles could be seen. Positive staining for aromatase was observed in the granulosa cells of the large antral follicle and Graafian follicle (Fig. 1a). Some luteal cells of the corpus luteum were weakly positive against the aromatase antibody.

The immunoreaction for 17 α -lyase was positive in

Fig. 1 a-c. Light microscopic immunohistochemistry of aromatase (a), 17 α -lyase (b), and laminin (c) from the Day 3 hamster ovary. **a.** Granulosa cells of large antral follicles (L) are positively stained for aromatase. **b.** Theca interna cells of antral follicles are positive against 17 α -lyase antibody. **c.** Each follicle is surrounded by a continuous line showing the presence of laminin. $\times 110$

Fig. 2 a-c. Immunohistochemistry of aromatase (a), 17 α lyase (b), and laminin (c) from the Day 4 (9:00 pm) hamster ovary. A large Graafian follicle and a small antral follicle are seen. **a.** Granulosa cells of preovulatory large Graafian follicle (L) are positively stained for aromatase. Granulosa cells of small antral follicle (S) are negative against aromatase antibody. **b.** Some theca interna cells of the Graafian follicle (L) are weakly reacted against 17 α -lyase antibody, but theca interna cells of small antral follicles (S) are immunonegative. An oocyte (arrow) can be observed in the Graafian follicle. **c.** The Graafian follicle (L) is surrounded by an irregular and fragmented immunoreaction for laminin, but an oocyte is still recognized (arrow). A small antral follicle (S) is surrounded by a continuous immunoreactive line for laminin. $\times 120$

Figs. 1 and 2. Legends on the opposite page.

Fig. 3 a-c. Immunohistochemistry of aromatase (a), 17 α -lyase (b), and laminin (c) from a Day 1 hamster ovary. A newly formed corpus luteum (C) can be seen. **a.** Luteal cells of the corpus luteum are strongly positive against the aromatase antibody. **b.** All the cells are negative for 17 α -lyase. **c.** The corpus luteum is invaded by numerous fragmented substances immunoreactive for laminin. $\times 120$

Fig. 4 a-c. Immunohistochemistry of aromatase (a), and 17 α -lyase (b) from Day 10 of the pregnant hamster ovary. **a.** Granulosa cells of antral follicles (A) are positively stained for aromatase. Luteal cells of the pregnant corpus luteum (P) are weakly positive for aromatase. **b.** Theca interna cells of antral follicles are positively stained for 17 α -lyase, but all the cells around the pregnant corpora lutea are negative against 17 α -lyase antibody. $\times 110$

the theca interna cells of the secondary, antral, and Graafian follicles (Fig. 1b), but that reaction was entirely negative in the granulosa cell of any follicle.

All the follicles were surrounded by their own continuous basal lamina showing a positive immunoreaction to the anti-laminin antibody (Fig. 1c).

5:00 pm on Day 4 (proestrus): In all ovarian sections

examined, 5 or 6 large preovulatory Graafian follicles appeared at this stage. Only the granulosa cells of these Graafian follicles showed a strongly positive staining against the aromatase antibody, but the granulosa cells of the secondary and antral follicles close to these Graafian follicles were devoid of the immunoreactivity for aromatase. No positively stained cells for aromatase were detected in the

Fig. 5. Electron micrograph of a part of a large preovulatory Graafian follicle from 5:00 pm of the Day 4 (proestrus) ovary. **a.** In the cytoplasm of the granulosa cell, which is positive for aromatase, sER, rER, lipid droplets, oval mitochondria with lamellar cristae and free ribosomes are seen. **b.** In the theca interna cell which is positive for 17 α -lyase, well-developed sER, round mitochondria with tubular cristae, lipid droplets, and rER are seen. a: $\times 21,500$, b: $\times 19,000$

Fig. 6. Electron micrograph of the secondary follicle from 5:00 pm of the Day 4 (proestrus) ovary. **a.** A granulosa cell which is negative for aromatase contains free ribosomes, a lipid droplet, round mitochondria with lamellar cristae, and elongated rER. **b.** A theca interna cell which is negative for 17 α -lyase shows rER, and round or elongated mitochondria with lamellar or tubular cristae. a: $\times 20,000$, b: $\times 23,500$

corpus luteum at this stage.

