

Immunolocalization of aromatase, 17 α -hydroxylase and side-chain-cleavage cytochromes P-450 in the human ovary*

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Summary. Immunohistochemical localization of cholesterol side-chain-cleavage, 17 α -hydroxylase and aromatase cytochromes P-450 was performed in 35 morphologically normal human premenopausal ovaries by using specific antibodies against the enzymes. In well-developed ovarian follicles in the late stages of follicular growth, immunoreactivity of P-450_{AROM} was only seen in granulosa cells while P-450_{17 α} and P-450_{SCC} activity was confined to theca interna cells, confirming that follicular oestrogen is produced in granulosa cells by the aromatization of androgens derived from the theca interna cells. In the corpus luteum, this functional differentiation is maintained, since immunoreactivity of P-450_{AROM} was exclusively present in luteinized granulosa cells while that of P-450_{17 α} was present in luteinized theca cells. Immunoreactivity of P-450_{SCC} was present in both types of cells in the corpus luteum.

Keywords: immunohistochemistry; human ovary; aromatase; 17 α -hydroxylase; side-chain-cleavage enzyme

Introduction

The production rates of various sex-steroid hormones change episodically during the ovarian cycle, corresponding to the stage of development of follicles and corpus luteum and subsequent plasma concentrations of pituitary gonadotrophins. To obtain a better understanding of sex-steroid hormone biosynthesis in the ovary, it is important to know the localization of steroidogenic enzymes, particularly in determining which cell types of the ovarian follicle and corpus luteum express these enzymes. In human ovarian follicles, reports on the steroidogenic capacities of isolated granulosa and theca cells led to the two-cell theory, stating that the theca cells produce the C₁₉-steroids, androstenedione and testosterone, and the granulosa cells aromatize those C₁₉-steroids and produce ovarian oestrogens (McNatty *et al.*, 1979; Hillier, 1981; Lipsett, 1986). In the human corpus luteum, some of these differences between granulosa and theca interna cells are considered to be preserved after ovulation and luteinization (Channing, 1969; Lipsett, 1986; Ohara *et al.*, 1987). However, there is still no direct demonstration of which cells of the intact ovarian follicle

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and corpus luteum are responsible for specific sex-steroid hormone production, particularly oestrogen synthesis, in the ovaries of humans and other animals. In the ovarian steroidogenic pathway, cholesterol side-chain cleavage cytochrome P-450 (P-450_{SCC}), 17 α -hydroxylase cytochrome P-450 (P-450_{17 α}) and aromatase cytochrome P-450 (P-450_{AROM}) are all important regulatory enzymes and catalyse respectively the conversion of cholesterol to pregnenolone, of pregnenolone to androgens and of androgens to oestrogens. To determine the cellular localization of those steroidogenic enzymes in ovarian steroidogenesis, immunohistochemical analysis of P-450_{SCC}, P-450_{17 α} and P-450_{AROM} was performed in 35 morphologically normal human ovaries.

The results have been reported in abstract form (Sasano *et al.*, 1988b).

Materials and Methods

Antibodies. P-450_{AROM} was purified from human term placenta and monoclonal antibodies directed against purified P-450_{AROM} were obtained from mice, as previously described (Mendelson *et al.*, 1985). P-450_{17 α} was purified from pig testis according to the method of Nakajin & Hall (1981) and a specific IgG fraction against purified P-450_{17 α} was raised in rabbits. Essentially, the immunochemical features of this IgG fraction obtained were the same as those described previously (Rodgers *et al.*, 1986a; Mason *et al.*, 1986). P-450_{SCC} was purified from bovine adrenocortical mitochondria according to the method of Takikawa *et al.* (1978), and a specific IgG fraction against this purified P-450_{SCC} was obtained from rabbits after immunization with purified P-450_{SCC} (Sugano *et al.*, 1985; Sasano *et al.*, 1988a).

Tissues for immunohistochemistry

Human ovaries were obtained from 35 patients who had oophorectomy with or without hysterectomy performed for various gynaecological disorders at the Tohoku University Hospital, Sendai Nihon Red Cross Hospital and Tohoku Rosai Hospital (all in Sendai, Japan). All the patients were premenopausal and had no clinical endocrine abnormalities. The ovaries were obtained after routine gross pathological examination and microscopic examination of haematoxylin and eosin-stained slides revealed no significant abnormalities.

