Expression of enzyme associated with steroid hormone synthesis and local production of steroid hormone in endometrial carcinoma cells

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Abstract

Endometrial carcinoma is a common malignancy of the genital tract. In the present study, we examined the expression of human steroidogenic acute regulatory (StAR) protein, P450 side-chain cleavage enzyme (P450 scc), 17 α -hydroxylase/17,20-desmolase (P45017 α) and aromatase (P450 arom) in endometrial carcinoma cells to clarify the ability of these cells to produce steroid hormones. The results of RT-PCR analysis showed that StAR, P450 scc and P45017 α genes were expressed in endometrial carcinoma cells. To examine the protein expression of StAR and P450 scc, we performed Western blotting using extracts from carcinoma cells. StAR protein and P450 scc were detected in both HHUA and HOUA-1 cells. The production of pregnenolone in

HHUA cells increased 2.4-fold with transfection of a StAR expression vector and 4.3-fold with transfection of an F2 side-chain cleavage system. The RT-PCR product of 3 β -hydroxysteroid dehydrogenase was present in endometrial carcinoma cells. In endometrial carcinoma cells, the production of progesterone also increased with over-expression of StAR and the F2 system. Results of steroid metabolic assays showed that endometrial carcinoma cells produced not only 17 α -hydroxyprogesterone but also androstenedione. Endometrial carcinoma cells express enzymes that are associated with the production of steroid hormones. Locally produced steroid hormones may have effects on tumor proliferation and tumorigenesis.

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Introduction

Endometrial carcinoma arises from the endometrium, which is affected by steroid hormones. Endometrial carcinoma cells are also affected by steroid hormones. Prolonged estrogen stimulation, such as that caused by nulliparity, late menopause, obesity and diabetes mellitus, has been identified as a risk factor for the development of endometrial carcinoma (Kelsey *et al.* 1982). Progesterone, which has differential actions on the normal endometrium, is effective for the treatment of endometrial carcinoma (Kelley & Baker 1961). Selection of progesterone treatment depends on the expression of estrogen and progesterone receptors in cancer cells. However, there is some controversy regarding the choice of progesterone treatment because metastasis of early-stage carcinoma can occur in some cases in which progesterone treatment has been effective due to qualitative or quantitative changes in estrogen receptors (ER) or progesterone receptors (PR) in both ovulatory and post-menopausal women (Runowicz *et al.* 1990). There is no relationship between levels of circulating steroid hormones and the expression

of ER and PR in endometrial carcinoma (Spona *et al.* 1979).

The first step in the biosynthesis of steroid hormones is the conversion of cholesterol into pregnenolone. The rate-limiting process is the transport of cholesterol from the outer mitochondrial membrane to the inner membrane, where P450 side-chain cleavage enzyme (P450 scc) and its associated electron transport proteins reside. Steroidogenic acute regulatory (StAR) protein promotes the intra-mitochondrial transport of cholesterol and controls the rate-limiting step in steroidogenic tissues. StAR protein is synthesized as a 37 kDa precursor that is imported into mitochondria, where cleavage of the mitochondria targeting sequence occurs to yield a 30 kDa StAR protein (Strauss *et al.* 1999). The StAR gene is mutated in subjects with congenital lipoid adrenal hyperplasia, resulting in marked impairment of adrenal gonadal steroid synthesis (Lin *et al.* 1995). StAR expression is restricted to steroid-producing cells of the adrenal gland, testis and ovary (Sugawara *et al.* 1995).

Steroid hormones are produced from cholesterol in tissues other than the adrenal gland and gonads. It has

recently been demonstrated that the central nervous system, vascular wall and heart synthesize the steroid hormones pregnenolone, progesterone, dehydroepiandrosterone, corticosterone and aldosterone (Le Goasogne *et al.* 1987, Takeda *et al.* 1996, Kayes-Wandover & White 2000). Although enzymes necessary for the synthesis of steroid hormones have been found in these tissues by RT-PCR analysis, the levels of expression of steroidogenic enzyme genes were lower than those in the adrenal gland (Kayes-Wandover & White 2000). Steroid hormones synthesized in local tissues are thought to be biologically active with an autocrine or paracrine action (Le Goasogne *et al.* 1987, Takeda *et al.* 1996, Kayes-Wandover & White 2000).

