Estrogen metabolizing enzymes in endometrium and endometriosis

H. Dassen1,2, C. Punyadeera1,2,6, R. Kamps1,3, B. Delvoux3, A. Van Langendonckt4, J. Donnez4, B. Husen5, H. Thole5, G. Dunselman1,3,8 and P. Groothuis1,3,7

1Research Institute GROW, University Hospital Maastricht/University Maastricht, Peter Debyelaan, The Netherlands; 2Department of Pathology, University Hospital Maastricht/University Maastricht, Peter Debyelaan, The Netherlands; 3Department of Obstetrics and Gynaecology, University Hospital Maastricht/University Maastricht, Peter Debyelaan 25 HX 6229, The Netherlands; 4Department of Gynaecology, Université de Catholique de Louvain, Brussels, Belgium; 5Solvay Pharmaceuticals Research Laboratories, Hannover, Germany

BACKGROUND: Estradiol (E2) is an important promoter of the growth of both eutopic and ectopic endometrium. The findings with regard to the expression and activity of steroidogenic enzymes in endometrium of controls, in endometrium of endometriosis patients and in endometriotic lesions are not consistent. METHODS: In this study, we have looked at the mRNA expression and protein levels of a range of steroidogenic enzymes [aromatase, 17β-hydroxysteroid dehydrogenases (17β-HSD) type 1, 2 and 4, estrogen sulfotransferase (EST) and steroid sulfatase (STS)] in eutopic and ectopic endometrium of patients (n = 14) with deep-infiltrative endometriosis as well as in disease-free endometrium (n = 48) using real-time PCR and immunocytochemistry. In addition, we evaluated their menstrual cycle-related expression patterns, and investigated their steroid responsiveness in explant cultures. RESULTS: Aromatase and 17β-HSD type 1 mRNA levels were extremely low in normal human endometrium, while mRNAs for types 2 and 4 17β-HSD, EST and STS were readily detectable. Only 17β-HSD type 2 and EST genes showed sensitivity to progesterone in normal endometrium. Types 1 and 2 17β-HSD and STS protein was detected in normal endometrium using new polyclonal antibodies. CONCLUSIONS: In endometriosis lesions, the balance is tilted in favor of enzymes producing E2. This is due to a suppression of types 2 and 4 17β-HSD, and an increased expression of aromatase and type 1 17β-HSD in ectopic endometrium.

Keywords: endometriosis; steroidogenic enzymes; aromatase; estrogen metabolism; endometrium

Introduction

An estimated six percent of women suffer from endometriosis and have complaints such as chronic pelvic pain and some are even infertile because of this disease (Olive and Schwartz, 1993; Vessey et al., 1993; Kjerulff et al., 1996). Endometriosis is defined as the presence of endometrial glands and stroma in extra-uterine sites, mostly the pelvic peritoneum, ovaries and rectovaginal space (Olive and Schwartz, 1993; Zeitoun et al., 1999; Bulun et al., 2000, 2002a).

Endometriosis is an estrogen-dependent disease (Dizerega et al., 1980; Zeitoun et al., 1998). The primary source of estradiol (E2) is the ovary, and therapies aimed at suppressing ovarian function or antagonizing the actions of estrogens, have proven to be effective. It has long been known that the endometrium itself is a source of E2, estrone, estrone sulfate and estrone-3-sulfate (Tseng et al., 1982; Tseng, 1984b) and that elevated aromatase activity is associated with malignancies of the endometrium (Tseng et al., 1982; Yamaki et al., 1985). It took however, another 10 years for investigators to suspect that endometriotic lesions may be self-supporting as evidenced by aromatase overexpression (Yamamoto et al., 1993; Noble et al., 1996,1997). Aromatase overexpression has now indeed been confirmed in numerous reports at the transcript level (Noble et al., 1996; Bulun et al., 1997; Matsuzaki et al., 2006; Smuc et al., 2007), at the protein level (Kitawaki et al., 1997,1999; Murakami et al., 2006; Velasco et al., 2006), as well as at the activity level (Kitawaki et al., 1997; Noble et al., 1997; Murakami et al., 2006). The clinical relevance of these observations was illustrated by the fact that aromatase inhibitors were effective in the treatment of endometriosis, particularly in those cases where GnRH agonist treatment...
failed (Takayama et al., 1998; Ailawadi et al., 2004), supporting the notion that significant estrogen production continues at extraovarian sites, including adrenals, adipose tissue, skin and endometriotic lesions.

