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Characterization of 17β-hydroxysteroid dehydrogenase type 4 in human ovarian surface epithelial cells

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The human ovarian surface epithelium (hOSE) is a single layer of mesothelial-type primitive epithelial cells that are potential estrogen targets. It has been reported that hOSE cells can produce estrogen. However, the mechanisms that regulate estrogen level(s) in hOSE cells are not yet known. To elucidate the enzymes involved in these reactions, we examined gene expression of 17β-hydroxysteroid dehydrogenases (17β-HSDs) in primary hOSE (POSE) and OSE2a cells using RT–PCR. We found that POSE cells and cells of the immortalized hOSE line, OSE2a, bidirectionally converted estrone (E_1) and 17β-estradiol (E_2). Both cell types expressed mRNA for 17β-HSD type 1 (17β-HSD1), suggesting that the enzyme is involved in the E_1 to E_2 conversion. Interestingly, both cells expressed 17β-HSD4 mRNA but not 17β-HSD2 mRNA. We prepared an antibody against the carboxyl terminal of 17β-HSD4 (anti-17β-HSD4 antibody), which recognized the 80 and 48 kDa proteins in POSE and OSE2a cells based on immunoblot analysis. Furthermore, immunohistochemical study revealed the presence of 17β-HSD4 in hOSE cells in the human ovary. These results suggest that 17β-HSD4 is involved in estrogen inactivation and may protect against an excessive accumulation of E_2 in hOSE cells.

Key words: estrogen/ovarian surface epithelial cells/ovary/17β-HSD4

Introduction

The human ovarian surface epithelium (hOSE) that surrounds each ovary is a sheet of cuboidal mesothelial cells thought to arise from coelomic epithelium with retention of both epithelial and mesenchymal potential (Auersperg *et al*., 1984; Okamura and Katabuchi, 2001, 2005). The hOSE cells participate in formation of the ovarian cortex and share embryonic origins with granulosa cells (GCs) (Auersperg *et al*., 2001). In adult life, hOSE cells play an important part in the cyclic rupture of the Graafian follicle and formation of the corpus luteum. Unlike GCs, which proliferate, differentiate into lutein cells and die as the corpus luteum regresses, hOSE cells continually proliferate and recolonize the ovarian surface in the wake of each ovulation (Murdoch, 1996; Katabuchi and Okamura, 2003). The hOSE cells have been reported to be a possible source of ovarian epithelial neoplasms under the hypothesis that injury and repair of surface epithelial cells after cyclic ovulation can result in cells that accumulate multiple genetic alterations with development of oncogenic phenotypes (Fathalla, 1971; Godwin *et al*., 1992).

The hOSE has unique features that are absent from the immediately adjacent pelvic mesothelium, suggesting that local factors in the ovarian cortex may play a role in modifying the growth and morphology of this important cell type. Extracellular signals to which hOSE cells are likely to respond include steroid hormones, polypeptide growth factors, extracellular matrix components and cell adhesion molecules. Cultured hOSE cells are reported to produce growth factors, cytokines, cell adhesion molecules and proteolytic enzymes (Auersperg *et al*., 2001).

Several studies have suggested that locally produced estrogen is involved in development of epithelial endometriosis within the highly estrogenic ovarian environment (Noble *et al*., 1996; Nisolle and Donnez, 1997; Ohtake *et al*., 1999). Expression of estrogen receptors alpha (ERα) and beta (ERβ) mRNA in hOSE cells has been reported in normal ovaries and in various hOSE cell lines (Brandenberger *et al*., 1998; Lau *et al*., 1999), suggesting that estrogen regulates their function and growth.

Recent studies have demonstrated that estrogenic activity in placenta and endometrium is regulated by tissue-specific estrogen bioavailability (Mustonen *et al*., 1998; Madsen *et al*., 2004). The last step of estrogen synthesis is catalysed by 17β-hydroxysteroid dehydrogenases (17β-HSDs), which convert less active 17-keto-steroids to active 17β-hydroxy-forms. To date, 12 different isozymes of 17β-HSDs have been identified (Adamski and Jakob, 2001; Luu-The, 2001; Mindnich *et al*., 2004), differing in tissue distribution, catalytic preferences, substrate specificity, and subcellular localization and mechanisms of regulation. In this study, we investigated intracellular control of estrogen activity in hOSE cells and characterized isozymes of 17β-HSDs in hOSE cells both at the mRNA and protein level.

