Immunolocalization of 3 β -hydroxysteroid dehydrogenase in human ovary

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Summary. Immunohistochemical localization of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) was performed in 55 cases of morphologically normal human ovaries by using a specific polyclonal antibody against purified human placental 3 β -HSD. In small developing follicles, immunoreactivity was observed only in the theca interna but also became recognizable in the membrana granulosa with development of the follicle. At a late stage of folliculogenesis, the intensity of the 3 β -HSD activity in the membrana granulosa was nearly equal to that of theca interna in 2 or 3 large follicles examined. One to several layers of theca interna cells just beneath membrana granulosa did not demonstrate any immunoreactivity of 3 β -HSD or that of cytochrome P-450 17 α -hydroxylase. These unstained theca interna cells did not appear to be directly involved in ovarian steroidogenesis and might be designated as 'enzymically inactive theca interna cells'. Marked immunoreactivity was observed in luteinized theca and granulosa cells of the corpus luteum.

Keywords: immunohistochemistry; human ovary; 3 β -hydroxysteroid dehydrogenase; steroidogenesis

Introduction

Progesterone is synthesized from pregnenolone via two-step reaction, i.e. the dehydrogenation of 3 β -hydroxy-5-ene-steroid and isomerization of 3-oxo-5-ene-steroid, and this two-step reaction is catalysed by a single enzyme, 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase (3 β -HSD; EC 1.1.1.51) (Ishii-Ohba *et al.*, 1986). In ovarian steroidogenesis, this enzyme plays an important role, especially through the synthesis of progesterone and androstenedione from the less hormonally active precursors, pregnenolone and dehydroepiandrosterone (DHEA), respectively. Many biochemical studies have been reported on this enzyme. However, biochemical studies generally have difficulties in demonstrating the localization of the enzyme, particularly in determining which cell types of the ovarian follicle and corpus luteum express 3 β -HSD. The activity of 3 β -HSD has been histochemically demonstrated on freshly frozen sections of non-pathological human ovaries (Deane *et al.*, 1962; Scully & Cohen, 1964; Fienberg & Cohen, 1965; Novak *et al.*, 1965; Jones *et al.*, 1968) but histochemical study of 3 β -HSD is well known to be associated with a variety of methodological difficulties such as preparation of tissues, selection of substrates and duration of incubation time and interpretation of results can sometimes be very difficult (Sasano, 1975).

We have demonstrated immunohistochemically aromatase, 17 α -hydroxylase and side-chain-cleavage cytochromes P-450 in non-pathological human ovaries (Sasano *et al.*, 1989a) and in a sex-cord stromal tumour of the human ovary associated with a variety of abnormalities in sex-steroid metabolism (Sasano *et al.*, 1989b) by using specific antibodies to respective cytochromes P-450.

Immunolocalization of steroidogenic enzymes can demonstrate expression of the enzymes directly on formalin-fixed paraffin wax-embedded specimens and so correlation of the presence of the enzymes with morphological analysis was relatively easy compared with histochemical studies which use relatively thick cryostat sections. A purified form of 3β -HSD has been recently isolated from a human placenta (Thomas *et al.*, 1988) and a specific polyclonal antibody has been raised in a rabbit by Lorence *et al.* (1990). Based on the same principle, we have now examined the immunolocalization of 3β -HSD in the human ovary at various stages of the cycle.

Materials and Methods

Ovaries and placenta. After histological review, neutral formalin-fixed and paraffin wax-embedded blocks of human ovaries were retrieved from the surgical pathology file (between 1985 and 1989) of the Tohoku Rosai Hospital, Sendai, Japan. All 55 patients were premenopausal, ranging from 29 to 45 years old, and did not have apparent clinical endocrine abnormalities before operation. Unilateral or bilateral oophorectomy was performed with or without hysterectomy for various gynaecological disorders, including carcinoma of the uterine cervix and endometrium, leiomyoma uteri, exclusion of clinically suspected ovarian neoplasms and others. Haematoxylin and eosin stained slides of these ovaries did not reveal any significant morphological abnormalities. The ovaries examined include 18 with a well-developed corpus luteum and 3 with relatively well-developed large follicles measuring 10, 10 and 13 mm in greatest dimension on the sections. Human placentae were obtained from 3 cases of uncomplicated full-term normal spontaneous vaginal delivery. Gross and microscopic examinations did not show any pathological findings. Specimens were routinely processed by being fixed in neutral formalin for 24 h and embedded in paraffin wax.