The immunoreaction for 17α -lyase was strongly positive in certain theca interna cells of only those Graafian follicles whose granulosa cells showed a strongly positive staining for aromatase, but the reaction was entirely negative in any theca interna cells in other kinds of follicles, except for Graafian follicles.

9:00 pm on Day 4 (proestrus) (Fig. 2): The general image of the ovary was similar to that observed in the evening (5:00 pm) of Day 4, though some preovulatory Graafian follicles with an oocyte were larger in size. Only the granulosa cells of the large preovulatory Graafian follicle showed a strong immunoreactivity for aromatase (Fig. 2a), but all the theca interna cells of any follicle were devoid of the immunoreactivity for 17α -lyase (Fig. 2b). Such large preovulatory Graafian follicles were surrounded by discontinuous basal lamina reacting to the anti-laminin antibody. Around the small antral follicle, continuous immunopositive staining for anti-laminin antibody could be observed (Fig. 2c).

10:00 am on Day 1 (estrus) (Fig. 3): Several newly formed corpora lutea were observed in each ovary, but there were no mature Graafian follicles. The granulosa lutein cells of these corpora lutea showed a strongly positive reaction for aromatase (Fig. 3a). However, the immunoreaction for 17α -lyase was completely negative in any cell (Fig. 3b). Discontinuously and fragmentally positive immunoreactivity against the anti-laminin antibody could be seen around the newly formed corpora lutea (Fig. 3c).

On any day of the estrous cycle, oocytes, peritoneal cells, and interstitial gland cells were entirely negative against the aromatase as well as 17α -lyase antibodies.

2. Pregnant hamsters (Fig. 4)

Large pregnant corpora lutea, follicles at various stages of development, and interstitial glands were easily seen in each ovary from pregnant hamsters at Day 10 of gestation. The immunoreaction for aromatase was strongly positive in the granulosa cells of antral follicles and weakly positive in the luteal cells of the pregnant corpora lutea at this stage (Fig. 4a). The theca interna cells of the secondary follicles were positively stained against 17α -lyase antibody, but no positively stained cells could be observed around the pregnant corpora lutea (Fig. 4b). The interstitial gland cells as well as oocytes were entirely negative to both of these antibodies.

Ultrastructure

Day 2 (the first day of diestrus): Many secondary and antral follicles were surrounded with continuous basal lamina. The granulosa cells of these follicles which were positively stained for aromatase had many lipid droplets, oval or elongate mitochondria with lamellar cristae, and abundant free ribosomes. Among these ribosomes, a few elements of rough endoplasmic reticulum (rER) and of smooth endoplasmic reticulum (sER) could be observed.

The theca interna is composed of two kinds of cells: fibroblast-like cells which are elongated in shape and rich in rER; and theca gland cells which show features of steroid producing cells such as developed sER, oval and round mitochondria with tubular cristae, and lipid droplets. At this stage, only a small number of theca interna cells (theca gland cells) could be found among the fibroblast-like cells.

5:00 pm on Day 4 (proestrus) (Fig. 5, 6): Some large preovulatory Graafian follicles surrounded by continuous basal lamina were observed in each ovary. The granulosa cells of these follicles were positively stained for aromatase. They had many lipid droplets, round and oval mitochondria with lamellar cristae, well developed rER, and newly formed sER (Fig. 5a). In some granulosa cells, sER were more prominent than rER in quantity.

Many theca interna cells in the large preovulatory Graafian follicles which were polygonal in shape and immunopositive for 17α -lyase were recognized among some fibroblast-like elongated cells. Near these cells, enlarged capillaries with erythrocytes in the lumen were seen. These theca interna cells had well developed sER, round mitochondria with tubular cristae, a few lipid droplets, elongated rER elements, and many free ribosomes (Fig. 5b).

The ultrastructures of the granulosa cells (Fig. 6a) and the theca interna cells (Fig. 6b) of the secondary follicle which were negative for both aromatase and 17α -lyase were similar to those of the cells of the secondary follicle on Day 2.