The net production of 17β-oestradiol is the result of a delicate balance between the synthesis and the inactivation of E2. Next to aromatase, the production of E2 is also mediated through the 17β-hydroxysteroid dehydrogenases (HSD) type 1, 3, 5, 7 and 12, as well as steroid sulfatase (STS), which converts the sulfated estrogens to biologically active estrogens (Matsuoka et al., 2002; Moeller and Adamski, 2006). Gene transcripts for types 1 and 7 17β-HSD and estrogen sulfatase (Fusi et al., 2005; Smuc et al., 2007) were found to be overexpressed in endometriotic lesions when compared with normal endometrium. In the human, aromatase produces mainlly estrone and not E2, which would support a key role for the type 1 17β-HSD as well.

Another key protein, which is believed to be involved in a rate-limiting step in steroid biosynthesis, is the steroidogenic acute regulatory protein (StAR). Even though StAR is not an enzyme, it is responsible for the transport of the substrate for steroid synthesis, cholesterol, across the mitochondrial membrane. StAR is highly overexpressed in endometriotic lesions (Tsai et al., 2001).

The conversion of E2 into less active metabolites in endometrium tissue is believed to be mediated for a large part by 17β-HSDs types 2, 4 and 8, which form by an oxidative reaction androstenedione and estrone (Miettinen et al., 1996; Husen et al., 2001; Luu-The, 2001; Mensah-Nyagan et al., 2001; Yang et al., 2001), and by the estrogen sulphotransferase (EST), which conjugates sulfate groups to the 3-hydroxyl position (Qian and Song, 1999).

The 17β-HSDs types 4 and 8 are constitutively expressed in normal human endometrium (Husen et al., 2001; Punyadeera et al., 2003). No information is available with regard to the expression levels of these two genes in endometriosis. EST is also expressed in normal endometrium (Utsunomiya et al., 2004). It is expressed in the secretory phase only, suggesting that it is regulated by progestins. In the study of Smuc et al. (2007) no differences were observed in the expression of EST in normal endometrium and ovarian endometriosis.

The type 2 17β-HSD is believed to be one of the most important E2-inactivating enzymes in the endometrium (Tseng and Gurpide, 1974,1975). There is clear evidence that transcription of the 17HSD2 gene in normal endometrium is regulated by progesterone (Tseng and Gurpide, 1975; Kauppila, 1984; Casey et al., 1994; Mustonen et al., 1998; Zhang et al., 2006), even though some inconsistent findings have been reported (Kitawaki et al., 2000; Matsuizaki et al., 2006).

Such contrasting reports were also published with regard to the expression of 17β-HSD type 2 in eutopic and ectopic endometrium of endometriosis patients. The general consensus is that the expression of type 2 17β-HSD is reduced or absent in eutopic and ectopic endometrium of endometriosis patients (Husen et al., 2001; Bulun et al., 2002a), and that 17β-HSD type 2 gene expression is insensitive to progestins (Vierikko et al., 1985; Zeitoun et al., 1998), indicating that endometriotic tissue may have reduced capacity to inactivate E2. Kitawaki et al. (2000), however, described exactly the opposite: in endometrium of patients 17β-HSD type 2 expression was increased in secretory endometrium of endometriosis patients. Also Smuc et al. (2007) did not observe differences in type 2 17β-HSD expression levels between normal endometrium and endometriosis. Similar discrepancies were reported for aromatase. Kitawaki et al. (1997,1999) and Matsuizaki et al. (2006) found no aromatase expression in endometrium of disease-free women, whereas Tseng and coworkers (1982,1984b) clearly showed aromatase activity in normal cycling endometrium.

The inconsistent findings with regard to the expression and activity of steroidogenic enzymes in endometriosis can attribute to a variety of factors including the use of different technologies, the quality of the samples (i.e. is the endometriotic tissue contaminated with normal tissue from the local environment) and the lesion types studied. More efforts are needed to address these issues.

Most studies have focussed on adenomyosis, leiomyomas and ovarian and peritoneal endometriosis, but little information is available about steroidogenic enzymes in the severe deep-infiltrative type of endometriosis. In the current study, we evaluated the expression of aromatase, STS, EST and the types 1, 2 and 4 17β-HSD in eutopic endometrium and endometriotic tissues of patients with deep-infiltrative endometriosis. In addition, expression was assessed in endometria from disease-free women. Hormonal regulation of the expression of these enzymes was also investigated in explant cultures of normal human endometrium.

**Materials and Methods**

**Tissues**

Endometrial tissue was collected from 48 women of 26–52 years of age with regular menstrual cycles, who underwent surgery for benign indications other than endometriosis. The tissue was collected by hysteroscopy (Pipelle catheter, Unimar Inc., Prodimed, Neuilly-Enthelle, France). The women were documented not to be on any kind of steroid medication. All women signed an informed consent, as required by the protocol approved by the Medical Ethical Committee of the University Hospital Maastricht.