Materials and methods

Cell and tissue collection and preparation

The hOSE cells were prepared from ovaries of 12 women undergoing abdominal total or radical hysterectomy at Kumamoto University Hospital as approved by the University Ethics Committee. Informed consent was

obtained before collecting hOSE cells. Patients' characteristics are summarized in Table I. All ovaries were grossly normal, and no pathologic lesions were observed on histological examination. Preparation of hOSE cells was performed by published methods (Nakamura *et al*., 1994). Briefly, after collagenase digestion under aseptic conditions, hOSE cells were scraped with a surgical blade. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (1:1 mixture) (Invitrogen, Carlsbad, CA, USA) containing 20% fetal bovine serum (FBS; Thermo Trace, Carlsbad, CA, USA), 100 U/ml penicillin G (Invitrogen) and 100 μg/ml streptomycin (Invitrogen) in a humidified 5% CO_2 incubator. After a few days of incubation, viable hOSE cells were detached from the dish with 0.125% trypsin and 0.01% EDTA and collected by centrifugation. Cells from each dish were resuspended in medium and split into four new culture dishes.

At seeding after dispersal with trypsin-EDTA, the viable cell number was $0.1–2.7 \times 10^6$ per ovary. The cells were subsequently passaged up to three times when cell number was too small for the following examination. It takes approximately 4 days to be confluent. The lifespan was approximately several weeks at longest and varied among the samples. However, in some cases, the cells did not reach subconfluent, and these cells were not employed for the following experiments. We employed these primary, passaged, non-immortalized hOSE cells for the following studies as POSE cells. Because the number of POSE cells was small and the lifespan in culture was limited, we had established immortalized hOSE cell lines using simian virus 40 (SV40) large T antigen, as previously reported (Nitta *et al*., 2001). One cell line, OSE2a, was derived from a reproductive-age patient, and these cells showed luteinizing hormone-dependent growth (Tashiro *et al*., 2003). They retained an epithelial character as shown by morphological, immunocytochemical and ultrastructural studies that yielded the same findings as those of POSE cells *in vivo* (Nakamura *et al*., 1994). OSE2a cells were cultured in DMEM/F-12 medium (1:1 mixture) containing 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin in a humidified 5% CO_2 incubator. THP-1 was obtained from the Health Science Research Resources Bank (Number JCRB0112.1; THP-1, Osaka, Japan). The THP-1 cell line, which was established from human acute monocytic leukaemia cells, reportedly expresses 17β-HSD4 (Jakob *et al*., 1995). THP-1 cells were cultured in RPMI 1640 medium with 10% FBS, 50 U/ml penicillin G and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator.

Estrogen production in POSE and OSE2a cells

Subconfluent POSE cells were trypsinized and seeded at 1×10^5 cells per well in four chamber microtiter plates (Nunc, Nunclon, Denmark) in DMEM/F-12 medium with 1% FBS. The POSE cells from a patient were used for a single experiment.

As for OSE2a cells, confluent cells were subcultured in four-well microtiter plates at 1×10^5 cells per well in serum-free DMEM/F-12 medium.

When the cells became confluent, of 50 ng/ml androstenedione, E_1 or E_2 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in propyleneglycol containing 10% ethanol was added to the medium as substrate, and the cells were incubated for an additional 24 h. The culture medium in the four-well plates was collected and stored at −20°C until measurement of estrogen was performed.

Total RNA isolation and RT–PCR

Cultured cells were washed twice with phosphate-buffered saline (PBS), and total RNA was prepared using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was quantified by measuring absorbance at 260 nm. Single-stranded cDNA was reverse transcribed from 2 μg total RNA with a Superscript II reverse transcriptase (Invitrogen) at a final concentration of 2.5 U in buffer containing 25 mM Tris–HCl, pH 8.3, 37.5 mM KCl, 4 mM $MgCl_2$, 4 mM dithiothreitol (DTT), 0.5 mM dNTPs and 2.5 μM oligo (deoxythymidine) primer (Invitrogen). Incubation was performed at 42°C for 50 min, followed by incubation at 70°C for 15 min Total RNA was digested with ribonuclease H (Life Technologies, St. Paul, MN, USA) at 37°C for 20 min. PCR was performed using 0.5 mL Easy Start (Molecular BioProducts, San Diego, CA, USA) containing *Taq* DNA polymerase (Invitrogen). PCR primers were designed based on published sequences. PCR was carried out after denaturation of the sample at 95°C for 5 min. Each cycle consisted of denaturation at 95°C for 45 s, annealing for 45 s and extension at 72°C for 45 s. After amplification, the final 7 min extension step was carried out at 97°C. Primer sequences, annealing temperature and PCR cycle number and the expected size of each product are summarized in Table II. PCR amplification was performed in a PC-707 thermocycler (Program Temp Control System PC-707, Astec, Tokyo, Japan). PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

