Steroid receptors ERα, ERβ, PR-A and PR-B are differentially expressed in normal and atrophic human endometrium

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Summary. Objectives: The endometrium expresses estrogen (ER) and progesterone receptor (PR), which are related to autocrine and paracrine processes that respond to estrogen and progesterone. Therefore, the aim of this study was to evaluate the distribution pattern of ERα, ERβ, PR-A and PR-B with monoclonal antibodies in normal human endometrial tissue. Study Design: Human endometrial tissue was obtained from 84 premenopausal and 11 postmenopausal patients and immunohistochemically analysed with monoclonal antibodies against ERα, ERß, PR-A and PR-B. Results: ERα, PR-A and PR-B declined significantly (p<0.001, p<0.05, p<0.05 respectively) in glandular epithelium from proliferative to late secretory phase. The ERß immunohistochemical reaction showed a similar significant declining pattern (p<0.05), although the staining intensity was lower than that of ERα. While ERα, ERß and PR-B decrease significantly in atrophic endometrial tissue compared to proliferative endometrium, a significant up-regulation of PR-A was observed compared to late secretory phase (p<0.05). Conclusion: ERα, ERß, PR-A and PR-B were expressed in normal human endometrium with a cyclical variation during the menstrual cycle. In normal postmenopausal endometrial tissue, a down-regulation of ERα, ERß and PR-B occurs with a subsequent higher expression of PR-A. These results show the presence of steroid receptors in human epithelium, indicating that these cells respond to estrogen and progesterone, thus playing a significant role in endometrial physiology.

Key words: Endometrium, Immunohistochemistry, Estrogen receptor alpha (ERα), Estrogen receptor beta (ERß) progesterone receptor A (PR-A), Progesterone receptor B (PR-B)

Introduction

The endometrium is one of the most dynamic tissues in humans, undergoing proliferative and secretory changes that are thought to be primarily controlled by the ovarian steroid hormones estrogen and progesterone. These hormones in human endometrium are thought to act through the estrogen receptor (ER) and progesterone receptor (PR) (Snijders et al., 1992). In several studies both receptors were analysed in paraffin-fixed normal human endometrial tissue (Garcia et al., 1988; Lessey et al., 1988; Snijders et al., 1992; Jones et al., 1995; Mylonas et al., 2004a, 2005) and isolated glandular epithelial cells (Mylonas et al., 2000) using immunohistochemical assays.

For several years it was generally believed that only single ER and PR receptors existed. However, the discovery of a new ER with specificity for estrogens has created new insights in the estrogen signalling system (Kuiper et al., 1998). The novel receptor ERß is highly homologous to the classical ER (ERα), which can bind estradiol with a high affinity and stimulate transcription of an ER target gene (Kuiper et al., 1997). Additionally, PR is expressed as two major isoforms, PR-A and PR-B, which arise from alternative transcriptional starting sites within the same gene. PR-A encodes 769 amino acids and lacks the first 164 amino acids of the N-terminus of the PR-B (933 amino acids) (Horwitz et al., 1983; Krett et al., 1988; Graham et al., 1995; Graham and Clarke, 2002). Although PR-A and PR-B are expressed as two major isoforms, PR-A and PR-B, which arise from alternative transcriptional starting sites within the same gene. PR-A encodes 769 amino acids and lacks the first 164 amino acids of the N-terminus of the PR-B (933 amino acids) (Horwitz et al., 1983; Krett et al., 1988; Graham et al., 1995; Graham and Clarke, 2002). Although PR-A and PR-B were thought to occur in similar amounts, it is now clear that they are differentially expressed and thus have distinct functions in several human tissues, including human endometrium (Graham et al., 1995; Leslie et al., 1997; Kumar et al., 1998; Graham and Clarke 2002).

Although ER and PR can be measured in paraffin-fixed normal human endometrial tissue (Garcia et al., 1988; Lessey et al., 1988; Snijders et al., 1992; Jones et al., 1995; Mylonas et al., 2004a, 2005), there were several difficulties in the visualisation of ERß and PR-B.
due to the lack of appropriate monoclonal antibodies. Recently, the immunohistochemical expression of ERβ in human endometrium was demonstrated using polyclonal (Lecce et al., 2001) and monoclonal antibodies (Mylonas et al., 2004a, 2005; Taylor et al., 2005). However, several conflicting data and issues regarding the expression of ERβ in human endometrial tissue still remain. For the PR, it is now clear that commercially available antibodies against PR might not have equal affinity to PR-A and PR-B, especially using immunohistochemistry. Since PR-B is not recognized by many used antibodies, the possibility of an underreporting PR-B expression should be considered (Mote et al., 2001).

Therefore, the aims of this study were the evaluation of the distribution patterns of ERα, ERβ and PR-A and PR-B using recently available commercial monoclonal antibodies in normal human pre- and postmenopausal endometrial tissue.

Materials and methods

Tissue samples

Samples of normal human endometrium were obtained from 84 premenopausal, non-pregnant patients undergoing gynaecological surgery either by D&C or hysterectomy for benign diseases. All pathological endometria were excluded from this study. Endometrium samples were classified according to anamnestical and histological dating (Dallenbach-Hellweg and Poulsen, 1985; Kurman 2002) into proliferative (day 1-14, n=46), early secretory (day 15-22, n=18) and late secretory phase (day 23-28, n =20) as previously described (Jones et al., 1995; Mylonas et al., 2000). Additionally, eleven (n=11) samples of atrophic human endometrial tissue from postmenopausal patients with no hormonal substitute that underwent hysterectomy or D&C for benign diseases, mainly urogenitary prolaps, were analysed.