It is well known that there is a close relationship between the prognosis of patients with endometrial carcinoma and the degree of differentiation of carcinoma cells. It has been reported that there is a correlation between the expression of ER and PR and the degree of tumor differentiation (McCarty *et al.* 1979). HHUA cells, which are highly differentiated endometrial carcinoma cells, express both ER and PR whereas HOUA cells, which are undifferentiated endometrial carcinoma cells, express neither ER nor PR (Ishiwata *et al.* 1984). In the present study, we examined the expression of StAR protein and other P450 enzymes associated with steroid hormone synthesis in HHUA and HOUA-1 cells to clarify the ability of endometrial carcinoma cells to produce steroid hormones.

Materials and Methods

Plasmid constructs

Human StAR cDNA was cloned into the pSV-SPORT 1 expression vector plasmid by inserting the *Sall*/*NotI* resting cutting sites as previously described (Sugawara *et al.* 1995). Expression vectors for bovine adrenodoxin (pCDADX) were kindly provided by Michael Waterman (Vanderbilt University, Nashville, TN, USA). The human P450 scc-adrenodoxin reductase-adrenodoxin fusion enzymes of the cytochrome P450 scc system, called the F2 system, were kindly provided by Dr Walter L Miller of the University of California, San Francisco, CA, USA.

Cell culture

Human HHUA highly differentiated endometrial carcinoma cells, HOUA-1 undifferentiated endometrial carcinoma cells and Hep G2 liver carcinoma cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Human adrenocortical carcinoma H295R cells were a gift from Dr Mitsuhiro Okamoto, Osaka University Medical School (Osaka, Japan). The cells were grown in 35 mm plastic dishes. HHUA, HOUA-1 and Hep G2 cells were

cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 50 µg/ml gentamycin. H295R cells were grown in DMEM/F12 containing 2% ULTROSER G (BioSeptra, Cergy-Pontoise, France) and 1% ITS Premix (Becton Dickinson and Co, Franklin Lakes, NJ, USA).

Transfection and steroid hormone assays

HHUA, HOUA-1 and Hep G2 cells were cultured at 40%–60% confluence in a medium with serum. One-tenth of a milliliter of serum-free medium contained F2, pSport StAR or pCDADX with 3 µl Fugene 6 (Roche Molecular Biochemicals, Mannheim, Germany) per 1 µg DNA. Dishes were treated with 20 µM trilostane (provided by Mochida Pharmaceutical Co Ltd, Tokyo, Japan) at 6 h after transfection to inhibit the enzyme activity of 3β-hydroxysteroid dehydrogenase (3β-HSD), which transforms pregnenolone to progesterone (Potts *et al.* 1978). In some dishes, 22R-hydroxycholesterol (5 µg/ml), which is a more soluble pregnenolone precursor and is an intermediate in the cholesterol side-chain cleavage reaction, was added to the culture medium. This hydroxysterol bypasses the translocation mechanism of cholesterol by which cholesterol is transported from the outer mitochondrial membrane to the inner membrane (Toaff *et al.* 1982). The culture medium was collected 48 h after transfection for RIA of pregnenolone as described previously (Sugawara *et al.* 1995). Progesterone was measured using a Progesterone Correlate-EIA Kit (Assay Designs Inc, Ann Arbor, MI, USA). Pregnenolone assays were performed using endometrial carcinoma cells without co-transfection of expression plasmids to evaluate pregnenolone accumulation in the medium. The culture medium with 20 µM trilostane was collected at various times after medium change. To evaluate pregnenolone and progesterone accumulation in the culture medium, trilostane was not added to the culture medium. Pregnenolone and progesterone were assayed in culture media collected at various times after medium change.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from HHUA, HOUA, Hep G2 and H295R cells. Complementary DNA synthesis was carried out at 37 °C for 60 min using 150 pmol oligo dT as a primer, 1 µg total RNA and 200 units SUPERScript II RNase H (Life Technologies Inc/BRL, Washington, DC, USA). Twenty microliters volume of reaction mixture for reverse transcriptase contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 20 mM dithiothreitol and 0.5 mM each of dATP, dCTP, dGTP and dTTP. Next, PCR reactions were carried out with 1 µl reverse transcription (RT) reaction product using the following primers. We designed oligonucleotide primers for amplification of StAR (Sugawara *et al.* 1995)