Pig ovary, cow ovary and human term placenta were used as controls for P-450_{17 α} , P-450_{SCC} and P-450_{AROM}, respectively. The pig and cow ovaries were obtained at a local slaughterhouse, and two grossly and microscopically normal human placentas were obtained from normal spontaneous vaginal deliveries.

Preparation of tissues. Pig and cow ovaries were cut into small pieces and fixed in 4% paraformaldehyde (pH 7.4) or PLP (periodate-lysine paraformaldehyde, pH 7.4) for 24–48 h at 4°C, or in 100% methanol or 10% neutral formalin fixative for 48–72 h at room temperature. Human placentas were placed in 10% neutral formalin fixative for 18 h at room temperature. The specimens were then embedded in paraffin-wax blocks, cut into 2.5 μ m thick sections, and mounted on regular glass slides. A portion of each fixed ovary was frozen and sectioned in a cryostat at 6 μ m thickness. The sections were mounted on albumin-coated glass slides. No significant differences in the distribution of immunoreactivity were observed among the various fixatives mentioned or between paraffin wax-embedded and frozen-sectioned ovaries.

The human ovaries were fixed in 10% neutral formalin solution after surgery, after which paraffin wax-embedded sections of 2.5 μ m thickness were cut and mounted on regular glass slides.

Immunohistochemistry. After routine deparaffinization, the sections were put into methanol with 0.3% H₂O₂ for 30 min to block endogenous peroxidase activity, then washed in three changes of 0.01 M-PBS for 5 min each, and treated with 1% normal goat serum for 30 min. The immunohistochemical method employed in this study was the Biotin–Strept Avidin (B–SA) amplified method using the StrAvidGen B–SA immunostaining system (Biogenex Laboratories, Dublin, CA, USA). The sections were treated sequentially with the anticytochromes P-450 for 18 h at 4°C in a moist chamber (1:50 to 1:500 dilution), and with biotinylated goat anti-rabbit or anti-mouse immunoglobulin and peroxidase-conjugated streptavidin for 30 min each at room temperature in a moist chamber, with washing in cold PBS between incubations. A final wash was followed by immersion of the reacted sections for 5–10 min in a solution containing 0.05% Tris–HCl, pH 7.6, 0.66 mM-3,3'-diaminobenzidine and 2 mM-H₂O₂. The reacted sections were finally counterstained for nuclei with 1% methyl green and mounted with a glycerol–gelatin water-soluble medium. For immunostain controls, normal mouse IgG and 0.01 M-PBS, normal rabbit IgG and 0.01 M-PBS, and normal rabbit IgG, 0.01 M-PBS and antisera preincubated with excessive amounts of purified cytochrome P-450_{SCC} were used instead of the primary P-450_{AROM}, P-450_{17 α} and P-450_{SCC} primary antibodies.

Results

Human placenta, and pig and cow ovary

Immunoreactivity of P-450_{AROM} was observed exclusively in the cytoplasm of syncytiotrophoblastic cells and possibly of intermediate trophoblast of normal human placenta (Fig. 1) but not in