Forty-eight biopsies of normal human endometrium were collected. Twelve were collected in the menstrual phase (M phase), 18 were collected in the proliferative phase: early proliferative phase, n = 9; late proliferative phase (LP phase), n = 9. Another 18 were collected in the secretory phase: early secretory phase (ES phase), n = 8; mid-secretory phase (MS phase), n = 8; late secretory phase, n = 2. After macroscopic inspection of the uteri by a pathologist, endometrium tissue was collected. The endometrium was dated according to clinical information with respect to the start of the last menstrual period, which was reconfirmed by histological examination of the tissue (Noyes et al., 1975).

Endometrium and endometriotic lesions were collected in 14 women of 22–39 years of age who underwent laparoscopic surgery to remove rectovaginal endometriosis. The endometrium was collected by pipelle biopsies during the operation (Pipelle catheter, Unimar Inc.). The collected tissues were frozen in Tripure Isolation...
Reagent TM (Roche, Basel, Switzerland) at −80°C until RNA isolation.

From each biopsy, part of the tissue was fixed in 10% buffered formalin for histology and immunohistochemistry and another part of the tissue was snap frozen in lysis buffer (Tripure Isolation Reagent™, Roche) and stored at −80°C for RNA isolation.

**Explant culture**

Endometrium tissues from M phase \((n = 8)\) and LP phase \((n = 8)\) biopsies were collected and transported to the laboratory in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 medium on ice. The tissue was minced into pieces of 2–3 mm\(^3\). Twenty-four explants were placed in Millicell-CM culture inserts (pore size of 0.4 μm, 30 mm diameter, Millipore, Billerica, MA, USA) which were placed in 6-well plates containing phenol red-free DMEM/Ham’s F12 medium (1.5 ml) (Life Technologies, Grand Island, NY). The medium was supplemented with L-glutamine (1%), penicillin and streptomycin (1%). The tissue was cultured for 24 h. At the end of the experiment, part of the explant was collected in formalin and embedded in paraffin, the rest was collected in lysis buffer (SV Total RNA Isolation Kit, Promega, Madison, WI, USA) and stored at −80°C until RNA isolation.

Previous experiments have shown that collagenase activity remains very low in proliferative endometria during the first 24 h of culture (Cornet et al., 2002), and that the tissue viability is not affected after 24 h of culture (Marbaix et al., 1992). Treatments included: control (0.1% ethanol); \(E_2\) (1 nM); progesterone (1 nM) and \(E_2\) and progesterone (1 nM each). The steroid hormones were gifts from Organon pharmaceuticales (Oss, The Netherlands).

**RNA isolation from normal endometrium**

Total cellular RNA from explants and uncultured endometrium was extracted using the SV total RNA isolation kit (Promega) according to the manufacturer’s protocol, with slight modifications. The concentration of DNase-1 during DNase treatment of the RNA samples was doubled and the incubation time was extended by 15 min in order to completely remove genomic DNA. Total RNA was eluted from the column in 50 μl RNase-free water and stored at −80°C until further analysis. The quality of the RNA samples was determined with a spectrophotometer and agarose gel electrophoresis. All the samples analysed gave RNA to DNA ratios higher than 1.5. Although primer/probes were designed to overlap exon boundaries, a PCR for a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed on the RNA samples to verify that the samples were free of genomic DNA.

**RNA isolation from endometriotic tissue**

Prior to RNA isolation, the tissue was thawed at 4°C, cut into small pieces and homogenized twice for 30 s with an Ultra-Turrax homogenizer (Rose Scientific, Edmonton, Alberta, Canada). The samples were centrifuged 10 min at 12 000 g. To the supernatant 200 μl chloroform was added. The samples were vortexed 15 s and incubated at room temperature for 10 min. The samples were centrifuged 15 min at 12 000 g. The aqueous upper phase was collected and 500 μl isopropanol was added to precipitate the RNA. The samples were vortexed 10 s and incubated 10 min at room temperature. The samples were centrifuged 10 min at 12 000 g. The pellets were washed with 1 ml 75% ethanol. After air-drying, the pellets were collected in RNase-free water and incubated for 10 min at 60°C.

**cDNA synthesis**

For complementary DNA (cDNA) synthesis, total RNA (1 μg) was incubated with random hexamers (1 μg/μl Promega) at 70°C for 10 min. The samples were chilled on ice for 5 min. To this mixture, a reverse transcriptase (RT)-mix consisting of 5x RT-buffer (4 μl), 5 mM dNTP mix (2 μl) (Pharmacia, Uppsala, Sweden), 0.1 M dithiothreitol (2 μl) (Invitrogen, Breda, The Netherlands) and superscript II reverse transcriptase (200 U) (Invitrogen) was added and the samples were incubated at 42°C for 1.5 h, after which the reverse transcriptase was inactivated by heating the samples at 95°C for 5 min. The cDNA was stored at −20°C until further use. In each real-time PCR reaction 50 ng of cDNA template was used.