(sense, 5'-GCAGCAGCAGCGGCGGCAGCAG-3'; antisense, 5'-ATGAGCGTGTGTACCAGTG-3'), and P450 scc (Matteson *et al.* 1986a) (sense, 5'-ATGCTGGAGGAAGTAGTG-3'; antisense, 5'-TCCTGCCAGCATCTCTGT-3'), P45017 α -hydroxylase/17,20-desmolase (P45017 α) (Matteson *et al.* 1986b) (sense, 5'-CATGCTGGACACACTGATGC-3'; antisense, 5'-CCTCCAGGCCTGGCGCACCT-3'), and P450 aromatase (P450 arom) (Simpson *et al.* 1987) (sense, 5'-CGGCCTTGTCGTATGGTCA-3'; antisense, 5'-GTCTCATCTGGGTGCAAGGA-3'). Fifty microliters of the PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs and 10 pmol each of the primers. The reaction was subjected to 35 cycles of denaturing at 94 °C for 45 s, annealing at 55 °C (P450 arom and StAR) for 45 s and extension at 72 °C for 1 min or subjected to 40 cycles of denaturing at 94 °C for 45 s, annealing at 57 °C for 45 s and extension at 72 °C for 1 min (P450 scc and P45017 α). There are two 3 β -HSD isoenzymes in humans, which are chronologically designated type I and type II. PCR was performed using the following primers for 3 β -HSD (Lorence *et al.* 1990, Rheume *et al.* 1991): sense, 5'-TGGAGCTGCCTTGTGACAGGA-3'; antisense, 5'-CATCTGGAATCAAGGCGGAG-3'. The reaction was subjected to 35 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. After the reaction, primers were removed using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Then, the PCR products (1/30) were subjected to 20 cycles under the same conditions using the following set of primers: sense, 5'-CAAGACCAA GCTGACAGTGC-3'; antisense, 5'-TATCATAGCT TTGGTGAGGCG-3'. RT-PCR products were then digested with HpaI to discriminate between type I and type II 3 β -HSD.

Western blotting

Cells collected into homogenization buffer consisting of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 10 mM EDTA were sonicated for 5 s. Disrupted cells were centrifuged at 600 g for 15 min, and the supernatant was used for Western blot analysis. Protein extracts (10 μ g) were electrophoresed in 12.5% polyacrylamide gel in the presence of 2% SDS and then transferred onto a nitrocellulose membrane or polyvinylidene difluoride (PVDF) membranes for immunodetection with rabbit anti-StAR serum or anti-P450 scc serum. Dr Jerome F Strauss III of the University of Pennsylvania, Philadelphia, USA generously provided human anti-StAR serum. Human anti-P450 scc was a gift from Dr Toshihiro Tajima of Hokkaido University School of Medicine, Sapporo, Japan. The signal was detected by chemiluminescence using ECL or ECL-Plus Western blotting

detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden).

Steroid metabolism assays

Steroid metabolism assays were performed by the method of Nelson and McAllister with slight modification (Nelson *et al.* 1999). Cell cultures were grown until subconfluence in 100 mm plastic dishes and were washed twice with DMEM/F12 serum-free medium. The medium was then replaced by DMEM/F12 with 2% ULTROSER G and 1% ITS Premix containing tritiated pregnenolone ([³H]-P5) (2.0 μ M). The medium was collected after 12, 24, 36 and 48 h of culture. Steroids were extracted from the medium with four volumes of dichloromethane. The residue was dissolved in methanol and subjected to reverse-phase HPLC using a TSKgel ODS-120T column of 4.6 mm internal diameter $\times$ 15 cm in length and with a pore size of 5 μ m in diameter (Toso, Tokyo, Japan). The HPLC system consisted of a Shimadzu LV-9A pump and an SPD-6A UV detector (Shimadzu Corporation, Kyoto, Japan). The gradient solvent delivery system consisted of 1:1 acetonitrile/methanol (A/M) and water (45:55) for 2 min followed by a 2-min linear gradient to 60% A/M for 2 min, a 21-min linear gradient to 68% A/M for 1 min and then a 3-min linear gradient to 100% A/M. Radioactivity was measured by a liquid scintillation counter. The retention times of authentic steroid standards were established for the nonreduced and reduced steroids at 240 and 210 nm respectively.

Data analysis

Values are presented as means $\pm$ S.E. Significance between experimental groups was determined by Student's unpaired *t*-test. *P* < 0.05 was taken as the level of significance.

