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Expression of 3β-Hydroxysteroid dehydrogenase and P450 side chain cleavage enzyme in the human uterine endometrium

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Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase; DAB, diaminobenzidine; DHEA, dehydroepiandrosterone; P450c11, 11β-hydroxylase; P450c17, 17-hydroxylase; P450c21, 21-hydroxylase; P450scc, P450 side chain cleavage enzyme; RT, reverse transcription

Abstract

The enzyme complex 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD) is involved in the biosynthesis of all classes of active steroids. The expression of 3β-HSD in human uterine endometrium during the menstrual cycle and decidua was examined in an effort to understand its role during ova implantation. 3β-HSD was weakly expressed in the glandular epithelium of the proliferative phase and moderately expressed in the glandular epithelium of secretory phase of the endometrium. In the decidua of the ectopic pregnancy, 3β-HSD was strongly expressed. The human uterine endometrial 3β-HSD was identified as being the same type as the placental 3β-HSD by RT-PCR and sequence analysis. In addition to the expression of 3β-HSD, P450scc was expressed in the decidua of the ectopic pregnancy. These results suggest that pregnenolone might be synthesized from cholesterol by P450scc *de novo* and then, it is converted to progesterone by 3β-HSD in the uterine endometrium. The data implies that the endometrial 3β-HSD can use not only the out-coming pregnenolone from the adrenal gland but also the self-made pregnenolone to produce progesterone. The *de novo* synthesis of progesterone in the endometrium might be a crucial factor for implantation and maintenance of pregnancy.

Keywords: 3β-HSD; decidua; P450scc; progesterone; uterine endometrium

Introduction

The periodic change of human uterine endometrium during the menstrual cycle depends on steroid hormones produced by the ovary (Tseng *et al.*, 1974; Buirchell and Hahnel, 1975; Pack *et al.*, 1978; Clarke *et al.*, 1982). Since the activity of enzymes involved in steroidogenesis and the periodic change of steroid hormone receptors were identified in human uterine endometrium, the endocrinological role of uterine endometrium has been more complicated (Lessey *et al.*, 1988; Tseng and Zhu, 1997; Matsuzaki *et al.*, 1999).

3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD, EC 1.1.1.145), which catalyzes the formation of Δ^4-3-ketosteroids from Δ^4-3-hydroxysteroids, plays an important role in biosynthesis of all classes of hormonal steroids, namely glucocorticoids, mineralocorticoids, progesterone, androgens, and estrogens (Readhead *et al.*, 1983). The enzymatic activity of human 3β-HSD is present in not only the classical steroidogenic tissues such as placenta, adrenal glands and gonads (Luu-The *et al.*, 1989; Lorence *et al.*, 1990; Rheaume *et al.*, 1991; Labrie *et al.*, 1992), but also in many peripheral tissues including prostate, breast, skin, liver, lung and heart (Abul- Hajj *et al.*, 1975; Milewich *et al.*, 1977; Lacoste *et al.*, 1990; Dumont *et al.*, 1992; Kayes-Wandover and White, 2000).

Enzymatic activity of 3β-HSD in human endometrial tissues was also reported (Seki *et al.*, 1987; Tang *et al.*, 1993). In particular, the presence of 3β-HSD was detected by Western blotting as a 42 kDa band in primary endometrium (Tang *et al.*, 1993). In addition to human endometrium, enzymatic activity of 3β-HSD was also reported in the myometrium and endometrium of rhesus monkeys (Martel *et al.*, 1994) and in the uteri of rats (Zaho *et al.*, 1991). Therefore, it was assumed that the 3β-HSD of the endometrium involved in the local metabolism of circulating steroids of adrenal origin. However, the expression of P450

side chain cleavage enzyme (P450scc, EC 1.14.15.6) which catalyzes the first and key regulatory reaction controlling the production of steroid hormones, was identified in the mouse decidua induced by implantation. Thus, it was presumed that, during the early phases of pregnancy, local progesterone synthesis in the maternal deciduas is important for successful implantation and/or maintenance of pregnancy in mice (Ben-Zimra *et al.*, 2002). So far, there has been no report of any evidence of *de novo* synthesis of progesterone in human uterine endometrium.