**Real-time PCR**

Primers and probes for 17β-HSD-1 (Hs00166219-g1), 17β-HSD-2 (Hs01579939-m1), 17β-HSD-4 (Hs00264973-m1), STS (Hs0165853-m1), EST (Hs00193690-m1) and aromatase (Hs00240671-m1) were purchased from Perkin-Elmer Applied Biosystems as pre-developed assays. Human cyclophilin A (Hs99999904-m1) was selected as an endogenous RNA control in order to normalize for the differences in the amount of total RNA added to each reaction. A pool of total RNA obtained from uncultured human endometrium tissues was included as positive control. All PCR reactions were performed using an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems). The thermal cycling conditions comprised an initial decontamination step at 50°C for 2 min, a denaturing step at 95°C for 10 min and 40 cycles of 15 s at 95°C followed by 1 min at 60°C. Experiments were performed for each sample in duplicate. Quantitative values were obtained from the threshold cycle number (Ct) at which the increase in signal associated with the exponential increase of the number of PCR products was first detected with the ABI Prism 7700 sequence detector software (Perkin-Elmer, Foster city, CA). The fold-change in expression was calculated using the ΔΔ Ct method, with cyclophilin A mRNA as an internal control (Livak and Schmittgen, 2001). For detailed description of the procedure please refer to the ABI user manual. (http://www.uk1.unifreiburg.de/core/facility/tagman/user_bulletin_2.pdf)

**Immunohistochemistry and western blotting**

Novel polyclonal antibodies (pAb) were generated against 17β-HSD type 1, type 2 and STS (prepared at Pineda Antikörper-Service, Berlin, Germany, on behalf of Solvay Pharmaceuticals). The pAb against type 1 17β-HSD (protein accession number NP_000404) was generated by injecting rabbits with a recombinant 17β-HSD type 1 peptide (h17HSD) which lacked 42 amino acids at the C-terminus (generously provided by Christina Fischer, University of Frankfurt, Germany). The pAb against type 2 17β-HSD (protein accession number NP_002144) was generated by injecting a cocktail of the three synthetic peptides, NENGPGAEELRRTC (peptide A), CNIAAGTSDKWEKLEKD (peptide B) and CARKHFGQDKPMPRALR (peptide C). The pAb against STS (protein accession number NP_00342) was generated by injecting a cocktail of three synthetic peptides, CWLEAEHAAASRPII (peptide A), CAVHEVESSKGEIHGGGS (peptide B) and CSSKGEIHGGGSNIYK (peptide C). The peptides were conjugated to a protein carrier (KLH). Three rabbits were injected with the peptide-conjugate cocktails for each antigen, and from each rabbit preimmune serum was collected. The generated antibodies were affinity purified using the immunizing peptides.

Specificity of the pAbs was demonstrated by western blotting and immunohistochemistry.
Figure 1: Western blot analysis (A) and immunohistochemistry (B) with the pAb against 17β-HSD type 1
The pAb shows a specific band at the expected molecular weight (A) and specific staining in subcutaneous tumors in immunodeficient mice generated from 17β-HSD type 1-transfected MCF-7 human breast cancer cells (B). Pre-incubation with the h17HSD1 fusion protein prevented antibody binding in both the western blot (A) and immunostaining (B). Lane 1, 10 µl (1 µg/µl) HSD1-containing tumor cytosol; lane 2, 20 µl homogenate (0.55 µg/µl) from a HSD1-positive-cell line (lane 2); lane 3, 10 µl homogenate (1 µg/µl) from a HSD1-negative-cell line