Table I. Patients and background and diagnosis

Patient	Age (year)	Parity	Diagnosis	Cycle day
1	52	2	Cervix carcinoma	Menopause
2	31	0	Cervix carcinoma	7
3	50	1	Fibroids	Perimenopause
4	44	1	Endometrial adenocarcinoma	40
5	49	2	Cervix carcinoma	Perimenopause
6	43	3	Cervical intraepithelial neoplasia III	31
7	48	2	Fibroids	28
8	33	0	Endometrial adenocarcinoma	Unknown
9	42	3	Cervix carcinoma	5
10	47	2	Fibroids	21
11	52	1	Cervix carcinoma	Menopause
12	51	0	Fibroids	26

Table II. PCR primers designed for the study 17β-hydroxysteroid dehydrogenase (17β-HSD) expression

Isozyme	Primer sequence	Temperature	Cycle	Size (bp)	Product GenBank Accession number
17β-HSD1	5′-CCTTCCACCGCTTCTACCAAT-3′ 5′-AAGCGCTCGGTGGTGAAGTA-3′	59	30	143	NM_000413
17β-HSD2	5′-TGGAAGGTGTGGATGTCCCGT-3′ 5′-CTGAGGAATTGCGAAGAACC-3′	55	30	593	NM_002153
17β-HSD4	5′-TCTATGATGGGTGGAGGATT-3′ 5′-GCGGCGTCCTATTTCCTCAA-3′	55	30	749	NM_000414

DNA sequencing

PCR products were purified using QIAquick PCR Purification Kit (Qiagen), according to the manufacturer's recommended protocol. Purified PCR products were quantified using an RNA/DNA calculator (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Each PCR product (100 ng) was sequenced on both strands using a thermal cycling method with fluorescent dye labelled-dideoxynucleotide terminators and *Taq* polymerase (Big Dye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster, CA, USA). The sequences were obtained using an automated DNA sequencer (ABI PRISM 310; Applied Biosystems). A database search was performed for obtained sequences using the Basic Local Alignment Search Tool (BLAST) 2.0 program (http://www.ncbi.nlm.nih.gov). All PCR products were sequenced to confirm that the cDNAs of interest had been amplified.

Protein extraction

Both cell types were cultured for 7 days at 37°C in DMEM/F-12 medium containing 10% FBS. Cells were washed two times in PBS, pH 7.2, lysed for 30 min on ice in a homogenization buffer containing 60 mM Tris–HCl, pH 7.5, 180 mM NaCl, 6 mM EDTA, 18 mM $Na_4P_2O_7$, 60 mM NaF, 1.2% NP-40, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml leupeptin and 1 μg/ml pepstatin A and clarified by centrifugation at 14 000 × *g* for 20 min at 4°C. Human endometrial tissue, the positive control for 17β-HSDs, was obtained from a surgically removed uterus for a gynaecological indication with permission from the patient and homogenized in a homogenization buffer as described above with a Teflon homogenizer and centrifuged at 14 000 × *g* for 5 min at 4°C to obtain supernatants.

Preparation of anti-17β-HSD4 antibody

A carboxyl-terminal peptide (C-peptide) corresponding to residues 724–736 (QKLQMILKDYAKL) of 17β-HSD4 was synthesized, purified by high-performance liquid chromatography and coupled to keyhole limpet hemocyanin (KLH) in collaboration with Transgenic (Kumamoto, Japan). Polyclonal antibody to 17β-HSD4 was prepared by immunizing rabbits with KLH-coupled C-peptide. Antiserum showed a high titer (up to 1:729 000) against the C-peptide by enzyme-linked immunosorbent assay (ELISA). The immunoglobulin G (IgG) fraction was prepared with a HiTrap Protein A HP affinity column (Amersham Biosciences, Piscataway, NJ, USA). The antibody was further purified by antigen-affinity chromatography. In control experiments, the antibody was preabsorbed with C-peptide (1:50) overnight at 4°C.