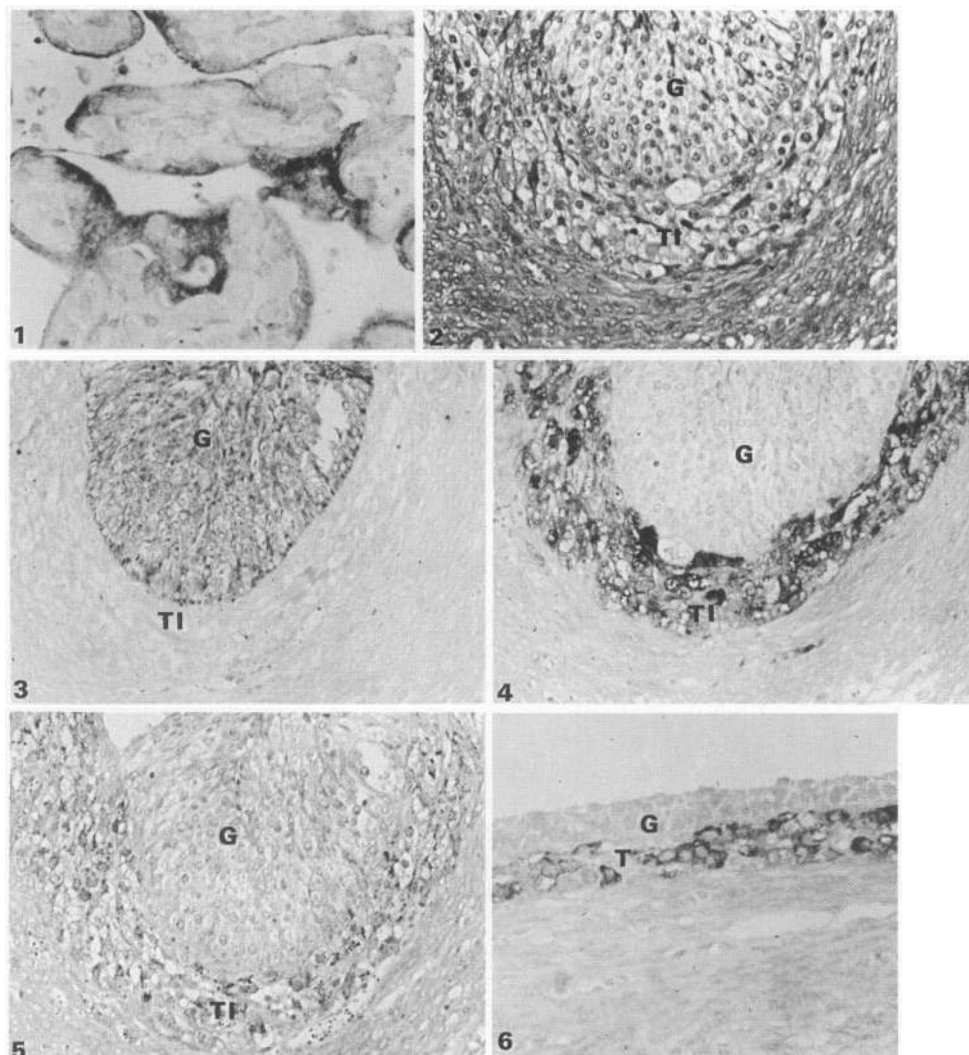


Fig. 1. Immunoreactivity of cytochrome P-450_{AROM} in human placenta from full term normal spontaneous vaginal delivery. The only positive staining is in syncytiotrophoblast and possible intermediate trophoblast. $\times 400$.

Fig. 2. Membrana granulosa (G) and theca interna (TI) of human large well-developed follicle, in the late stage of follicular growth. The follicle examined measured ~ 10 mm in greatest dimension on the original section. H&E, $\times 200$.

Fig. 3. Immunoreactivity of P-450_{AROM} in the membrana granulosa of the human ovarian follicle shown in Fig. 2. $\times 200$.

Fig. 4. Immunoreactivity of P-450_{17α} in the theca interna of the human ovarian follicle shown in Fig. 2. $\times 200$.

Fig. 5. Immunoreactivity of P-450_{SCC} in the theca interna of the human ovarian follicle shown in Fig. 2. Immunoreactivity is represented by dark dots in the theca interna. $\times 200$.

Fig. 6. Immunoreactivity of P-450_{17α} in atretic follicle. Immunoreactivity is not observed in the atrophied membrana granulosa (G) but is seen in theca cells (T). $\times 400$.

cytotrophoblastic cells, in decidual cells or stromal cells of the chorionic villi. Immunoreactivity of both P-450_{SCC} and P-450_{17 α} was present in theca interna cells but not in granulosa cells in the intermediate to large-sized ovarian follicles of the cow and pig ovaries. Cow and pig corpora lutea were not examined.

Human ovary

In small and not well developed follicles, immunoreactivity of P-450_{17 α} and P-450_{SCC} was observed in theca interna cells but not in granulosa cells and that of P-450_{AROM} was faintly present in granulosa cells. This difference in distribution of the different P-450 cytochromes became more pronounced in large well-developed follicles in the late stages of follicular growth (Fig. 2), in which immunoreactivity of P-450_{17 α} and P-450_{SCC} was exclusively present in the theca interna while that of P-450_{AROM} was observed in the membrana granulosa but not in the theca interna (Figs 3, 4 & 5). Immunoreactivity of P-450_{SCC} and P-450_{17 α} was retained in the theca of follicles undergoing atresia, including atretic cystic follicles (Fig. 6). No significant immunoreactivity of P-450 cytochromes was observed in the stromal cells, surface epithelium, primordial or preantral follicles. In the human corpus luteum (Fig. 7), immunoreactivity of P-450_{AROM} was present in luteinized granulosa cells (Fig. 8), while that of P-450_{17 α} was observed only in luteinized theca cells (Fig. 9). Immunoreactivity of P-450_{SCC} was present in both types of luteinized cells, but was more intense in luteinized granulosa cells in most cases (Fig. 10).