To confirm specificity of the 17β-HSD type 1 pAb, gels were loaded with 10 µl (1 µg/µl) HSD1-containing tumor cytosol (Fig. 1, lane 1), 20 µl homogenate (0.55 µg/µl) from a HSD1-positive-cell line (Fig. 1, lane 2) and 10 µl homogenate (1 µg/µl) from a HSD1-negative-cell line (Fig. 1, lane 3; for general characteristics of cells and tumors see Husen et al., 2006), and subsequently probed with the preimmune serum, the pAb or the pAb after pre-incubation with the immunizing peptides. Antibody binding was visualized with an alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (1:10 000, Sigma-Aldrich, St. Louis, MO, USA) and incubation with NBT/BCIP-substrate solution (Sigma-Aldrich). Specific binding of the pAb against type 2 17β-HSD was shown by loading the individual immunizing peptides, as well as lysates prepared from placenta. The pAb and preimmune serum were applied at a 1:5000 dilution. Antibody binding was visualized with anti-rabbit horseradish peroxidase (1:10 000; Dako, Glostrup, Denmark) and incubation in a chemiluminescent substrate (Supersignal West Pico Chemiluminescent Substrate, Pierce, Rockford, USA). Specific binding of the anti-STS pAb was demonstrated by loading lysates prepared from endometrium and placenta and purified arylsulfatase C (Sigma-Aldrich) on the gels. The affinity purified antibody was applied at a dilution of 1:1000. Antibody binding was visualized in the same way as for the type 2 17β-HSD.

For immunostaining, paraffin sections of 5 µm were cut from uncultured and cultured explants and from the deep-invasive endometriotic lesions and uterus from endometriosis patients. In addition, sections were prepared from placenta and breast cancer tissue to serve as positive tissue controls. The sections were deparaffinized 2 x 5 min in xylene and 2 x 5 min in 100% ethanol. The endogenous peroxidases were blocked by incubating 30 min in 0.3% H2O2 in methanol. For antigen retrieval, the sections were boiled in Tris–EDTA buffer (pH 9.0) in a microwave oven for 20 min. In the preliminary stainings, a 1:1000 dilution was used for the anti-17β-HSD type 1 pAb and preimmune serum. The Dako-Envision protocol (Dako Diagnostika, Hamburg, Germany) was used to visualize antibody binding. The preimmune serum and pAbs against 17β-HSD type 2 and STS were diluted 1:2000 and 1:1000, respectively. Antibody binding was visualized with the Chemate™ Envision kit (Dako Diagnostika).

After assessing specificity, the immunohistochemistry procedure was further optimized. The conditions used to stain the clinical specimens were the following. The anti-17β-HSD type 1 pAb and preimmune serum were used at 1:4000 and sections were incubated for 1 hr at room temperature. The anti-17β-HSD type 2 pAb and preimmune serum were incubated for 2 hr at room temperature at a dilution of 1:2000. The anti-STS polyclonal antibody and preimmune serum were diluted 1:1000 and sections were incubated overnight at 4°C. Antibody binding was visualized by incubating 30 min with Chemate™ Envision (Dako Dako Diagnostika) and staining with diaminobenzidine solution. The reaction was stopped in water. The sections were briefly counter-stained in haematoxylin, dehydrated and sealed in Entellan (Merck, Whitehouse Station, NJ, USA).

Staining intensity was scored in epithelial and stromal cells separately on a 5-point scale: negative (0); weak (1); weak-moderate (2); moderate (3); moderate-strong (4) and strong (5). In addition, the percentage of positively stained cells within each compartment was assessed.

Statistical tests
Statistical tests were carried out using the Statistical Package for the Social Sciences 11 (SPSS Inc., Chicago, IL) statistical analysis package. To evaluate whether expression levels varied significantly throughout the menstrual cycle, the non-parametric unpaired Mann–Whitney U-test was used to test for differences between these expression levels versus the expression level in the M phase. This was done for both the mRNA and protein data. This test was also used to test for differences in the expression of the estrogenic enzymes in normal endometrium versus eutopic and ectopic endometrium of endometriosis patients. The non-parametric Wilcoxon signed-rank test was used to test for differences between steroid-treated explants and controls at a confidence level of 95%.

Results
Validation of the antibodies against 17β-HSD types 1 and 2 and STS
With immunoblotting, a specific band at the expected molecular weight of 37 kDa was visible in the cytosol of the tumor cell line and cells known to express 17β-HSD type 1, which was not visible with the preimmune serum, and which disappeared after pre-incubation with the 17β-HSD type 1 fusion protein (Fig. 1A). Immunostaining in paraffin sections of breast cancer tissue also disappeared after pre-incubation of the pAb with excess h17HSD fusion protein (Fig. 1B) indicating the specificity of the generated pAb.

Only one of the three pAbs generated against type 2 17β-HSD showed cross reactivity with one of the three peptides (NENGP-GAEELRRTC, peptide A; Fig. 2A). A specific band was also present in the placenta lysate at the expected molecular weight of 43 kDa. The other pAbs only gave a non-specific band in placent al lysates around 75 kDa, which was also seen with the preimmune serum (results not shown). These antibodies also showed strong staining in the syncytiotrophoblast, which could not be displaced by any of the three peptides (Fig. 2B, polyclonal 2). The candidate antibody, however, showed immunostaining...
mostly in the vessels, which was completely abolished by pre-incubation with the immunizing peptides (Fig. 2B, polyclonal 1).