Immunoblot analysis

Protein concentrations of cell extracts and supernatant of endometrial tissues were determined by Bradford's method (Bradford, 1976) with bovine serum albumin (BSA) as standard. The same amounts of proteins were treated with sodium dodecyl sulphate (SDS) sample buffer (Laemmli, 1970) and boiled for 2 min. Samples containing 20 μg protein were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in 10% acrylamide and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) in 25 mM Tris, 193 mM glycine and 20% methanol by semidry method (Transblot®, Bio-Rad Laboratories, Hercules, CA, USA). The membrane was incubated overnight with the anti-17β-HSD4 antibody (1:500) or anti-17β-HSD4 antibody preabsorbed with C-peptide. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:10,000). Immunoreactive proteins were detected using an enhanced chemiluminescence detection kit (Amersham Biosciences), according to the manufacturer's protocol. A suitable antibody to 17β-HSD1 was not available for immunoblot analysis.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized in xylene and hydrated in a series of graded ethanol solutions, then microwaved in 10 mM citrate, pH 6.0, for 30 min for antigen retrieval (Shi *et al*., 1997). Sections were incubated in 3% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity. Slides were immersed, tissue face down, in 50 μL 2% BSA with antibody against affinity-purified anti-17β-HSD4 antibody (1:200), anti-17β-HSD4 antibody preabsorbed with C-peptide or control serum and incubated at room temperature in a humid chamber for 30 min. Following three consecutive washes in PBS for 5 min each, the sections were incubated at room temperature for 30 min with a peroxidase-conjugated goat anti-rabbit IgG antibody (EnVision+ System, DAKO, Carpinteria, CA, USA). Peroxidase activity was visualized by incubation with 3,3′-diaminobenzidine (DAB) reaction reagent (DAKO) for several seconds. The resulting slides were washed with water, dehydrated by a series of graded ethanols and mounted. Photographs were obtained under light microscopy VANOX-S (OLYMPUS, Tokyo, Japan).

Hormone assays

E_1 and E_2 were measured by Mitsubishi Kagaku Bio-clinical Laboratories using Direct estrone kit (DBC, London, Ontario, Canada) and DPC estradiol kit (Diagnostic Products, LA, CA, USA), respectively. Detectable limits in these assay conditions were 8 pg/ml. The cross reactivities of the estradiol kit to E_1 and the estrone kit to E_2 were reported by their laboratories to be below 1.03% and 0.7%, respectively. We also checked the cross reactivities by ourselves, 50 ng/ml E_1 in the E_2 kit and 50 ng/mL E_2 in the E_1 kit. The concentration of E_1 in the culture medium was below detectable levels just after we added 50 ng/ml E_2, and E_2 was not detected just after 50 ng/ml E_1 was added in the culture medium. Hormone assays were performed in duplicate or triplicate.

Statistical analysis

Values were expressed as the mean ± SD. Statistical analysis was performed using Student's *t*-test. In the case of multiple comparisons, significances of individual differences were assessed using the Scheffe test followed by a one-way analysis of variance (ANOVA). All of the analyses were completed using Statview 5.0.1 (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Estrogen production in POSE and OSE2a cells

We first examined whether POSE cells could produce E_1 and E_2 in the presence of corresponding substrate. Among 12 patients, we studied four preparations of POSE cells from the different individuals ($n = 4$; patients 1–4 in Table I) followed by statistical analysis. Values are represented as mean ± SD. Concentrations of E_1 and E_2 in culture medium containing 1% FBS were below detectable limits. The concentrations of E_1 and E_2 were below detectable level (8 pg/ml, respectively) just after (0 h) the addition of 50 ng/ml of E_2 and E_1, respectively. E_2 concentration in culture medium was increased to 3.4 ± 0.6 ng/ml ($P < 0.05$), when cells were incubated with 50 ng/ml E_1 for 24 h. When cells were incubated with 50 ng/ml E_2, E_1 concentration was increased to 2.3 ± 0.6 ng/ml ($P < 0.05$). These results indicated minimum variability in estrogen production in response to E_1 and E_2 despite different clinical conditions of the patients from whom the cells were obtained.

E_2 production in OSE2a cells subsequently increased with increasing the incubation time over 24 h (Figure 1A). When cells were incubated with 50 ng/ml E_2 as substrate, E_1 concentration was also increased up to 12 h. POSE cells also showed time-dependent conversion of both E_2 to E_1 and E_1 to E_2, respectively (data not shown). The number of POSE cells was too limited to compare the kinetics with OSE2a cells. These results clearly showed that bidirectional conversion between E_1 and E_2 occurred in POSE cells and in OSE2a cells and that this conversion was time dependent. Estrogen was not produced by POSE and OSE2a cells when 50 ng/ml androstenedione was added as substrate (data not shown). These results suggested that OSE2a cells mimicked the character of POSE cells and that OSE2a cells were useful for further studies of the physiological functions of hOSE.