Discussion

During the oestrous cycle, cyclic changes in the amounts of ovarian sex steroids are related to morphologically observed follicular and luteal development. Biochemically, this ovarian steroidogenesis is dependent on the availability of cholesterol as a substrate for further enzymic reactions and on the activities of specific steroidogenic enzymes. Those cyclic changes of ovarian sex-steroid hormone production may be considered as a reflection of the specific and total contents of the enzymes responsible for their biosynthesis (Rodgers *et al.*, 1986a).

For localization of steroidogenic enzymes in the ovary, various histochemical analyses of 3 β -hydroxysteroid dehydrogenase, glucose 6-phosphate dehydrogenase, and other enzymes have been reported (Novak *et al.*, 1965; Fienberg & Cohen, 1965), but advances in immunohistochemical techniques have made it possible to observe the distribution of the P-450 cytochromes directly. P-450_{SCC} has been immunohistochemically demonstrated in cultured rat granulosa cells (Goldring *et al.*, 1986), rat ovary (Zlotkin *et al.*, 1986), cow follicles (Rodgers *et al.*, 1986b) and cow corpora lutea (Rodgers *et al.*, 1986c), and P-450_{17 α} has been described in cow follicles (Rodgers *et al.*, 1986b). However, no immunohistochemical localization has been reported in human ovaries to our knowledge. Immunohistochemical localization of P-450_{AROM} in the ovary has not been published except for one report showing the presence of P-450_{AROM} in the theca interna (Matsuda *et al.*, 1984). In addition, immunohistochemical localization of P-450_{AROM} was reported in syncytiotrophoblast of the human placenta (Fournet-Dulguerov *et al.*, 1987), and confirmed in the present study. In our current investigation, the distribution of P-450_{17 α} and P-450_{SCC} was essentially similar to that in cow follicles (Rodgers *et al.*, 1986b). Within the follicles, immunoreactivity of P-450_{AROM} was only observed in the membrana granulosa. These immunohistochemical results are consistent with the biochemical findings and indicate good agreement between the type of steroidogenic enzyme expressed and the type of steroid hormones produced in the tissues of the human ovary (Lipsett, 1986). In particular, the exclusive localization of P-450_{AROM} in the membrana granulosa and of P-450_{17 α} in the theca interna directly confirms the two-cell theory in the human ovary, i.e. oestrogen synthesis by the granulosa cells requires androgen produced in the theca interna (McNatty *et al.*, 1979; Hillier, 1981; Lipsett, 1986). However, these results differ from those of Matsuda *et al.* (1984)

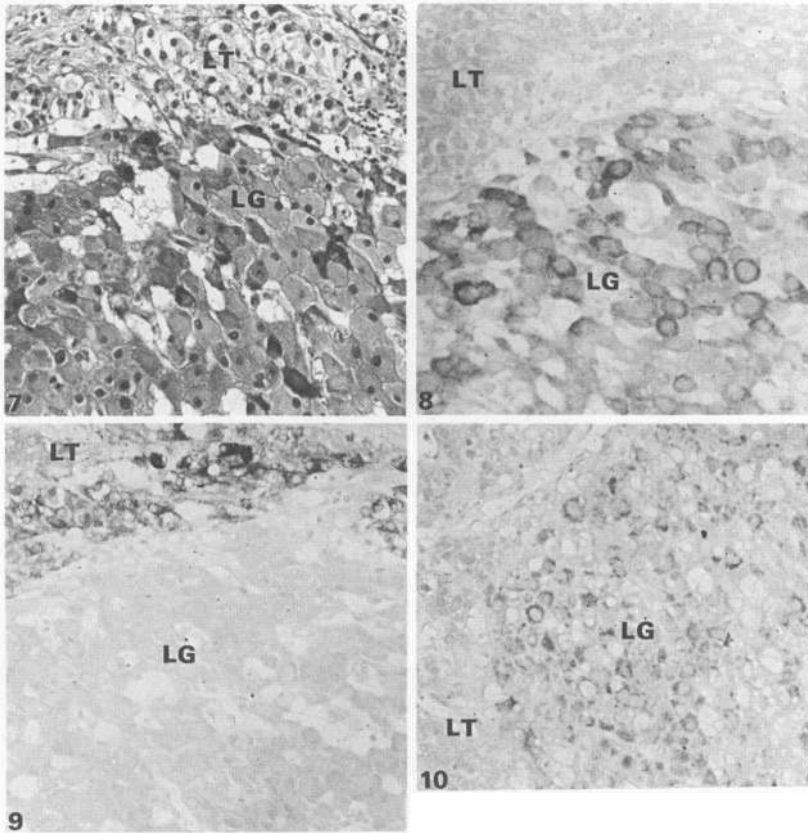


Fig. 7. Luteinized granulosa (LG) and theca (LT) cells in human corpus luteum. H&E, $\times 200$.