Results

Expression of steroidogenesis-associated enzymes in human endometrial carcinoma cells

RT-PCR analysis was performed to examine the gene expression of enzymes associated with synthesis of steroid hormones, including human StAR protein, P450 scc, P45017 α (P450c17) and P450 arom in the two human endometrial carcinoma cell lines, HHUA and HOUA-1, and in the two additional cell lines, Hep G2 and H295R cells. Hep G2 human liver carcinoma cells are non-steroidogenic cells, and H295R human adrenocortical carcinoma cells, which were used as a positive control for RT-PCR, are steroidogenic cells (Fig. 1). StAR RT-PCR products (975 bp) were present in both HHUA and HOUA-1 cells. StAR PCR products were also found to

Figure 1 Gene expression of enzymes associated with steroid hormones in carcinoma cells. RT-PCR was performed using mRNA extracted from HHUA cells (highly differentiated endometrial carcinoma cells), from HOUA-1 cells (poorly differentiated endometrial carcinoma cells), from H295R cells (human adrenal adenocarcinoma cells), and from Hep G2 cells (human liver adenocarcinoma cells). The sizes of the PCR products were 975 bp (StAR), 318 bp (P450 scc), 708 bp (P45017 α ; P450c17) and 987 bp (P450 arom). A positive control was amplified using mRNA from H295R cells. A negative control was amplified without RT reaction mixture.

be present in Hep G2 liver carcinoma cells. P450 scc RT-PCR products (318 bp) were present in both HHUA and HOUA-1 cells but not in Hep G2 cells. P45017 α RT-PCR products (708 bp) were present in HHUA, HOUA-1, and Hep G2 cells. P450 arom PCR products (987 bp) were not present in HHUA or HOUA-1 cells. Thus, the steroidogenesis-associated genes StAR, P450 scc and P45017 α , but not P450 arom, were expressed in endometrial carcinoma cells. Endometrial carcinoma cells

Figure 2 Expression of StAR and P450 scc proteins in endometrial carcinoma cells. Western blotting was performed using HHUA, HOUA-1, Hep G2 and H295R cells. Mitochondrial protein (15 μ g) was electrophoresed on 12% polyacrylamide gel containing 0.1% SDS and then immunoblotted onto a PVDF membrane with (a) StAR or (b) P450 scc antiserum. BeWo cell extract was used as a negative control for StAR protein. COS-1 cell extract was used as a negative control for P450 scc protein. Immunodetection was carried out using an ECL-Plus detection kit.

thus contain mRNA coding for enzymes involved in the synthesis of progesterone and androgens but not estrogens.

Expression of StAR and P450 scc proteins in endometrial carcinoma cells

StAR and P450 scc gene expression was detected in endometrial carcinoma cells by using RT-PCR. To confirm the protein expression of StAR and P450 scc in endometrial carcinoma cells, we performed Western blotting using mitochondrial extracts from cells. StAR protein was found to be present in HHUA cells and HOUA-1 cells (Fig. 2A). P450 scc protein was detected in HHUA and HOUA-1 cells (Fig. 2B). Although StAR protein was present, P450 scc protein was not present in Hep G2 cells.

Production of pregnenolone in carcinoma cells

To determine whether endometrial carcinoma cells synthesize steroid hormones and release steroid hormones into the culture medium, we measured the amount of pregnenolone in a culture medium with over-expression of StAR or the F2 P450 scc system. In HHUA cells, the production of pregnenolone was increased 2.4-fold with the StAR expression vector relative to that of mock transfection. With the F2 system, the production of pregnenolone increased 4.3-fold. With the ADX expression vector, the production of pregnenolone increased 2.9-fold. When 22R-hydroxycholesterol was added to the culture medium, the production of pregnenolone increased with the F2, StAR or ADX expression vectors. When 22R-hydroxycholesterol was added to the culture

medium, the production of pregnenolone was increased much more with the F2 system (Fig. 3A). The results suggest that the expression level of P450 scc was increased compared with the expression levels of other factors. In HOUA-1 cells, the production of pregnenolone did not increase with the F2, StAR, or ADX expression vector (Fig. 3B). Transfection with F2, StAR or ADX did not increase pregnenolone production in Hep G2 cells. When 22R-hydroxycholesterol was added to the culture medium, the production of pregnenolone was increased with the F2 system in Hep G2 cells (Fig. 3C). Protein extracts from transfected cells were analyzed using Western blotting. StAR and P450 scc system proteins were detected in transfected cells (Fig. 3D). To evaluate pregnenolone accumulation in endometrial carcinoma cells, we assayed pregnenolone in the medium at various culture times. Pregnenolone accumulation was observed with time-dependent increases in both endometrial carcinoma cell lines. The level of pregnenolone production in HHUA cells had increased after 12 h of culture. Accumulation of pregnenolone in HOUA-1 cells was observed at 48 h (Fig. 3E).