To check specificity of the STS pAb, western blot analysis was performed on purified microsomal steroid sulfatase (arylsulfatase C; Fig. 3A). Besides the expected bands at 63 kDa, other bands (~40 and 50 kDa) were visible as well. A similar pattern was observed when incubated with the preimmune serum (Fig. 3A) and lower dilutions of the pAb (results not shown), and these bands could not be blocked with the immunizing peptides. In lysates of endometrium and placenta tissues also the 63 kDa band is visible (Fig. 3B), however, the 40 and 50 kDa bands were more abundant. Based on the known size of the active enzyme and the post-translational modifications that could occur, it is not possible that the 40 and 50 kDa bands represent STS. In contrast, immunohistochemical staining was completely prevented after pre-incubation of the pAb with the immunizing peptide A, but not peptides B and C (Fig. 3C). This antibody appears therefore to be suitable for immunohistochemistry and not for western blotting.

**Expression of E₂ synthesizing and metabolizing enzymes throughout the menstrual cycle**

The expression of type 2 17β-HSD mRNA increased in normal endometrium during the secretory phase of the menstrual cycle compared with the proliferative phase of the cycle (Fig. 4). Transcript levels increased in the ES phase and remained high throughout the secretory phase. At the protein level, however, 17β-HSD type 2 expression was observed mostly in the glandular epithelium (Fig. 5). Levels peaked during the MS phase, the implantation window (Fig. 6). Little staining was also observed in the stroma, but expression in the stroma did not change throughout the menstrual cycle.

Aromatase gene transcript levels in normal endometrium were near the detection limit, and no cyclical variations were observed.
No expression of 17β-HSD type 1 mRNA was observed (results not shown). Surprisingly, however, prominent expression was observed at the protein level (Figs 5 and 6). These levels did not vary between stages of the cycle (Figs 5 and 6).

Transcript levels of 17β-HSD type 4 and STS were similar to that of 17β-HSD type 2, but the levels did not increase in the secretory phase (results not shown). Expression of STS protein did also not change during the menstrual cycle accept for the LS phase were it was significantly downregulated compared to the menstrual phase (Figs 5 and 6). STS protein was expressed in both epithelial and stromal cells.

EST transcript levels were lower \((P < 0.05)\) than that of 17β-HSD types 2 and 4, but expression levels increased significantly in the secretory phase (Fig. 4). Despite these differences in expression levels, a highly significant correlation was found between the cyclic variations in mRNA expression of 17β-HSD type 2 and EST (correlation 0.79, \(P < 0.0001\)).

**Steroid regulation of \(E_2\) synthesizing and metabolizing enzymes**

The production of aromatase, the 17β-HSD types 1 and 4 and STS mRNA in cultured endometrium explants prepared from M-phase and LP-phase explants was not affected by \(E_2\) or progesterone (data not shown).

The levels of EST and 17β-HSD type 2 gene transcripts were significantly induced by progesterone in both, M-phase and LP-phase explants versus control (Fig. 7). Treatment with \(E_2\) alone had no effect on the 17β-HSD type 2 and EST mRNA expression in M-phase and LP-phase explants. Tissues collected from the LP phase were significantly \((P < 0.05)\) more responsive than M-phase tissue to the action of progesterone for both EST and 17β-HSD type 2.

Steroid regulation at the protein level in these short-term cultures was not confirmed with the semi-quantitative immunohistochemistry.

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**Figure 5:** Representative photographs of 17β-HSD type 1, type 2 and STS protein expression in normal endometrium collected on menstrual cycle days (CD) 11, 17 and 21

**Figure 6:** Expression of 17β-HSD type 1, type 2 and STS protein throughout the menstrual cycle.

The staining intensity and the percentage of cells stained are presented and analysed separately. Data are presented as means ± SEM. *\(P < 0.05\) versus M phase.
E2 synthesizing and metabolizing enzymes in endometriotic tissues

Compared with normal endometrium, transcript levels of 17β-HSD type 1 and aromatase were significantly higher in the endometrium (P < 0.01) and endometriotic lesions (P < 0.01) of endometriosis patients compared with normal endometrium (Fig. 8). Aromatase mRNA levels were found also to be significantly higher (P < 0.05) in the endometriotic lesions compared with the endometrium of endometriosis patients (Fig. 8). The 17β-HSD type 1 protein is expressed in all cells, but staining intensity is significantly lower in epithelial (P < 0.01) and stromal cells (P < 0.01) of the eutopic and ectopic endometrium of the endometriosis patients compared with the normal endometrium (Figs 9a and 10). Transcript levels of 17β-HSD type 2 were significantly lower (P < 0.01) in the endometriotic lesions than in the endometrium of patients and controls. No significant differences

Figure 7: Effects of E2 and progesterone (P) on gene transcript levels of 17β-HSD-2 and EST in M-phase and LP-phase explants. Data are presented as means ± SEM. *P < 0.05 versus control; **P < 0.01 versus M-phase explants.