Identification of mRNA for 17β-HSD subtypes in POSE and OSE2a cells

As conversion between E_1 and E_2 is conducted by different isozymes in various tissues, we focused on the isozymes responsible for conversion

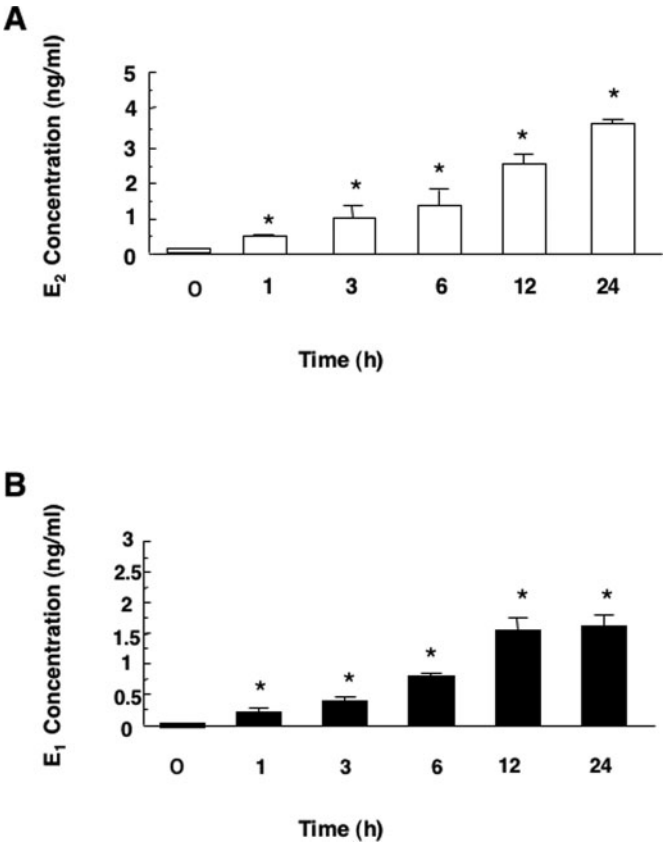

Figure 1. Time-course effects of (**A**) E_1 and (**B**) E_2 on the concentrations of (**A**) E_2 and (**B**) E_1 in OSE2a cells. At the indicated times, the culture media were collected for E_1 or E_2 assay as described in Materials and methods. Values are expressed as the mean ± SD (SD of the mean of triplicate determinations). *, $P < 0.05$ versus 0 h treated control.

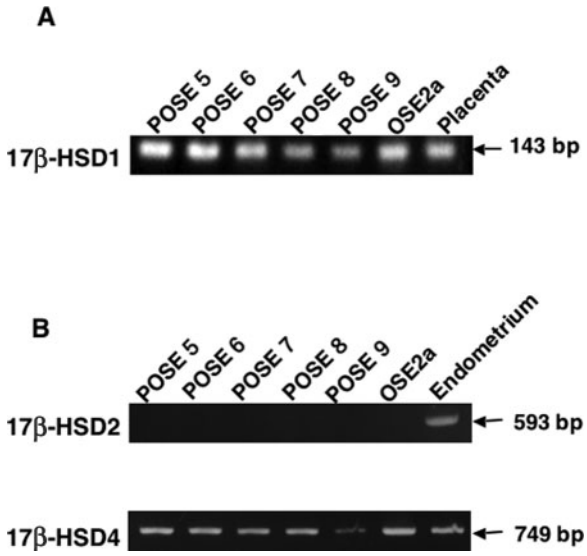

Figure 2. RT–PCR analysis of mRNA of (**A**) 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) and (**B**) 17β-HSD2 and 17β-HSD4 in primary human ovarian surface epithelium (POSE) cells from five patients (patients 5–9: POSE 5–9) and OSE2a cells. Total RNA was prepared from (**A**) human placenta (2 μg) or (**B**) proliferative phase endometrium (2 μg) as the positive control.

between E_1 and E_2 in hOSE cells. First, we examined isozyme expression in POSE cells from five patients ($n = 5$; patients 5–9 in Table I) and OSE2a cells by RT–PCR. When we used a primer pair for 17β-HSD1, a 143 base pair (bp) band was detected in all five POSE cell preparations, OSE2a cells and placenta were employed as a positive control (Figure 2A). We could not detect PCR product for 17β-HSD2 in any hOSE cell types, whereas it was clearly detected in endometrium (Figure 2B). Instead, a 749 bp PCR product corresponding to 17β-HSD4 was amplified in all hOSE cell types at levels comparable with those obtained with endometrium (Figure 2B). Direct sequencing of PCR products from five POSE cell preparations and OSE2a cells confirmed that 143 and 749 bp fragments corresponded with 17β-HSD1 and 17β-HSD4, respectively.