Antibodies. The details of the anti- 3β -HSD are described in detail elsewhere by Lorence *et al.* (1990). A New Zealand White rabbit was treated with purified human placental 3β -HSD, suspended in Freund's adjuvant as described previously (Thomas *et al.*, 1988) and the primary antibody was obtained as an IgG-enriched fraction of antiserum. It was shown by Western blot analysis that the antibody reacted specifically with a protein of M_r 45 000 in human placental microsomes and could be used to isolate, characterize and express a cDNA clone encoding human placental 3β -HSD (Lorence *et al.*, 1990). The production, characterization and immunohistochemical application of polyclonal anti-P-450_{17 α} has been described (Sasano *et al.*, 1989c; Lund *et al.*, 1988).

Immunohistochemistry. After routine deparaffinization, the sections were put into methanol with 0.3% H_2O_2 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 used in this study was the Biotin-streptavidin (B-SA) amplified method using the StrAvidGen B-SA immunostaining system (Biogenex Laboratories, Dublin, CA, USA). The sections were treated sequentially with the antibodies for 18 h at 4°C in a moist chamber (1:50 to 1:500 dilution), and with biotinylated goat anti-rabbit 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 min in a solution containing 0.05% Tris-HCl, pH 7.6, 0.6 mM-3, 3'-diaminobenzidine and 2 mM- H_2O_2 at room temperature. The sections which did not demonstrate immunoreactivity by an immersion for 5 min were put in the solution above for up to 10 min. The reacted sections were finally counterstained for nuclei with 1% methyl green and mounted with a glycerol-gelatin water-soluble medium. For immunostain controls, 0.01 mM-PBS and normal rabbit IgG were used instead of the primary antibodies.

Results

Placenta

Immunoreactivity of 3β -HSD was observed exclusively in the cytoplasm of syncytiotrophoblastic cells and possibly of intermediate trophoblast of normal full-term human placenta (Fig. 1). Immunoreactivity was not present in cytotrophoblastic cells, in decidual cells or stromal cells of the chorionic villi.

Ovary

No immunoreactivity was observed in primordial follicles within the ovarian cortex, in primary and secondary or preantral follicles examined. In tertiary or antral follicles, discernible immunoreactivity was seen in several layers of theca interna cells (Fig. 2). At this stage of folliculogenesis, 3β -HSD was not expressed in membrana granulosa cells (Fig. 2). With the development of follicles,

Fig. 1. Immunoreactivity of 3 β -HSD in human placenta from full-term normal spontaneous vaginal delivery. Immunoreactivity was observed in syncytiotrophoblast and possibly intermediate trophoblast. $\times 400$.

Fig. 2. Immunoreactivity of 3 β -HSD in the theca interna (TI) of a maturing follicle (tertiary or antral). No immunoreactivity was observed in the membrana granulosa (MG) and cumulus oophorus (CO). $\times 400$.

immunoreactivity became recognizable in the membrana granulosa. In 3 well-developed large follicles in late stages of follicular growth (Figs 3 & 4), pronounced immunoreactivity of 3 β -HSD was observed in the theca interna and membrana granulosa cells (Fig. 5). The intensity of immunoreactivity was generally stronger in theca interna than membrana granulosa, but was nearly equal between theca interna and membrana granulosa in some parts of 2 of the 3 well-developed follicles (Fig. 6). Interestingly, 1 (Fig. 5) to 5 (Fig. 6) layers of theca interna cells which are directly beneath membrana granulosa did not show any immunoreactivity of 3 β -HSD in the 3 well-developed

follicles examined. Immunostaining of P-450_{17 α} in serial sections of these follicles revealed that these theca interna cells were negative for P-450_{17 α} (Fig. 7). No morphological differences were observed between enzyme positive and negative theca interna cells in haematoxylin–eosin stained serial sections (Figs 3 & 4). In atretic follicles, especially in atretic cystic follicles, immunoreactivity was observed in the membrana granulosa and theca interna but was much stronger in the latter (Fig. 8).