Our research was designed to examine the presence of 3β-HSD in the endometrium at the level of protein and gene expression by Western blotting analysis, immunohistochemistry and reverse transcription RT-PCR. To determine whether progesterone might be synthesized *de novo* or not and whether other steroids such as mineralocorticoids and glucocorticoids might also be synthesized in human uterine endometrium, we have also examined the expression of P450scc, 17-hydroxylase (P450c17, EC 1.14.99.9), 21-hydroxylase (P450c21, EC 1.14.99.10) and 11β-hydroxylase (P450c11, EC 1.14.15.4) by RT-PCR.

Materials and Methods

Collection of tissues

The human uterine endometrium were obtained from proliferative phase, secretory phase and ectopic pregnancy. The human placental tissues were also obtained immediately after delivery.

RT-PCR analysis

Total RNA was prepared by using RNeasy Mini Kit (Qiagen, Chatsworth, CA). Oligonucleotide primers used for RT-PCR (Table 1) were located in two different exons of each gene to avoid amplification of any contaminating genomic DNA. The PCR primer pairs for 3βHSD-820 were designed with common sequences to two types of human 3β-HSD isoform, and the reverse primer for 3βHSD-628 was specific to human placenta type 3β-HSD. After PCR, the 820 bp product for 3βHSD-820 primer was cloned into pGEM-T easy vector system (Promega), and sequenced by ABI Prism Big-Dye method (Perkin Elmer) using T7 primer and Sp6 primer.

Western blot analysis

Anti-3β-HSD antibodies were kindly provided by Dr Luu-The, Laboratory of Molecular Endocrinology, CHUL Research Center, Quebec, Canada. Placental and endometrial tissue samples were homogenized in lysing solution (1% sodium deoxycholate, 0.1% sodium decyl sulfate, and 0.8 mM phenylmethylsulfonyl fluoride in PBS). Homogenates were centrifuged at 1,500 *g* for 15 min. Protein concentrations were determined using the Bio-Rad Protein Assay reagent with bovine serum albumin as a standard. Protein extracts (6 μg for placenta and 25 μg for endometrium) were electrophoresed in an 12% SDS-polyacrylamide gel under reducing conditions, electroblotted onto an Immobilon-P membrane (Millipore), and detected with the anti-3β-HSD antibodies in the previously described manner (Lim *et al.,* 2002).

Histologic and immunohistochemical stain

The specimens from patients were fixed in 10% buffered formalin and embedded in the paraffin. Four micrometer sections were cut and hematoxylin eosin stain was performed. For immunostaining, four micrometer sections were cut on the Probe-on plus slide (Research genetics, AL), heated at 60°C for 30 min, and deparaffinized by xylene. Next, tissue sections were rehydrated in phosphate-buffered saline (PBS) solution, and then the slides were blocked in 3% hydrogen peroxide for 10 s. The slides were washed twice in Immuno/DNA buffer solution (Research genetics, AL) and then incubated in protein blocker solution (Research genetics, AL) for 3 min. The sections were incubated overnight at 4°C with the 3β-HSD antibodies which was diluted to 1:200 by antibody diluent (Research genetics, AL) and incubated with the universal secondary antibody (Research genetics, AL) for 8 min. The sections were labeled with peroxidase-conjugated streptoavidin (Research genetics, AL) for 10 min, and incubated in diaminobenzidine (DAB) for 10 min and washed in Immuno/DNA (Research genetics, AL). Finally, the slides were counterstained with Mayer's hematoxylin, washed in distilled water, and mounted by universal mount (Research genetics, AL).