3 β -HSD expression in endometrial carcinoma cells

We examined the gene expression of 3 β -HSD to determine the ability of endometrial carcinoma cells to metabolize pregnenolone to progesterone. RT-PCR was performed using mRNA extracted from HHUA, HOUA-1, Hep G2 and H295R cells. PCR products (644 bp) obtained by amplifying 3 β -HSD transcripts were present in HHUA, HOUA-1, Hep G2 and H295R cells (Fig. 4A). The primers selected for the analysis do not discriminate between type I and type II 3 β -HSD transcripts. RT-PCR products were digested with HpaI, which selectively cut type I 3 β -HSD PCR products, because the HpaI restriction site is not present in type II 3 β -HSD PCR products. HpaI digestion of RT-PCR products from the type I 3 β -HSD gene resulted in the production of two fragments, a 508-bp fragment and a 136-bp fragment (Fig. 4B). H295R cells have type I and type II 3 β -HSD gene expression (Lorence *et al.* 1990, Rheume *et al.* 1992). Digestion of PCR products from H295R produced fragments of 644 bp, 588 bp and 136 bp. Digestion of PCR products from HHUA cells and Hep G2 cells produced fragments of 508 bp and 136 bp. PCR products of HOUA-1 cells were not digested with HpaI (Fig. 4C). These results indicated that type I 3 β -HSD was present in HHUA cells and that type II 3 β -HSD was present in HOUA-1 cells. 3 β -HSD genes were expressed in endometrial carcinoma cells.

Production of progesterone in endometrial carcinoma cells

To further investigate the metabolism of pregnenolone, we examined the production of progesterone in endo-

metrial carcinoma cells without trilostane in the culture medium. Progesterone production was significantly increased with the F2 system transfected into HHUA cells with or without the addition of 22R-hydroxycholesterol to the culture medium. Progesterone production was not increased when the StAR expression vector, pStAR, was transfected into HHUA cells without the addition of 22R-hydroxycholesterol to the culture medium (Fig. 5A). Although the production of pregnenolone did not significantly increase with F2 transfection, pStAR transfection increased the progesterone production in HOUA-1 cells. When F2 or pStAR was transfected into HOUA-1 cells, the production of progesterone was significantly increased with the addition of 22R-hydroxycholesterol to the culture medium (Fig. 5B). The production of progesterone in Hep G2 cells was significantly increased with the addition of 22R-hydroxycholesterol to the culture medium when F2, pStAR, or pCDAX was transfected (Fig. 5C). To evaluate progesterone accumulation in endometrial carcinoma cells, we assayed pregnenolone and progesterone in culture media after various periods of culture. In HHUA cells, the level of pregnenolone had increased at 24 h of culture and then decreased, whereas the level of progesterone had decreased at 24 h of culture, increased at 36 h and then gradually decreased again (Fig. 5D). Unlike the situation in HHUA cells, the level of pregnenolone in HOUA-1 cells had decreased at 48 h of culture and then increased. Progesterone accumulation was observed with time-dependent increases in HOUA-1 cells (Fig. 5E).

Pregnenolone metabolism in endometrial carcinoma cells

To further examine the metabolism of pregnenolone, we performed steroid metabolism assays. The production levels of 17 α -hydroxycholesterol (17 OHP4), androstenedione and progesterone (P4) in HHUA cells increased from 12 h and peaked at 24 h after the addition of [3 H]-P5 to the culture medium. The production of 17 OHP4 was increased much more than that of P4. The production levels of all of these steroids began to decrease at 36 h after the addition of [3 H]-P5 to the culture medium (Fig. 6A). In HOUA-1 cells, the production of P4, 17 OHP4 and androstenedione gradually increased from 12 h and the production of P4 rapidly increased from 24 h after the addition of [3 H]-P5 to the culture medium. Unlike the situation in HHUA cells, the production of progesterone did not decrease after 36 h in HOUA-1 cells. The production levels of 17 OHP4 and androstenedione increased with the progress of time and peaked between 36 and 48 h (Fig. 6B). In Hep G2 cells, the production levels of P4, 17 OHP4 and androstenedione increased from 12 h, peaked at 24 h, and then decreased. The production of P4 increased more than that of 17 OHP4 and androstenedione (Fig. 6C).