Fig. 3. Membrana granulosa (MG), theca interna (TI) and theca externa (TE) of well-developed large follicle in the late stage of folliculogenesis. The follicle measured 13 mm in the greatest dimension on the section. H & E, $\times 400$.

Fig. 4. Membrana granulosa (MG) and theca interna (TI) of a well-developed ovarian follicle in the late stage of folliculogenesis. The follicle measured 10 mm in the greatest dimension on the section. H & E, $\times 200$.

Fig. 5. Immunoreactivity of 3 β -HSD was present in the membrana granulosa (MG) and theca interna (TI) in the ovarian follicle shown in Fig. 3. Intensity of immunoreactivity was nearly equal between MG and TI. No immunoreactivity was observed in theca externa (TE). One layer of theca interna cells next to the membrana granulosa did not show any immunoreactivity (*). $\times 400$.

Fig. 6. Immunoreactivity of 3 β -HSD in the membrana granulosa (MG) and theca interna (TI) in the ovarian follicle shown in Fig. 4. Immunoreactivity of TI cells was much stronger than that of MG. Up to 5 layers of theca interna cells facing the membrana granulosa did not show any immunoreactivity (*). $\times 200$.

No significant immunoreactivity was observed in the stromal cells including theca externa cells (Fig. 5) and surface epithelium. No foci of stromal luteinization were observed in the ovaries examined. In the well-developed corpus luteum (Fig. 9), immunoreactivity of 3 β -HSD was present in luteinized theca and granulosa cells (Fig. 10), with nearly equal intensity in the two cell types.

Fig. 7. Immunoreactivity of P-450_{17α} in the theca interna (TI) of the follicle observed in Figs 4 and 6. Inner layers of theca interna cells (*) did not show immunoreactivity. Membrana granulosa (MG) was negative for immunostains. $\times 200$.

Fig. 8. Immunoreactivity of 3 β -HSD in the atrophied membrana granulosa (MG) and theca interna (TI) of an atretic follicle. $\times 400$.

Layers of luteinized theca cells facing luteinized granulosa cells did not demonstrate immunoreactivity of 3 β -HSD (Fig. 10). Morphological examination of serial sections showed no discernible differences between luteinized theca cells which expressed the enzyme and those that did not, other than location of the cells (Fig. 9).

Fig. 9. Luteinized granulosa (LG) and theca (LT) cells in a well-developed human corpus luteum. H&E, $\times 200$.

Fig. 10. Immunoreactivity of 3 β -HSD in the luteinized granulosa (LG) and theca (LT) cells in the human corpus luteum in Fig. 7. Several inner layers of luteinized theca cells (*) did not demonstrate immunoreactivity. $\times 200$.

Discussion

To obtain a better understanding of ovarian steroid metabolism, localization of steroidogenic components, i.e. to determine which types of cells in the ovarian follicle and corpus luteum produce which hormones, is as important as biochemical and physiological studies. Recent progress in the purification of the enzymes involved in steroidogenesis and subsequent production of antibodies

against the enzymes have made it possible to observe the distribution of expression of the steroidogenic enzymes in the ovary by immunolocalization. In the human ovary, we have previously reported immunolocalization of aromatase, 17α -hydroxylase and side-chain-cleavage cytochromes P-450, which are all important enzymes in ovarian steroidogenesis (Sasano *et al.*, 1989a). 3β -HSD also plays an important role in ovarian steroidogenesis through converting pregnenolone and DHEA to progesterone and androstenedione, respectively (Edwards *et al.*, 1976). Immunolocalization of 3β -HSD was reported in the bovine adrenal cortex (Ishimura *et al.*, 1988) but not in the ovaries of human or any other species to the best of our knowledge.