Figure 8: Gene transcript levels of steroidogenic enzymes in eutopic and ectopic endometrium from endometriosis patients and normal endometrium. Data are presented as means ± SEM. *P < 0.05, **P < 0.01 versus normal endometrium; #P < 0.05; ##P < 0.01 versus eutopic endometrium of the same patient.

Figure 9: Protein expression of 17β-HSD-1 (A), 17β-HSD-2 (B) and STS (C) in eutopic and ectopic endometrium from endometriosis patients and normal endometrium. Staining intensity and the percentage of stained cells are analysed and presented separately. Data are presented as means ± SEM. EP, epithelium; S, stroma. *P < 0.05; **P < 0.01 versus normal endometrium; #P < 0.05 versus eutopic endometrium of the same patient.
In this study, we have investigated the expression of six steroidogenic enzymes, including 17β-HSD type 1, type 2, and steroidal 17β-hydroxysteroid dehydrogenase (17β-HSD) type 1 and type 4, as well as steroidogenic acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase 3 (3β-HSD) in eutopic and endometriotic tissue when compared with normal endometrium. We found that the expression of these enzymes was significantly different between normal endometrium and endometriosis patients. Specifically, we observed that the 17β-HSD type 2 protein was expressed mostly in epithelial cells of the lesions and the normal endometrium. No difference in STS protein levels was observed between eutopic and endometriotic tissue when compared with normal endometrium (Fig. 9C).

The mRNA levels of 17β-HSD type 4 were significantly lower in the lesions and endometrium of endometriosis patients compared with the normal endometrium (Fig. 8). The 17β-HSD type 2 protein is expressed mostly in epithelial cells. However, the number of positive stromal cells was slightly increased (P < 0.05) in the eutopic endometrium of patients, whereas staining intensity was significantly reduced (P < 0.05) in stromal cells of the lesions compared with the normal endometrium (Figs 9b and 10).

The mRNA levels of 17β-HSD type 2 were found to be significantly increased in the eutopic endometrium of patients (Fig. 8). The 17β-HSD type 2 protein is expressed mostly in epithelial cells. However, the number of positive stromal cells was slightly increased (P < 0.05) in the eutopic endometrium of patients, whereas staining intensity was significantly reduced (P < 0.05) in stromal cells of the lesions compared with the normal endometrium (Figs 9b and 10).

No differences were observed between the STS mRNA levels in eutopic and endometriotic tissues of patients without endometriosis and endometrium of patients with endometriosis (Fig. 8). STS protein was expressed both by stroma and epithelium. STS protein levels were significantly lower (P < 0.05) in epithelial cells of endometriosis patients compared with those of normal endometrium (Fig. 9c). No difference in STS protein levels was observed between epithelial cells of the lesions and the normal endometrium (Fig. 9C).

EST mRNA levels were significantly higher (P < 0.05) in endometriotic tissue when compared with normal endometrium (Fig. 8).

**Discussion**

In this study, we have investigated the expression of six enzymes involved in the synthesis and inactivation of E2 in eutopic and endometriotic tissue of patients with deep-infiltrative endometriosis. Three pAb were generated to study types 1 and 2 17β-HSD and STS protein expression in the tissues. We show that there are parallels between deep-infiltrative endometriosis and the reports on adenomyosis, ovarian and peritoneal endometriosis, with regard to the expression of steroidogenic enzymes, but we also report some novel findings.

With regard to the E2 synthesizing enzymes, we found very low levels of aromatase and 17β-HSD type 1 mRNA in normal human endometrium, which is in line with earlier reports (Bulun et al., 1993; Matsuzaki et al., 2006). Aromatase and 17β-HSD type 1 expression was not responsive to E2 and progesterone in the explant cultures. This is in contrast with the findings of Tseng (1984a) who showed that aromatase activity in isolated endometrial stromal cells was induced up to 40-fold by progesterone, which was further enhanced 20–100-fold by E2. Whether this is due to the fact that in the tissue context, estrogen metabolism may be different than in isolated cell compartments, or that prolonged exposure is needed to induce aromatase expression remains to be elucidated.