Fig. 8. Immunoreactivity of P-450_{AROM} in luteinized granulosa (LG) cells. $\times 200$.

Fig. 9. Immunoreactivity of P-450_{17a} in luteinized theca (LT) cells. $\times 200$.

Fig. 10. Immunoreactivity of P-450_{SCC} in luteinized granulosa cells (LG). $\times 200$.

who found P-450_{AROM} to be present in the theca interna. Voutilainen *et al.* (1986) have reported that only minuscule amounts of P-450_{17a} mRNA were found in human granulosa cells, and the amount did not increase in response to any hormonal stimuli. Our results are also consistent with this report. The androgen produced in the theca interna is also considered to play an important role in follicular growth and development (Azzolin & Saiduddin, 1983).

Immunohistochemical localization of P-450_{SCC} in our study in well-developed follicles revealed the presence of immunoreactivity only in the theca interna but not in the membrana granulosa. Rogers *et al.* (1986b) demonstrated that immunoreactivity of P-450_{SCC} was present in the membrana granulosa in bovine ovarian follicles by immunofluorescent staining, but also reported that the intensity of immunoreactivity was far less than that of the theca interna, and was barely above background in the same follicles. It is well-known that granulosa cells have measurable amounts of P-450_{SCC} (Funkenstein *et al.*, 1983) and can produce progesterone in culture (Shemesh, 1979). The possibility that various factors of immunostaining contributed to a failure of detection of immunoreactivity of P-450_{SCC} in granulosa cells of follicles in the present study cannot be ruled out.

In the corpus luteum, luteinized granulosa cells are large polygonal cells with abundant cytoplasm, while luteinized theca cells are small (about half the size of luteinized granulosa cells), with less abundant, darkly staining cytoplasm (Clement, 1987). Both types of luteinized cells are derived from their corresponding counterparts in ovarian follicles. Those luteinized cells have been considered to retain their characteristics from the follicular stage (Channing, 1969; Lipsett, 1986); Ohara *et al.* (1987) have demonstrated that small and large human luteal cells have steroidogenic properties similar to those exhibited by follicular thecal and granulosa cells, respectively. Our current study of the localization of immunoreactivity of P-450_{17 α} and P-450_{AROM} is consistent with this theory. Our results also show that selective localization of enzyme activity can be attributed to selective localization of the protein, not to the presence of an endogenous inhibitor.

Mitochondria of granulosa cells are known to change from an elongated form with lamelliform cristae to a highly pleomorphic appearance with a dense matrix and tubular cristae during luteinization, and this change is considered to reflect increased progesterone synthesis (Crisp & Channing, 1972). Farkash *et al.* (1986) recently demonstrated by ultrastructural immunohistochemistry of P-450_{SCC} in rat ovary that the transition of lamelliform to tubular mitochondrial cristae was associated with the expression of P-450_{SCC}. The prominent immunoreactivity noted in the present study of P-450_{SCC}, which is the mitochondrial cytochrome P-450 and is necessary for progesterone synthesis, in luteinized granulosa cells can also reflect increased progesterone production after ovulation.

Our present investigation, as well as that of the cow ovary (Rodgers *et al.*, 1986b), did not show the presence of P-450 cytochromes in stromal cells of the ovary. In the present study, all the ovaries were from non-pregnant premenopausal subjects and none showed stromal hyperthecosis.

Luteinized stromal cells, which increase in number during pregnancy and after the menopause, probably due to elevated concentrations of gonadotrophins (Fienberg & Cohen, 1965; Clement, 1987), were not found in significant numbers in the ovaries studied in the present report. Enzymically active stromal cells, which are characterized by their oxidative and other enzymic activities (Fienberg & Cohen, 1965; Clement, 1987), are also known to increase in number with age (Scully & Cohen, 1964). The stromal cells differentiate into theca cells at the periphery of developing follicles, and further investigations are needed to clarify the relationships between stromal and theca cells in steroidogenesis.

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