In human ovarian folliculogenesis, immunoreactivity of 3β -HSD was first seen in theca cells and became apparent in large well-developed follicles corresponding to later stages of follicular development. Histochemical analysis, which demonstrated enzyme activity by using a *p*-nitrophenyl-substituted ditetrazolium (nitro BT) as electron acceptor and indicator of activity localization and adding 5α -dihydro- 3β -hydroxysteroids and/or 3β -hydroxy-5-ene-steroids as substrates (Sasano, 1975), showed enzyme activity in theca interna cells but not in membrana granulosa and theca externa cells of developing human ovarian follicles (Deane *et al.*, 1962; Scully & Cohen, 1964; Novak *et al.*, 1965; Fienberg & Cohen 1965; Jones *et al.*, 1968). Our findings of 3β -HSD immunoreactivity in theca interna and externa cells were consistent with these histochemical studies but the presence of immunoreactivity in the membrana granulosa of well-developed follicles was not. However, McNatty *et al.* (1979) demonstrated that progesterone was produced in granulosa cells of human follicle. In addition, after ovulation, luteinized granulosa cells showed strong histochemical activity of 3β -HSD (Deane *et al.*, 1962; Scully & Cohen, 1964; Novak *et al.*, 1965; Fienberg & Cohen, 1965; Jones *et al.*, 1968) as well as strong immunoreactivity of the enzyme in the corpus luteum in our study. It is, therefore, not entirely surprising that 3β -HSD protein was induced and expressed in the membrana granulosa cells of pre-ovulatory human follicles. Discrepancy of immunoreactivity from histochemical activity of 3β -HSD may be explained by the possibility that 3β -HSD induced in the membrana granulosa in well-developed follicles is inactive due to inhibitory mechanisms, but further investigation is necessary.

The absence of immunoreactivity of 3β -HSD and P-450_{17 α} in one to several layers of theca interna cells located just beneath the membrana granulosa in well-developed large follicles was unexpected. These theca interna cells might be considered not to play, at least direct, roles in ovarian steroidogenesis because of the absence of expression of steroidogenic enzymes. We, therefore, designate these cells as 'enzymically inactive theca interna cells'. The presence of this type of theca interna cell has not been previously reported as far as we are aware. Previous histochemical studies described heterogeneity of enzyme activity in the theca interna but did not report the presence of 'enzymically inactive cells' located beneath the membrana granulosa. This is not surprising considering difficulties with detailed simultaneous morphological observations on relatively thick frozen sections in histochemical studies. Several layers of luteinized theca cells located next to luteinized granulosa cells also did not show immunoreactivity of 3β -HSD. The metabolic characteristics of 'enzymically inactive theca interna cells' are therefore considered to be retained after ovulation. The possibility that these 'enzymically inactive theca interna cells' merely represent heterogeneity of expression and/or degenerated theca cells and/or even artefactual changes through immunostaining procedures including the use of formalin-fixed and paraffin wax-embedded specimens cannot be completely ruled out. However, the biological significance of this observation warrants further investigations especially in relation to follicular oestrogen synthesis.

Our findings of distribution of immunoreactivity of 3β -HSD in the corpus luteum are nearly consistent with previous histochemical investigations of the human ovary (Deane *et al.*, 1962; Scully & Cohen, 1964; Novak *et al.*, 1965; Fienberg & Cohen, 1965; Jones *et al.*, 1968). Jones *et al.* (1968) postulated that 3β -HSD in luteinized granulosa and theca cells were different forms of the enzyme based on the difference of histochemical reactions when adding Δ^4 and Δ^5 steroids as substrates. However, recent studies strongly indicated that only one protein is present in humans for the biosynthesis of both C19 and C21 Δ^4 -3-ketosteroids from their corresponding Δ^5 - 3β -hydroxy-

steroids, i.e. the pregnene and androstene activity of 3 β -HSD is expressed at a single catalytic site on one enzyme (Thomas *et al.*, 1988; Lorence *et al.*, 1990). In addition, anti 3 β -HSD antibody detected immunoreactivity in luteinized theca and granulosa cells in our study. It is therefore highly unlikely that different forms of the enzyme are present in luteinized theca and granulosa cells. The marked increase of immunoreactivity of 3 β -HSD in luteinized granulosa cells is clearly associated with progesterone synthesis in the luteal phase as was observed in the change of distribution of P-450_{scc} (side-chain cleavage) (Farkash *et al.*, 1986; Sasano *et al.*, 1989a) and of ultrastructural morphology of mitochondria (Crisp & Channing, 1972; Farkash *et al.*, 1986) in membrana granulosa after ovulation. Our present investigation, using an antibody against 3 β -HSD derived from human placenta, clearly defines the localization of expression of the enzyme in the human ovary.

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