Immunoblot analysis of 17β-HSD4 in POSE and OSE2a cells

For the next step, we developed an anti-17β-HSD4 antibody to identify enzyme at the protein level. When immunoblot analysis was carried out with cell extracts from POSE cells from three patients ($n = 3$; patients 10–12 in Table I) and OSE2a cells, two proteins with apparent molecular masses of 80 and 48 kDa were recognized by an affinity-purified anti-17β-HSD4 antibody (Figure 3A). None of these proteins were detected when antibody was preabsorbed with C-peptide used as an antigen (Figure 3B). The antibody reacted with proteins with similar size in THP-1 cells and endometrium, both of which were employed as positive controls for 17β-HSD4 expression (Figure 3C). The 80 kDa protein was more abundant in OSE2a cells than in

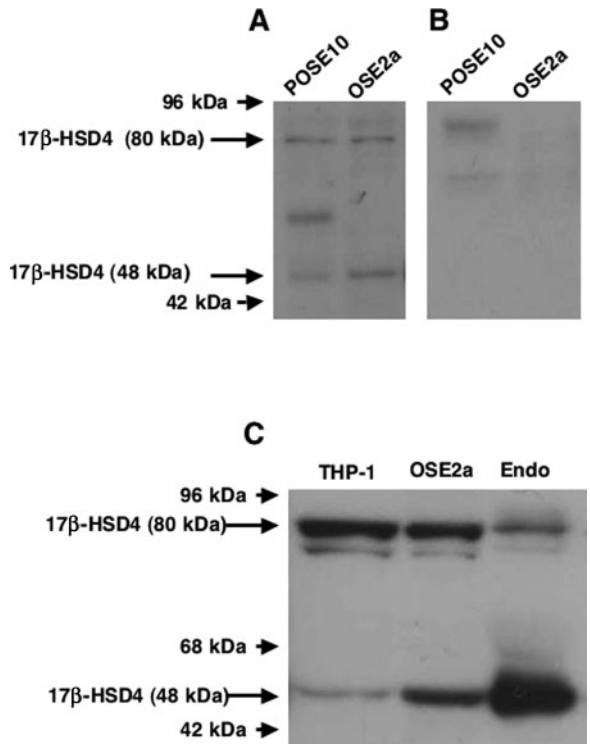

Figure 3. Immunoblot analysis of 17β-hydroxysteroid dehydrogenase type 4 (17β-HSD4). Cell extracts (20 μg protein) from primary human ovarian surface epithelium (POSE) cells, OSE2a cells, THP-1 cells and endometrium (Endo) were subjected to the immunoblot analysis with (**A** and **C**) affinity-purified anti-17β-HSD4 antibody or (**B**) preabsorbed antibody. We did experiments with three samples (patients 10, 11 and 12) with reproducible results and showed the representative data of one sample (patient 10: POSE 10). The positions of the 80 and 48 kDa proteins are indicated.

endometrium. In contrast, the 48 kDa protein was most abundant in endometrium among all samples. The antibody cross-reacted with proteins of apparent lower molecular masses than 80 kDa (Figure 3A

and C). It is not clear at present whether these extra bands were because of non–specific interactions with the antibody or degraded forms of 17β-HSD1. We could not examine the expression of 17β-HSD1 at the protein level, because a suitable antibody to 17β-HSD1 was not available.

Immunohistochemical staining

In control experiments, anti-17β-HSD4 antibody immunostained endometrium strongly (Figure 4B), whereas no immunostaining was observed using preabsorbed antibody (Figure 4C). In human ovary, hOSE cells were strongly immunostained with anti-17β-HSD4 antibody (Figure 4E). Immunostaining was not observed when consecutive sections were immunostained with preabsorbed antibody (Figure 4F). Furthermore, the ovarian stroma was slightly immunostained with anti-17β-HSD4 antibody. OSE2a cells were also immunostained with anti-17β-HSD4 antibody (data not shown). From these results, we concluded that 17β-HSD4 is expressed in hOSE cells in the human ovary as well as OSE2a cells.

Discussion

Various reports suggest that hOSE cells are capable of synthesizing E_2 (Ivarsson *et al*., 2001). In this study, E_2 was not produced in POSE and OSE2a cells when androstenedione was added as substrate for aromatase (p450arom), whereas E_2 was converted from E_1. These results suggest that E_2 production in quiescent hOSE cells depends on the presence of extracellular E_1.

17β-HSD1 was the first 17β-HSD to be purified and cloned (Luu-The *et al*., 1989). In various tissues, 17β-HSD1 preferentially catalyses E_1 to E_2 conversion. Recent reports suggest that increased E_2 production in endometrial cells contributes to the pathogenesis and development of endometrial hyperplasia and adenocarcinoma (Utsunomiya *et al*., 2001). Our findings strongly suggest that the conversion of E_1 to E_2 which are catalysed by 17β-HSD1 is a natural feature for hOSE.