Figure 3 Production of steroid hormones in endometrial carcinoma cells. The amount of pregnenolone in the culture medium was measured using RIA. Cells were cultured in 3.5 cm culture dishes that each contained 2 ml culture medium. The F2 fusion SCC and ADX system, StAR expression vector (pStAR) and ADX expression vector (pCDADX) were transfected into (a) HHUA cells, (b) HOUA-1 cells and (c) Hep G2 cells. Trilostane (a 3 β -HSD inhibitor) was added to each culture medium at 6 h after transfection, and 22R-hydroxycholesterol (22OH cholesterol) was added to some dishes 24 h after transfection. At 48 h after transfection, the amount of pregnenolone in the medium was measured. The results are presented as means $\pm$ S.E. from three separate experiments.

*Significant difference for basal pregnenolone compared with mock transfection, significant difference for pregnenolone with 22R-hydroxycholesterol compared with mock transfection: * $+P < 0.05$, ** $+P < 0.01$. (d) Ten micrograms protein extract were electrophoresed in 12.5% polyacrylamide gel in the presence of 2% SDS for immunodetection with rabbit human anti-StAR serum (lower panel) or anti-P450 scc serum (upper panel). (e) Production of pregnenolone was assayed in HHUA and HOUA-1 endometrial carcinoma cells. Cells were cultured to about 60% confluence in 35 mm culture dishes. The cells were incubated with 20 μ M trilostane for the times indicated, and then immunoassay for pregnenolone was performed. The results are presented as means $\pm$ S.E. from three separate experiments.

Figure 4 3 β -HSD expression in endometrial carcinoma cells. (a) RT-PCR was performed using mRNA extracted from HHUA, HOUA-1, Hep G2 and H295R cells. The size of the PCR product was 644 bp (type I and type II 3 β -HSD). The first PCR reaction was subjected to 35 cycles and then to 20 cycles. (b) The *HpaI* restriction site is present in the type I 3 β -HSD gene. Digestion of type I 3 β -HSD (644 bp) produced fragments of 508 bp and 136 bp. PCR products of type II 3 β -HSD were not digested with *HpaI*. (c) One-fifth of the PCR products were digested with *HpaI* restriction enzyme.

Discussion

Locally produced steroid hormones play important roles in tissues even if their amounts are small. Steroid hormones that act as modulators of the signal transducer of synapse are synthesized in tissues of the central nervous system (CNS) by conversion of cholesterol (Baulieu 1997). RT-PCR analysis has shown that the *StAR* gene is co-expressed with P450 scc and 3 β -HSD in the CNS (Furukawa *et al.* 1998). As in CNS tissues, the *StAR* gene was found to be present together with P450 scc and P45017 α in HHUA and HOUA-1 endometrial carcinoma cells. HHUA and HOUA-1 cells have enzymes for synthesizing androstenedione from cholesterol via pregnenolone, progesterone and 17 α -hydroxyprogesterone. In our study, expression of the aromatase gene was not detected by RT-PCR analysis, although the aromatase gene has been shown to be expressed in stromal cells in endometrial carcinoma tissues (Watanabe *et al.* 1995, Sasano *et al.* 1996). In cancerous tissues of patients with endometrial carcinoma, it seems that androstenedione is converted into estradiol.

The fusion protein F2 is composed of the human P450 scc system (Harikrishna *et al.* 1993). Transfection of the F2 system resulted in increases in pregnenolone production in

HHUA cells and Hep G2 cells but not in HOUA-1 cells. The amount of pregnenolone could only be measured by *StAR* or the F2 system gene overexpression in HHUA cells, suggesting that the expression levels of *StAR* protein and P450 scc enzyme in endometrial carcinoma cells are low and that they do not contribute to sufficient production of steroid hormones for detection of steroid hormones in the serum of patients. The production of pregnenolone was not increased by F2 or p*StAR* transfection in HOUA-1 cells. Pregnenolone may be transformed into other steroidal derivatives or bind uncharacterized receptors, including pregnane X receptor (Lehmann *et al.* 1998), to utilize them in HOUA-1 cells.