Results

Western blot analysis for 3β-HSD

Human placental 3β-HSD (type I) which was expressed strongly in human placenta was already identified by molecular cloning and Western blot analysis (Luu-The *et al.*, 1989; Lachance *et al.*, 1990; Lorence *et al.*, 1990). The protein of 3β-HSD was detected by Western blotting as a 42 kDa band in a human endometrial tissue (Tang *et al.*, 1993). We examined whether the endometrial 3β-HSD could cross-react with anti-placental 3β-HSD antibody which was provided by Dr Luu-The. The antibody detected the endometrial 3β-HSD as a 42 kDa band which was the

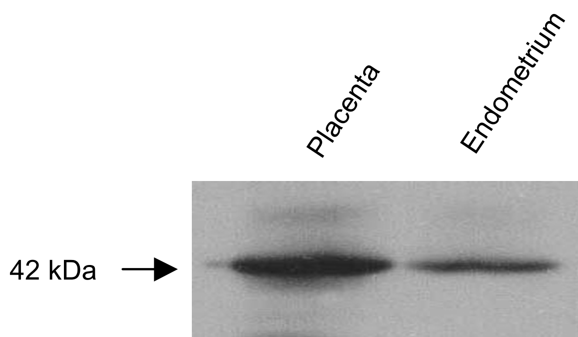

Figure 1. Western blot analysis. The samples (containing 6 μg or 25 μg protein) extracted from the human placenta and uterine endometrium were electrophoresed in an SDS-polyacrylamide gel under reducing conditions, and detected with the anti-3β-HSD antibody as described under Materials and Methods.

same band as the placental 3β-HSD (Figure 1).

Immunohistochemistry for 3β-HSD

We examined the change of 3β-HSD expression according to the morphological change of uterine endometrium using immunohistochemistry. When the chorionic villi which express type I 3β-HSD (Mason *et al.*, 1997), was applied as a control, the cytotrophoblasts and syncithiotrophoblasts were stained strongly with anti-3β-HSD antibody (Figure 2A). We prepared the endometrial tissues of the proliferative and secretory phase from the normal woman and decidua from the woman with ectopic pregnancy to prevent contamination of any chorionic villi, and then the samples were immunostained with anti-3β-HSD antibody. The glandular epithelium of the proliferative phase was very weakly, the glandular epithelium of the secretory phase was moderately, and the decidua was strongly