In contrast to the low levels of 17β-HSD type 1 mRNA in the normal human endometrium, we did however detect 17β-HSD type 1 protein. The fact that we were unable to detect 17β-HSD type 1 mRNA transcripts in the endometrium of disease-free women, suggests that these transcripts either degraded quickly or are synthesized in lower quantities. Reports on the expression of 17β-HSD type 1 in normal endometrium are conflicting. Miettinen et al. (1996) reported low expression of 17β-HSD type 1 mRNA in endometrium, whereas Casey et al. (1994) found no expression of 17β-HSD type 1 mRNA in normal human endometrium, and Utsunomiya et al. (2001) found no 17β-HSD type 1 protein expression in normal endometrium. In this study, we used a new pAb and we clearly demonstrated expression of type 1 17β-HSD in normal endometrium. Steroidal regulation, however, was not observed in the explant cultures. The presence of type 1 17β-HSD protein agrees with the observed E2 conversion into estrone in normal proliferative endometrium (Delvoux et al., 2007).

Aromatase and type 1 17β-HSD mRNA levels were higher in eutopic and endometriotic tissue of patients with endometriosis than in the endometrium of controls, which is in agreement with the findings of others (Noble et al., 1996; Matsuzaki et al., 2006). Surprisingly, however, 17β-HSD type 1 protein levels were lower in epithelial and stromal cells of endometrium and lesions of endometriosis patients compared with the normal endometrium. For 17β-HSD type 1 mRNA, two isoforms have been described, 1.3 and 2.3 kb (Miettinen et al., 1996). Only the 1.3 kb mRNA is correlated with 17β-HSD type 1 protein expression and activity (Miettinen et al., 1996). In normal human endometrium, the 1.3 kb 17β-HSD type 1 mRNA expression is low in normal human endometrium, but the 2.3 kb mRNA is abundantly present (Miettinen et al., 1996). Expression of the type 1 17β-HSD gene may therefore not necessarily reflect changes at the functional protein level. The type 1 17β-HSD is still considered a rate-limiting enzyme in the production of E2, since the major product of the aromatase activity in humans is estrone and not E2 (Simpson et al., 1994), and is therefore still an interesting alternative target for therapy.
The endometrium is also a source of drug metabolizing enzymes usually present in the liver. Reddy et al. (1981) showed that human endometrial tissue was able to metabolize \( \text{E}_2 \) into the catecholestrogens 2-hydroxy- and 4-hydroxyestradiol.
It has now been shown that the human endometrium expresses CYP3A4, known to convert estrone into 16α-hydroxyestrone and E2 into 2-hydroxyestradiol (Hukkanen et al., 1998), and CYP1B1, known to convert E2 into 4-hydroxyestradiol (Tsuchiya et al., 2004). Furthermore, the human endometrium expresses catechol-O-methyltransferase, which converts 2-hydroxy- and 4-
hydroxyestradiol into 2- and 4-methoxyestradiol (Salih et al., 2007). Another group of potential E2-inactivating enzymes was also identified in the endometrium, the uridine diphospho-
glucuronosyltransferases (UGTs) (Lepine et al., 2004). The endo-
metrium appears to be a rich source of UGTs, which are able to
conjugate both estrogens and catecholestrogens on different po-
sitions. However, their relevance with regard to the development of endometrial pathologies is not known.

Summary
We confirm that aromatase and 17β-HSD type 1 expression is higher in deep-invasive endometriosis than in eutopic and normal endometrium. Even though no 17β-HSD type 1 gene transcripts were detected in normal endometrium, we did observe a clear expression of the 17β-HSD type 1 protein, which was not elevated in the ectopic endometrium. Expression of the STS was prominent at both the mRNA and protein level, but was not elevated in endometriotic tissues. Type 2 17β-HSD gene transcript levels were lower in deep-invasive endometriosis. Type 2 17β-HSD protein levels, however, were not lower in lesion compared with the eutopic or normal endometrium. We show for the first time that the E2-inactivating enzyme 17β-HSD type 4 is expressed in deep-invasive endometriosis, and that it is also down-regulated in endometriosis. Together with the continuous pre-

sence of aromatase and the substantial levels of STS, this may result in an increase in the local production of E2 in the endometriotic lesions. Inventarization of the net metabolic con-
versions in the whole endometriotic tissue, would aid in the selection of the appropriate combination of enzyme inhibitors to optimize medical therapy of endometriosis.

In normal endometrium both the 17β-HSD type 2 and EST were responsive to progesterone. The fact that in deep-infil-
tration endometriosis only the expression of type 2 17β-HSD was suppressed and not that of EST, suggests local and selective dysregulation of type 2 17β-HSD expression.

Funding
This study was funded by Solvay Pharmaceuticals Research Laboratories, Hannover, Germany.

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