There is accumulating evidence that conversion between E_2 and E_1 is conducted by 17β-HSD isozyme(s) in intact and tumour cells. It has been suggested that conversion from E_1 to E_2 is conducted by 17β-HSD1 and that conversion from E_2 to E_1 is conducted by 17β-HSD2 and 17β-HSD4. It has been reported that 17β-HSD1 and 17β-HSD2 mainly regulate E_2 tissue levels, modulating estrogenic action in human breast tissue (Söderqvist *et al*., 1998) and endometrium (Casey *et al*., 1994). Several reports have suggested that substantial changes in estrogen metabolism occur in malignant breast tissue (Poutanen *et al*., 1992; Spiers *et al*., 1998) and in endometrial abnormalities (Utsunomiya *et al*., 2001). Although 17β-HSD2 has been known as the major enzyme for E_2 to E_1 conversion in estrogen target tissues including placenta and endometrium (Mustonen *et al*., 1998; Madsen *et al*., 2004), we did not detect mRNA for 17β-HSD2 in POSE or OSE2a cells under our experimental conditions. Interestingly, 17β-HSD4 mRNA was clearly detected in both hOSE cells, suggesting that 17β-HSD4 is the catalyst in hOSE cells.

Immunoblot analysis using affinity-purified anti-17β-HSD4 antibody detected two major proteins with apparent molecular masses of 80 and 48 kDa in POSE and OSE2a cells, as well as in THP-1 and endometrium. Furthermore, immunohistochemistry with anti-17β-HSD4 antibody clearly showed that 17β-HSD4 was present in OSE cells in human ovary sections *in vivo*, as well as in OSE2a cells. Given the absence of 17β-HSD2 in hOSE cells, it is highly possible that 17β-HSD4 plays the major role in conversion of E_2 to E_1. To our knowledge, this is the first report on the presence of 17β-HSD4 in hOSE cells. The ovarian stroma was slightly immunostained with anti-17β-HSD4 antibody. The physiological meanings of this result are not clear at present. The expression of gene encoding several enzymes involved in gonadal steroidogenesis has been reported in the ovarian stroma (Barbieri, 1992; Jabara *et al*., 2003). Therefore, it is possible that 17β-HSD4 plays some physiological roles in ovarian stroma.

Initially, 17β-HSD4 was reported as an enzyme converting E_2 to E_1 in porcine endometrium (Adamski *et al*., 1992). Its physiological function may be protection against excessive E_2 in estrogen target tissues, such as endometrium. It has been reported that 17β-HSD4 is widely distributed throughout non-steroidogenic tissues and cells including THP-1 cells (Martel *et al*., 1992; Jakob *et al*., 1995). In these tissues, 17β-HSD4 has been reported to be involved in β-oxidation of very long chain fatty acids (van Grunsven *et al*., 1998, 1999; Baes *et al*.,

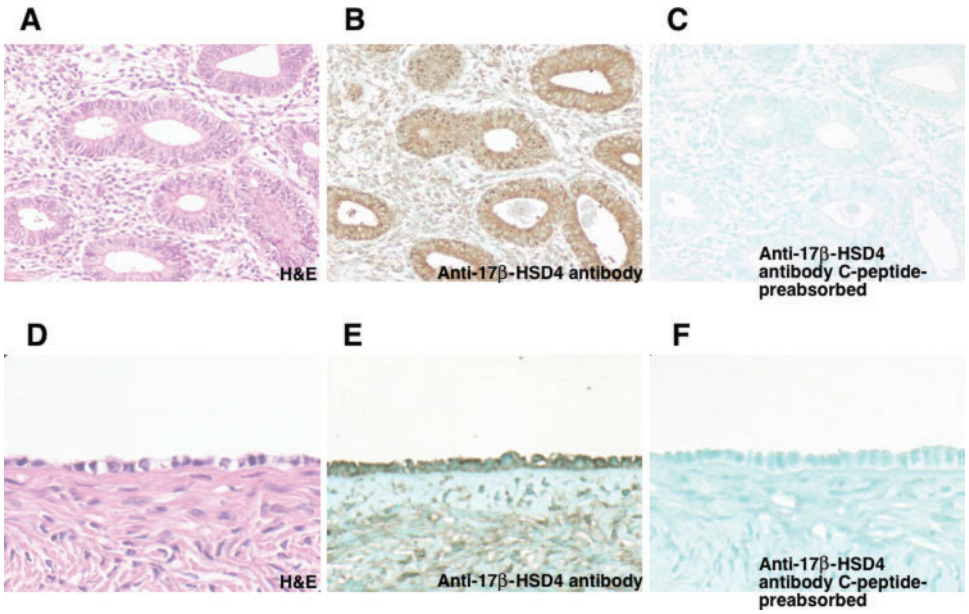

Figure 4. Immunohistochemical staining of human (**A**–**C**) endometrial tissue or (**D**–**F**) non-cancerous ovary with (**B** and **E**) anti-17β-hydroxysteroid dehydrogenase type 4 (anti-17β-HSD4) antibody or (**C** and **F**) preabsorbed antibody. Tissues were counterstained with methyl green. Hematoxylin and eosin (H&E) staining is shown in both tissues (**A** and **D**). Images were taken at 200× (**A**–**C**) and 400× (**D**–**F**).