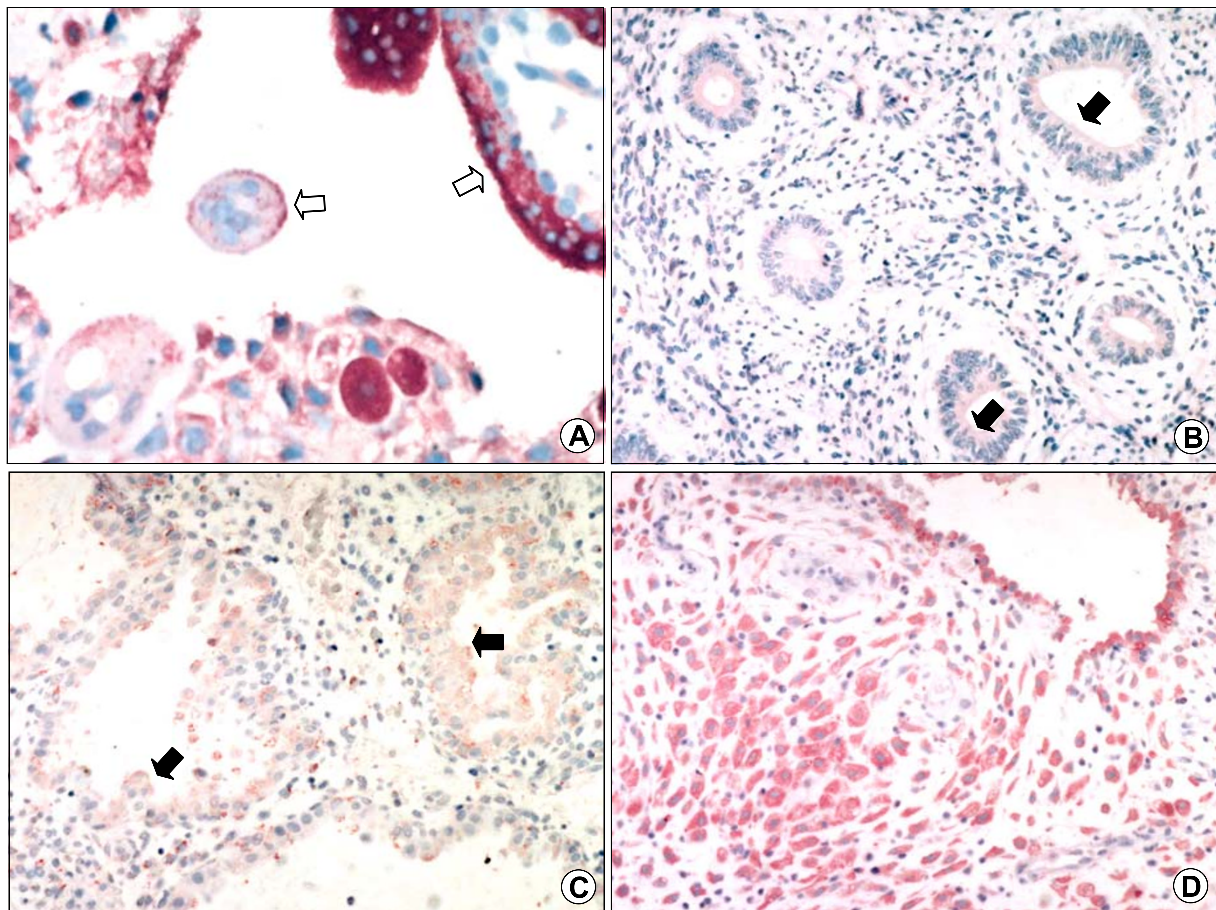

Figure 2. Immunohistochemistry of 3β-HSD. The cytotrophoblasts and syncithiotrophoblasts of the chorionic villi (A) and the decidua (D) were strongly stained, the glandular epithelium of the secretory phase of the endometrium (C) was moderately stained, and the glandular epithelium of the proliferative phase of the endometrium (B) was very weakly stained with the anti-3β-HSD antibody. The cytotrophoblasts and syncithiotrophoblasts were indicated by open arrows and the glandular epithelium of the proliferative and secretory phase of the endometrium were indicated by closed arrows.

stained with anti-3β-HSD antibody, respectively (Figure 2). Hence, the expression of 3β-HSD was enhanced with increase of progesterone concentration in uterine endometrium.

Expression of 3β-HSD in human uterine endometrium

We next confirmed the expression of 3β-HSD on mRNA level and determined which type of 3β-HSD is expressed in uterine endometrium. We designed two primer sets for RT-PCR; one was the common to type I and II 3β-HSD, namely 3-βHSD-820, and the other, 3βHSD-628, was specific to type I 3β-HSD (Table 1). Using the 3βHSD-820 primer set, the 820 bp band was obtained in the endometrial sample. The band is the same as the 820 bp band obtained in placenta sample which is expressed type I 3β-HSD. In the case of 3βHSD-628 primer set, the same 628 bp band as those of placenta sample was also detected in the endometrial sample (Figure 3). The 820 bp band was subcloned into the pGEM-T easy vector system and sequenced. The nucleotide sequences of the 820 bp were completely identical to those of the type I 3β-HSD (data not shown).

Expression of P450scc in Human Uterine endometrium

Next, to see whether progesterone might be synth-

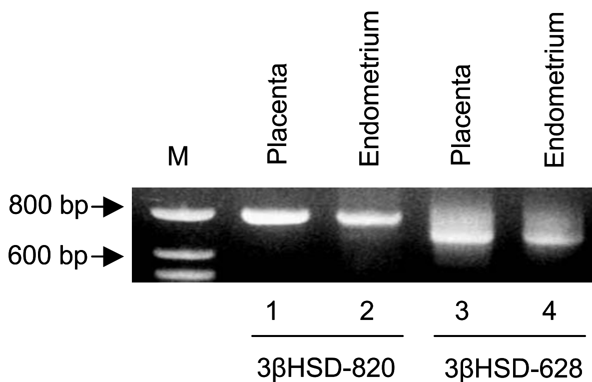

Figure 3. RT-PCR analysis of 3β-HSD. The same band in the endometrial sample as that of placenta sample was obtained with 3β-HSD-820 primer set which is common to type I and II 3β-HSD. The 628 bp band was also detected in the placenta and the endometrial sample by the type I specific 3β-HSD-628 primer set.