DISCUSSION

In this study, we examined the localization of aromatase and 17α -lyase in the ovaries of both normally estrous cycling and pregnant hamsters and their relation to ultrastructural aspects of these cells. We showed that the immunoreaction for aromatase was positive on all days of the estrous cycle in both the granulosa and corpora luteal cells of the hamster

ovary, while the positive immunoreaction for 17 α -lyase was recognized from Day 2 to the morning (10:00 am) of Day 4 in theca interna cells, the immunopositive cells began to decrease in number in the evening (5:00 pm) of Day 4, and only a few cells of the theca interna showed a positive reaction before ovulation (9:00 pm). We observed that on the evening and night of Day 4, the immunoreaction for aromatase was positive only in the granulosa cells of the several Graafian follicles, and was negative in the granulosa cells of the other antral follicles near these Graafian follicles. In the evening (5:00 pm) of Day 4, the positive staining for 17 α -lyase was confined to the theca interna cells of the Graafian follicles whose granulosa cells were positively stained for aromatase.

We suggest that only the several Graafian follicles which showed immunoreactivities for both aromatase and 17 α -lyase in the evening of Day 4 might ovulate in this cycle. CHIRAS et al. (1978) reported that many secondary follicles grew into antral follicles between estrous (Day 1) and diestrus (Day 2, 3), but only several Graafian follicles could ovulate in each estrous cycle restrictedly in normal cycling hamster ovaries. Our suggestion well supports his data. It is believed that there might be a certain mechanism which restricts the number of follicles maturing into the Graafian follicles, and only the Graafian follicles could synthesize estrogen *de novo* and ovulate in the proestrus stage of the ovary.

JOHNSON (1987) reported in his biochemical study that ovarian estrogen production and the plasma 17 α -lyase level change periodically in the cycling hamster; that is to say, the estrogen production and the plasma level of 17 α -lyase increase at diestrus and drastically decrease in the afternoon of proestrus (before ovulation), but the plasma level of aromatase dose not change. His data well coincide with our results. Our immunohistochemical findings showing a rapid loss of 17 α -lyase from the ovary just before the ovulation clearly account for the rapid decrease in estrogen production in the hamster ovary.

It has been reported that side-chain cleavage enzymes (ZLOTKIN et al., 1986; RODGERS et al., 1986; LE GOASCOGNE et al., 1989; SASANO et al., 1989), 3 β -hydroxysteroid dehydrogenase (PUPKIN et al., 1966; BJERSING, 1967; DUPONT et al., 1990; COUET et al., 1990), 17 α -hydroxylase/C₁₇₋₂₀ lyase (SASANO et al., 1989; ISHIMURA et al., 1990), and 17 β -hydroxysteroid dehydrogenase (YOSHINAGA-HIRABAYASHI et al., 1987) are immunohistochemically localized in the theca interna cells, whereas aromatase (ISHIMURA et al., 1989; ZHONG et al., 1989; YOSHINAGA-HIRABAYASHI et al., 1990), in the granulosa cells. Since ovaries at Day 10 of the pregnant hamster showed a positive

immunoreactivity for aromatase and 17 α -lyase in the secondary follicle, the pregnant hamster ovary is presumed to have an ability for *de novo* synthesis of estrogen. On the other hand, in the guinea pig ovary at the mid and late stages of pregnancy, the immunoreaction for 17 α -lyase is positive in the theca interna cells of the secondary follicles, but that for aromatase is negative in any cell (unpublished data). In the pregnant rat ovary, aromatase was positive in the granulosa cells of the secondary follicles (YOSHINAGA-HIRABAYASHI et al., 1990), but 17 α -lyase was negative in any cell of the ovary (unpublished data). These findings suggest that the ovaries from both pregnant guinea pigs and rats can not biosynthesize estrogen *de novo*. There are considerable differences among animal species in the localization of estrogen synthesizing enzymes in the cycling and pregnant ovaries. As to the significance of the immunostainability of the pregnant hamster corpora lutea, further study is needed.

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Dr. Tomoko HIRABAYASHI
Department of Anatomy
Osaka University Medical School
2-2 Yamadaoka, Suita, Osaka-fu
565 Japan

平林 朋子
565 吹田市山田丘 2-2
大阪大学医学部
第三解剖学教室