Androgen receptor and 5α-reductase are expressed in pelvic endometriosis

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The aim of this study was to evaluate whether androgen receptor (AR) and the enzymes that convert testosterone into the more potent androgen dihydrotestosterone, 5α-reductases (5α-R1 and 5α-R2) are expressed in pelvic endometriosis. The study involved 21 infertile women who underwent laparoscopy and were divided into two groups: control (n = 13) and endometriosis (n = 8) according to the histological and laparoscopic findings. Endometrial and endometriotic implant biopsies were performed. By reverse transcription polymerase chain reaction and immunohistochemistry, AR, 5α-R1 and 5α-R2 messenger RNA and protein were detected in biopsies of pelvic endometriosis, as well as in the eutopic endometrium of both groups. These findings suggest that active androgens may be formed within the endometriotic tissue and that both local and systemic androgens have the potential to act on endometriotic cells.

Keywords 5α-reductase, androgen receptor, androgens, endometriosis, gene expression.

The role of androgens in uterine physiology has not been established yet. Androgen receptor (AR) has been identified in the human endometrium, but its location and regulation are still controversial. Androgens appear to counteract estrogen effects on cellular proliferation and also appear to play a role in the mechanisms of endometrial physiology and disease. However, the local regulation and expression of the enzymes responsible for androgen metabolism in human endometrium is poorly understood. For instance, normal and cancerous endometrium seem to express two isoforms of 5α-reductase, an enzyme responsible for the conversion of testosterone into dihydrotestosterone (DHT), the most potent androgen. Whether this enzyme is expressed in endometriosis has not been reported so far.

In the light of the above evidence, the aim of the present study was to investigate the gene expression and protein localisation of AR and 5α-reductases type 1 (5α-R1) and type 2 (5α-R2) in the eutopic and ectopic endometrial tissues of women with endometriosis. Due to the need of laparoscopic confirmation of either presence or absence of endometriosis and to avoid the interference of cyclic changes in androgen metabolism, the study was focused on infertile women on the follicular phase of menstrual cycle.

Methods

The study population consisted of women undergoing complete diagnostic workup for couple infertility, seen consecutively in a tertiary care university hospital. Inclusion criteria were (i) the need of diagnostic laparoscopy and (ii) no use of hormonal medication in the previous 3 months. Women with tubal obstruction and pelvic adhesion were not excluded, but none of the participants had uterine fibroids, adenomyosis or...
active pelvic inflammatory disease. The study protocol was approved by the local Ethics Committee, and the written informed consent was obtained from each woman.

The study enrolled 21 women with ages ranging from 18 to 43 years who were divided into two groups: a control group, without any clinical or laparoscopic evidence of endometriosis (n = 13) and a group with confirmed pelvic endometriosis (n = 8), comprising ovarian (n = 4) and extraovarian (n = 4) disease. The control and endometriosis groups were similar by age (mean age 33 and 32 years, respectively), cycle stage (median cycle day 6th versus 7th day), cycle length (28.6 versus 28.5 days), body mass index (24 versus 22 kg/m²) and hip circumference (77 versus 73 cm).

Blood samples were drawn after an overnight fast from an antecubital vein for determination of serum testosterone and sex hormone binding globulin (SHBG) concentrations, which were in the normal range and were similar in both the control and endometriosis groups (mean serum testosterone 0.64 versus 0.52 ng/ml; SHBG 47 versus 43 nmol/l).

Women underwent laparoscopy on the first half of the menstrual cycle when eutopic endometrium was obtained from both groups by aspirative biopsy and samples of the endometriotic lesions were collected contemporaneous. The laparoscopic evaluation was always performed by the same surgical team, composed of three experienced surgeons, including one of the authors (M.M.C.). In the endometriosis group, all women had stage III or IV according to the revised American Society of Reproductive Medicine classification of endometriosis. Diagnosis was confirmed by histological examination of the endometriotic lesions. Only one implant per woman was tested, and standardisation was achieved by sampling the largest one. The endometrial samples were dated histologically using Noyes criteria as corresponding to cycle days 4–15 and 4–14 in the control and in the endometriosis groups, respectively.

Fresh tissue samples were divided into two portions: one was immediately frozen in liquid nitrogen and stored at –80°C until messenger RNA (mRNA) extraction, and the other was fixed in 10% formalin for histological diagnosis and immunohistochemistry.