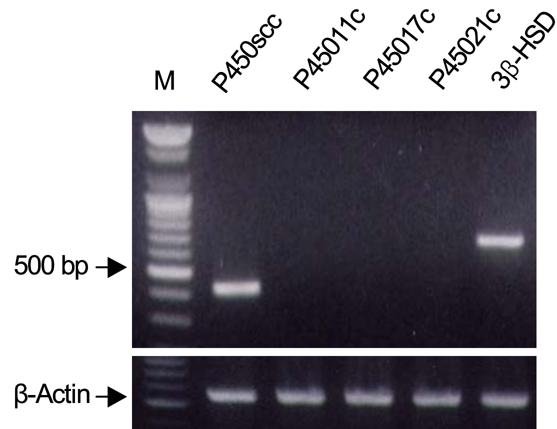

Figure 4. RT-PCR analysis of steroidogenic enzymes. I μg of total RNA was used for first strand cDNA synthesis. PCR amplification was performed with the specific primer set for P450scc, P45011c, P45017c, P45021c and 3β-HSD (Table 1). The PCR products of P450scc and 3β-HSD primer set were detectable in the uterine endometrium of ectopic pregnancy.

Table 1. Sequences of oligonucleotides.

Gene	5'→'3 Oligonucleotide sequence	Strand	Exon	Product size (bp)
P450scc	CAG TGG CAC TTG TAT GAG ATG	+	6	420
	TTG GTC ATC TCT AGC TCA GCG	-	8	
3βHSD-820	AGT GAT TCC TGC TAC TTT GG	+	1	820
	TAG CTT TGG TGA GGC GTG TC	-	3	
3βHSD-628	AGT GAT TCC TGC TAC TTT GG	+	1	628
	CTA GCA GAA AGG AAT CGG CT	-	3	
P450c17	GTA TCG CCT TCG CTG ACT CTG	+	2	632
	ATT GTG CAG CAG GAA GGC CAG	-	5	
P450c21	TGA GAT TCA GCA GCG ACT GC	+	6,7	417
	GTG AAG GCC TGC AGC AGT CG	-	8	
P450c11	TTC CAG TAC GGC GAC AAC TG	+	4,5	565
	CCA AGG CAC TGG CGC ATG CC	-	8	

esized *de novo* in the uterine endometrium, we examined the expression of genes for steroidogenic enzymes, P450 scc, P450c17, P450c21 and P450c11, as well as 3β-HSD using RT-PCR. The specific primers used for RT-PCR (Table 1) were located in two different exons of each gene to avoid amplification of any contaminating genomic DNA. In addition to 3β-HSD, only P450scc mRNA was detectable in the uterine endometrium sample after 30 PCR cycles (Figure 4). This result suggests that pregnenolone might be synthesized from cholesterol *de novo* by P450scc and the pregnenolone could be converted to progesterone by 3β-HSD in the uterine endometrium.

Discussion

As a result of endocrine stimulation, regular morphologic changes occur in human uterine endometrium. The concentration of progesterone in the secretory phase is higher than that in the proliferative phase of menstrual cycles, while vice versa for the concentration of estradiol. During the secretory phase of menstrual cycles, endometrial stromal cells differentiate into decidual cells which play a crucial role in implantation and maintenance of pregnancy. Decidualization is thus a progesterone-induced differentiation of the estrogen-primed endometrial stromal cells, and a key factor in regulating decidualization of endometrial stromal cells might be progesterone originated from ovarian sources (Irwin *et al.*, 1989; Fujimoto *et al.*, 1996; Brar *et al.*, 1997).

Enzymatic activity of 3β-HSD was detected in human uterine endometrial tissues. In addition to the enzymatic activity of 3β-HSD in human endometrium, the presence of 17β-HSD, which controls the last step in the formation of all androgens and all estrogens in gonadal as well as extra-gonadal tissues, was also reported in the human endometrium (Pollow *et al.*, 1975; Seki *et al.*, 1987; Maentausta *et al.*, 1991; Mustonen *et al.*, 1998). Moreover, aromatase activity in the endometrium was also reported (Yamamoto *et al.*, 1993). Therefore, those results suggested that human endometrium could produce biologically active steroids from circulating precursors of adrenal origin. Because progesterone plays an important role in implantation and maintenance of pregnancy, the concentration of progesterone in the secretory phase is significantly higher than in the proliferative phase. Hence, the expression of endometrial 3β-HSD might be enhanced with the increase of circulating concentration of gonadotrophic hormones. However, some reports showed the different results about the enzymatic activity of 3β-HSD during the menstrual cycle (Seki *et al.*, 1987; Tang *et al.*, 1993).