It is possible to measure the amount of pregnenolone in HHUA cells by adding trilostane, a 3 β -HSD inhibitor, to a culture medium of the cells (Artemenko *et al.* 2001). Although pregnenolone was not detected in HOUA-1 cells even when trilostane was added to the culture medium, progesterone was detected without the addition of trilostane. Human type I 3 β -HSD is expressed in syncytial trophoblasts at high levels, and in sebaceous glands and breast and prostatic cancer cells, while type II 3 β -HSD is almost exclusively expressed in the adrenal cortex and gonads (Lorence *et al.* 1990). RT-PCR analysis showed that HHUA cells have a type I 3 β -HSD and that

Figure 5 Production of progesterone in endometrial carcinoma cells. The amount of progesterone in the culture medium was measured using EIA. Cells were cultured in 3.5 cm culture dishes that each contained 2 ml culture medium. F2, pStAR and pCDADX were transfected into (a) HHUA cells, (b) HOUA-1 cells and (c) Hep G2 cells. At 24 h after transfection, 22R-hydroxycholesterol (22OH cholesterol) was added to some dishes. At 48 h after transfection, the amount of progesterone in the medium was measured. The results are presented as means $\pm$ S.E. from three separate experiments. *Significant difference for basal pregnenolone compared with mock transfection, +significant difference for pregnenolone with 22R-hydroxycholesterol: * $P < 0.05$, ** $P < 0.01$ (d and e) Production of pregnenolone (open symbols) and progesterone (solid symbols) was assayed in (d) HHUA and (e) HOUA-1 endometrial carcinoma cells. Cells were cultured to about 60% confluence in 35 mm culture dishes. The culture medium was collected at various times after medium change. The results are presented as means $\pm$ S.E. from three separate experiments.

HOUA-1 cells have a type II 3β -HSD that converts pregnenolone into progesterone, and results of steroid assays showed that HHUA and HOUA-1 cells convert pregnenolone into progesterone. An interesting finding is that the metabolism of progesterone was much more rapid

in HHUA cells than in HOUA-1 cells. HHUA cells have both ER and PR, but HOUA-1 cells do not (Ishiwata *et al.* 1984). It is possible that HHUA-1 cells utilize intracellular progesterone and thus do not release progesterone into the culture medium. The good prognosis

Figure 6 Pregnenolone metabolism in endometrial carcinoma cells. (a) HHUA cells, (b) HOUA-1 cells and (c) Hep G2 cells were grown until subconfluence, and then 2.0 μ M tritiated pregnenolone ($[^3\text{H}]\text{-P5}$) were added to each culture medium. The media were collected at various times, and steroids were extracted from each medium with dichloromethane. Values are the means $\pm$ S.E. of radioactivity, expressed as a percentage of total activity of $[^3\text{H}]\text{-P5}$, from three separate experiments in which each treatment group contained three replicate cultures. Progesterone: open circles, solid line; 17 α -hydroxycholesterol: open triangles, dashed/dotted line; androstenedione: open box, dotted line.

of highly differentiated endometrial carcinoma may be associated with the utilization of pregnenolone and the regulation of the growth of carcinoma cells.

It is widely known that the development and growth of endometriotic lesions are dependent on estrogen (Dizerega *et al.* 1980). Although P450 arom is not expressed in normal endometrium, its expression has been detected in endometriotic tissues (Noble *et al.* 1996, Bulun *et al.* 2001). Estrogens are secreted from the ovary and from peripheral tissues (adipose and skin). Estrogens are also produced locally in endometriotic tissue itself. Expressions of genes associated with steroid hormone synthesis, i.e. StAR, P450 scc and P45017 α genes have recently been detected not only in the normal endometrium but also in endometriotic tissues (Tsai *et al.* 2001). It is possible that endometriotic tissues not only convert circulating androstenedione in plasma but also produce *de novo* steroid hormones from cholesterol (Tsai *et al.* 2001). Locally produced steroid hormones may play roles in estrogen-dependent growth of endometriotic lesions with an autocrine or paracrine action.

The present study has shown that endometrial carcinoma cells express steroidogenic enzymes, synthesize steroid hormones from cholesterol, and convert pregnenolone into androstenedione. Further study is needed to determine the ability of cells in endometrial carcinoma tissues from patients to produce steroid hormones and to clarify the relationship between production levels of steroid hormones and prognosis of endometrial carcinoma patients.

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