**RNA extraction and reverse transcription polymerase chain reaction**

Total RNA extraction and complementary DNA (cDNA) synthesis were carried out using phenol–guanidine isothiocyanate and the Superscript First-Strand System Synthesis for reverse transcription polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The primers used for polymerase chain reaction (PCR) reactions (5’ to 3’) were: AR (GenBank locus AH002624), sense GCA TGG TGA GCA GAG TGC CCT ATC, antisense TCC CAG AGT CAT CCC TGC TTC AT, fragment size = 365 base pairs (bp); 5α-R1 (GenBank locus BC00673), sense TGG CGC TTC TCT ATG GAC TT, antisense GGA AGC AAC ACT GCA GTT GA, fragment size = 369 bp and 5α-R2 (GenBank locus NM000348), sense TAC TTC TGG GCC TCT TCT GCC, antisense TTT CAT CAG CAT TGT GGG AGC, fragment size = 567 bp. In addition, a 659 bp fragment of β2-microglobulin gene sequence was amplified to control for cDNA quality in each sample. The β2-microglobulin primers (GenBank locus NM000408) were: sense ATC CAG CGT ACT CCA AAG ATT CAG and antisense AAA TTG GAA GTT AAC TTA TGC ACC C.

The primers were designed to span intron–exon borders so as to prevent amplification of any contaminating genomic DNA. Computer analysis performed to compare the synthesised primers with the human sequences in the gene database of the National Center for Biotechnology Information using BLAST showed no significant homology with all other genes. Sequence homology among the different primers used in the present study was also avoided, excluding possible cross-reactions.

For optimisation of the PCR reactions, known positive cDNA samples were amplified for 20–45 cycles, and the experiments were performed within the linear range. Final PCR conditions were as follows: 35 cycles (1 minutes 94°C, 1 minutes 55°C, 1 minutes 72°C, 5 minutes 72°C) for AR, 35 cycles (45 seconds 72°C, 45 seconds 60°C, 90 seconds 72°C, 10 minutes 72°C) for 5α-R1 and 5α-R2 and 30 cycles (1 minutes 94°C, 1 minutes 55°C, 1 minutes 72°C, 5 minutes 72°C) for β2-microglobulin. cDNA from dissociated cells of human prostate gland was used as a positive control for all PCR reactions. Negative controls were the products of first-strand synthesis reaction carried out in the absence of RT.

The products were subjected to electrophoresis on 1.5% agarose gel stained with ethidium bromide, photographed under ultraviolet light and the optical density of the bands was measured by an image-processing system (ImageMaster VDS, Pharmacia Biotech, Uppsala, Sweden). The samples in which a band could not be detected by the densitometer were considered to have a negative result.

**Immunohistochemistry**

Immunohistochemistry was performed by the avidin–biotin–peroxidase technique using the Vectastain® Universal Elite ABC kit (Vector Laboratories, Burlingame, CA, USA), which contains a secondary antibody made in goat that recognises rabbit and mouse primary antibodies. Samples were fixed with a 10% paraformaldehyde solution, paraffin embedded and cut into 4 μm sections. The sections were then dewaxed in xylene and rehydrated in decreasing concentrations of ethanol and water. Antigen retrieval was enhanced by boiling the slides for 10 minutes in 0.01 M citrate buffer, pH 6.0, followed by incubation at room temperature and phosphate buffered saline (PBS) washing. After exposure to 1% H2O2 in methanol to block endogenous peroxidase, sections were treated with...
normal goat serum for 30 minutes to suppress nonspecific binding.

The primary antibodies used in the study were: anti-5α-R1 (sc-20658; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a rabbit polyclonal antibody raised to a recombinant protein corresponding to amino acids 61–165 mapping near the amino terminus of 5α-R1 of human origin; anti-5α-R2 (sc-20659; Santa Cruz Biotechnology), a rabbit polyclonal antibody raised against amino acids 61–160 of human 5α-R2 and anti-AR (Ab-3; Oncogene Research Products, San Diego, CA, USA), a mouse monoclonal antibody raised to a recombinant protein containing amino acids 331–572 of human AR. The primary antibodies were diluted 1:50 (AR and 5α-R1) or 1:10 (5α-R2) and incubated with the sections for 24 hours at 4°C.

Sections were treated with biotinylated secondary antibody solution (1:100) for 30 minutes at room temperature. After washing with PBS, ABC reagent was added for more 30 minutes, followed by a new wash in PBS. After the peroxidase developing with 3,3′-diaminobenzidine/hydrogen peroxide, sections were counterstained with haematoxylin, dehydrated and mounted. Positive controls were specimens of prostate for AR and breast cancer for 5α-R1. Negative controls were endometrial samples in which the primary antibody was replaced by nonimmune serum or by nonspecific rabbit immunoglobulin G (Oncogene Research Products).

**Results**

Figure 1A shows representative products of RT-PCR for AR, 5α-R1, 5α-R2 and β2-microglobulin gene expression. Only two samples of eutopic endometrium from the endometriosis group were not suitable for RT-PCR analysis. The specific fragment corresponding to AR was amplified in all but one endometriotic sample and in all samples of eutopic endometrium. The median ratio between AR and β2-microglobulin densitometric units was 0.8, 0.6 and 0.7 in the control, eutopic and ectopic endometrial samples, respectively. There was no correlation between serum testosterone levels and the endometrial expression of AR (Spearman’s correlation coefficient = −0.379, \( P = 0.147 \)).