If the endometrium acts actively as a paracrine or autocrine tissue, it was assumed that the level of 3β-HSD concentration in endometrium should be changed during the menstrual cycle and maintained highly during gestation. In this study, we have examined the expression of 3β-HSD in human uterine endometrium during the menstrual cycle and in decidua by immunohistochemistry. We estimated the expression level of 3β-HSD depending on the staining level. The expression of 3β-HSD in the proliferative phase was very low, whereas the expression in the secretory phase was increased (Figure 2). 3β-HSD was expressed more strongly in the decidua of ectopic pregnancy. This result is in accordance with the increase of progesterone concentration in the secretory phase of menstrual cycles. The 3β-HSD expressed in the uterine endometrium was identified as being the same type as the placental 3β-HSD (type I) by RT-PCR and sequencing analysis (Figure 3).

Next, to see whether progesterone might be synthesized *de novo* and whether other sterioids such as aldosterone and cortisol might be synthesized in uterine endometrium, we examined the expression of genes which encoded steroidogenic enzymes as well as 3β-HSD. The first and rate-limiting step in the biosynthesis of all steroid hormones is the conversion of cholesterol to pregnenolone by the mitochondrial cytochrome P450scc. If P450scc may be expressed in uterine endometrium, pregnenolone might be synthesized from cholesterol *de novo*. Once pregnenolone is produced from cholesterol, it may undergo 17β-HSD hydroxylation to 17-hydroxypregnenolone, or it may be converted to progesterone by 3β-HSD. Both pregnenolone and progesterone may be 17β-hydroxylated to 17-hydroxypregnenolone and hydroxyprogesterone, respectively, and these steroids may then undergo the scission of C17, 20 carbon bonds to yield dehydroepiandrosterone (DHEA) and androstenedione by a single enzyme, P450c17, respectively (Nakajin *et al.*, 1981). If the expression of P450c17 gene is not detectable, pregnenolone is converted to mineralocorticoids; if P450c17 gene is expressed and P450c21 and P450c11 gene is expressed, pregnenolone is converted to the glucocorticoid, cortisol. We have examined the expression of these steroidogenic enzymes: P450scc, 3β-HSD, P450c17, P450c21 and P450c11, using RT-PCR in uterine endometrium of the ectopic pregnancy. The specific primers used for RT-PCR (Table 1) were located in two different exons of each gene to avoid amplification of any contaminating genomic DNA. In addition to 3β-HSD, only P450scc mRNA was detectable in uterine endometrium sample after 30 PCR cycles (Figure 4). Up to date, because the enzyme activity of 3β-HSD, 17β-HSD and P450aro was detected in the uterine endometrium where there were believed to use merely the circulating steroids of adrenal origin for synthe-

sizing progesterone and estrogen. However, this result suggests that during the early phase of pregnancy, the endometrial 3β-HSD can use not only the out-coming pregnenolone from the adrenal gland but also the self-made pregnenolone to produce progesterone. Ben-Zimra *et al.* suggested that the expression of P450scc and 3β-HSD type VI gene in mouse deciua is important for successful implantation and/or maintenance of pregnancy. They also proposed that the local production of progesterone acts as an immunosuppressant at the maternofetal interface preventing the rejection of the fetal allograft (Ben-Zimra *et al.,* 2002). In this context, the expression of p450scc and 3β-HSD gene in human uterine endometrium has a significant meaning for implantation and maintenance of pregnancy. Although we couldn't confirm the expression of P450scc in the endometrium during the menstrual cycle, the *de novo* synthesis of progesterone might be especially important for regulating the decidualization of endometrial stromal cells. To address this question experiments are underway in our laboratory.

Acknowledgement

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