2000), branched fatty acids (van Veldhoven *et al*., 1996; Dieuaide-Noubhani *et al*., 1997) and bile acid synthesis (Dieuaide-Noubhani *et al*., 1996). Inactive mutations in the 17β-HSD4 gene lead to a fetal form of Zellweger syndrome (de Launoit and Adamski, 1999). It is not clear at present whether or not 17β-HSD4 is involved in lipid metabolism in hOSE cells. It has been suggested that 17β-HSD4 is capable of converting E_2 to E_1 by *in vitro* studies (Dinkel *et al*., 2002). Taken together with this results, it is highly possible that 17β-HSD4 is involved in conversion of E_2 to E_1 in intact hOSE cells. One report (Husen *et al*., 2000) stated that expression of 17β-HSD4 decreased in endometrial cell lines HEC-1A and RL95-2 when the cells were treated with FBS. It has also been reported that progesterone, peroxisome proliferators and dexamethasone are able to induce 17β-HSD4 expression in endometrium and THP-1 cells (Jakob *et al*., 1995; Kaufmann *et al*., 1995). However, these effects were not observed in OSE2a cells in our preliminary experiments (unpublished data). Regulation of 17β-HSD4 expression in hOSE cells is an important project for future study.

Human 17β-HSD4 gene (*HSD17B4*) encodes an 80 kDa protein containing three catalytically active domains: the N-terminal short chain alcohol dehydrogenase reductase (SDR), the central hydratase domain and the C-terminal sterol carrier protein 2 (SCP2)-like domain (Figure 5) (Leenders *et al*., 1998). Full-length 17β-HSD4 is easily cleaved between the SDR and hydratase domains plus SCP2-like domain in peroxisomes (Markus *et al*., 1995; Corton *et al*., 1996; Jiang *et al*., 1996, 1997). Based on the protein structure of human 17β-HSD4, the immunoreactive 48 kDa protein we observed appears to be the hydratase plus SCP2-like domain cleaved from the 80 kDa protein. Although 17β-HSD4 is a member of the SCP2 gene family, including SCP2 and SCPx (Ohba *et al*., 1994), the function of the SCP2-like domain is still controversial. It may be required for import of 17β-HSD4 into peroxisomes, or it may act as a molecular chaperone in peroxisomes to protect intact enzyme from denaturation (Bun-ya *et al*., 2000). It has been reported that antibody against the N-terminal portion of porcine 17β-HSD4 reacts with an 80 and a 32 kDa protein in porcine myometrium (Kaufmann *et al*., 1995). Recently, one group (Brown *et al*., 2004) developed an antibody against equine 17β-HSD4 and found that the antibody reacted with an 80 and a 45 kDa protein in equine preovulatory follicles. Taken together with this results, the 45 kDa protein in equine follicles must be the cleaved fragment from the full-length 17β-HSD4. In addition, we found that the proportion of the 80 and the 48 kDa protein differed among the examined cells and endometrium, suggesting that the cleavage is under tissue-specific control. Elucidation of the role of the 48 kDa protein is necessary for future study. Our anti-17β-HSD4 antibody is suitable for immunoblot and immunohistochemical analyses and useful for these studies.

In conclusion, hOSE cells can convert E_1 and E_2 bidirectionally using 17β-HSD1 and 17β-HSD4, respectively. These results suggest that the physiology of hOSE cells are controlled under both extracellular and intracellular estrogen activation/inactivation.

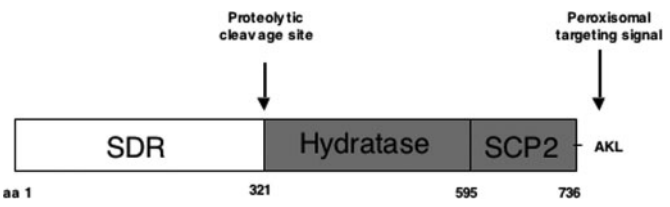

Figure 5. Structural features of human 17β-hydroxysteroid dehydrogenase type 4 (17β-HSD4). Three catalytically active domains, the reported proteolytic cleavage site and peroxisomal targeting signal are indicated. SDR, short chain alcohol dehydrogenase reductase; SCP2, sterol carrier protein 2.

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