The cDNA fragment corresponding to 5α-R1 was detected in 10/13 endometrial samples of the control group, in 5/6 samples of eutopic endometrium from women with endometriosis and in all samples of endometriotic tissue. The median ratio between 5α-R1 and β2-microglobulin densitometric units was 0.7, 0.6 and 0.6 in the control, eutopic and ectopic endometrial samples, respectively. 5α-R2 transcript was detectable in only one sample of control endometrium and in two samples of eutopic endometrium and in two samples of endometriotic tissues (Figure 1A). Neither 5α-R1 nor 5α-R2 expression in the endometrium correlated with serum testosterone levels (\( r = −0.037 \) and 0.231, respectively).

The specificity of the PCR products was confirmed by the expected molecular weight and sequencing of the amplified fragments and also by absence of amplification of the negative control (Figure 1A). The β2-microglobulin bands were equally expressed in the endometrial tissue of cases and controls, as well as in endometriotic tissue. The implants which did not show AR, 5α-R1 or 5α-R2 gene expression were confirmed to be negative by repeating the PCR, while their cDNA integrity was confirmed by detecting β2-microglobulin product.

Protein localisation by immunohistochemistry showed nuclear staining corresponding to AR both in the stroma and in the glandular epithelium of eutopic endometrium of both groups, as well as in endometriotic implants (Figure 1B). While the pattern of AR localisation was not disrupted in endometriotic lesions compared with normal and eutopic endometrium, the intensity of AR immunostaining in endometriosis tissue was variable, ranging from absent to strong (the later is shown in Figure 1B). 5α-R1 immunostaining was localised to the cytoplasm of glandular and stromal cells of eutopic endometrium and endometriosis. The intensity of 5α-R1 immunostaining was variable between samples but not particularly different between endometriosis and normal endometrium (Figure 1B). The localisation of 5α-R2 was cytoplasmic in both glands and stroma, with lesser intensity than 5α-R1 and the same aspect in control, eutopic and ectopic endometrium. Negative controls, as expected, did not stain (Figure 1B).

**Discussion**

To our knowledge, this is the first study to investigate 5α-R1 and 5α-R2 gene expression in endometriosis. While the proteins were present in both endometriotic glands and circumjacent stroma, 5α-R1 gene transcripts were clearly detectable in most of the biopsies evaluated. The presence of 5α-R1 mRNA and protein suggests a possible role for this enzyme in the regulation of androgen metabolism in normal endometrium and endometriosis. This role might be related to the conversion of testosterone to DHT, which takes place inside the target cell and leads to a more potent stimulation of AR.

Since androgens have antiproliferative effect on cells of endometrial origin,6 the expression of 5α-R1 in endometriotic lesions may be viewed as a potential regulatory mechanism limiting the implant growth. This interpretation is also coherent with the same enzyme being expressed in endometrioid endometrial cancer.7 The apparent paradox that endometrial cancer is associated with increased androgen levels is more likely to be explained by an increase in estrogens derived from androgen aromatisation and unopposed by progesterone, as seen in postmenopausal women.7

In the present study, we have observed that AR mRNA and protein are expressed in human endometrium and endometriosis.

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Androgen receptor and 5α-reductase in endometriosis

The receptor was localised in the nuclei of glandular epithelial cells, as well as in stromal cells. The presence of AR in both eutopic and ectopic endometrium suggests a possible but yet unrevealed role for androgens in the pathophysiology of endometriosis. Androgens are known to counteract estrogen proliferative action on the endometrium, and thus, the lack of AR could favour endometriotic implant growth. However, in this study, we have demonstrated AR transcription in seven of eight specimens of endometriotic tissue examined, suggesting that pelvic endometriosis lesions are predominantly positive to AR.

Taken together, these findings suggest that active androgens may be formed within the endometriotic tissue and that both local and systemic androgens have the potential to act on endometriotic cells. This may explain why danazol effectively inhibits human endometrial cell growth in vitro, whereas gestrinone, being a less androgenic compound, does not. This might also support the clinical use of androgenic agents, such as danazol, in local delivery systems such as intrauterine systems and vaginal preparations designed to treat pelvic endometriosis. The finding of variable levels of expression of AR, 5α-R1 and 5α-R2 suggests that the effects of androgens on the endometrium may be heterogeneous and that some women may have less endometrial sensitivity to androgens. Hence, these molecules could be assessed when planning individualised hormone therapies for endometriosis if they are validated in future studies as useful prognostic markers.

**Conclusion**

Endometriotic lesions of women with stage III or IV disease express AR, 5α-R1 and 5α-R2, and thus are potentially capable of responding to androgens.
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