Immunohistochemistry

Immunohistochemistry was performed using a combination of microwave-oven heating and the standard streptavidin-biotin-peroxidase complex using the mouse-IgG-Vectastain Elite ABC kit (Vector Laboratories, Burlingame, California, USA) as previously described (Mylonas et al., 2004a, 2005). Mouse monoclonal antibodies used for the experiments are listed in Table 1. For positive controls sections of human breast cancer tissue and normal colon were used, while human ileum served as negative control tissue.

Briefly, paraffin-fixed tissue sections were dewaxed using xylol for 15min, rehydrated in an alcohol row, and subjected to antigen retrieval on a high setting for 10min in a pressure cooker in sodium citrate buffer (pH 6.0), containing citrate acid 0.1M and sodium citrate 0.1M in distillate water. After cooling, the slides were washed twice in PBS. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 20 min. Non-specific binding of the primary antibodies was blocked by incubating the sections with diluted normal serum (10ml PBS with 150µl horse serum, provided by Vectastain Elite ABC kit) for 20min at room temperature. Sections were then incubated at room temperature for 60min with the primary antibodies. ERα and PR-A were diluted in dilution-medium (Dako, Glostrup, Denmark), while ERβ and PR-B were diluted in PBS. After washing with PBS, the slides were incubated in diluted biotinylated serum for another 30 min at room temperature (10 ml PBS, 50µl horse serum, provided by Vectastain Elite ABC kit). After incubation with the avidin-biotin peroxidase complex (diluted in 10ml PBS, reagent ABC provided by Vectastain Elite ABC kit) for another 30 min and a repeated washing step with PBS, visualisation was performed with substrate and chromagen 3,3’-diaminobenzidine (DAB; Dako, Glostrup, Denmark) for 8-10min. The slides where counterstained further with Mayer’s acidic hematoxylin and washed in an alcohol multiple-row (50-98%). After xylol treatment the slides were covered. Negative controls were performed by replacing the primary antibody with normal mouse serum. Positive controls for ERα, PR-A and PR-B include human invasive breast cancer. The ERβ antibody was tested positive on human colon tissue. Positive cells showed a brownish colour and negative controls as well as unstained cells were blue.

Evaluation and statistical analysis

The intensity and distribution of the specific

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>ERα</td>
<td>1D5</td>
<td>mouse IgG1</td>
<td>1:150</td>
<td>Immunotech, Hamburg, Germany</td>
</tr>
<tr>
<td>ERβ</td>
<td>PPG5/10</td>
<td>mouse IgG2a</td>
<td>1:50</td>
<td>Serotec, Oxford, United Kingdom</td>
</tr>
<tr>
<td>PR-A</td>
<td>10A9</td>
<td>mouse IgG2a</td>
<td>1:50</td>
<td>Immunotech, Hamburg, Germany</td>
</tr>
<tr>
<td>PR-B</td>
<td>SAN27</td>
<td>mouse IgG1</td>
<td>1:50</td>
<td>NovoCastra, Newcastle, United Kingdom</td>
</tr>
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ER: estrogen receptor, PR: progesterone receptor.
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Immunohistochemical staining reaction was evaluated using a semi-quantitative method (IRS-score) as previously described (Remmelt and Stegner, 1987) and previously used in the evaluation of inhibin/activin subunits (Mylonas et al., 2004b, 2006), CA-125 (Mylonas et al., 2003b) as well as endometrial steroid receptor expression (Mylonas et al., 2000, 2004a, 2005). The slides were examined by two independent observers, including a gynaecological pathologist (N.S.). Since stromal cells expressed also these steroid receptors but with a very variable intensity, only glandular epithelial cells were evaluated for statistical analysis. At least 3 representative fields between the endometrial basalis layer and luminal epithelium were selected and approximately 200 cells of these areas were counted. In 32 cases of the 369 evaluated slides (8.67%) there was a discrepancy between the two observers and these slides were again evaluated by both together. The level of agreement between the two observers was 91.33%.

The IRS score was calculated as follows: IRS=SIxPP, where SI is the optical stain intensity (graded as 0 = no, 1 = weak, 2 = moderate, and 3 = strong staining) and PP the percentage of positive stained cells. The PP was estimated by counting out approximately 200 cells and it was defined as 0 = no staining, 1 = <10%, 2 = 11-50%, 3 = 51-80%, and 4 = >81%. The Mann-Whitney rank-sum test was used to compare the means of the different IRS scores (SPSS, Chicago, IL, USA). Significance was assumed at p<0.05.

Results

Both glandular epithelial and stromal cells demonstrated nuclear immunoreactivity for ERα, ERβ, PR-A and PR-B, confirming previous results (Garcia et al., 1988; Lessey et al., 1988; Snijders et al., 1992; Jones et al., 1995; Mylonas et al., 2004a, 2005; Leslie et al., 2005). While the staining reaction of ERα in biopsies in the proliferative phase was intense (Fig. 1a), it decreased continuously from early (Fig. 1b) till late secretory phase (Fig. 1c) confirming previous results (Garcia et al., 1988; Snijders et al., 1992; Jones et al., 1995; Mylonas et al., 2004a, 2005). Atrophic endometrial tissue also expressed ERα, but with a lower intensity than proliferative endometrial tissue (Fig. 1d). ERβ immunostaining patterns were also expressed in endometrial glandular and stromal cells. However, ERβ immunostaining intensity was lower compared to the ERα staining reaction. The intensity of positive reacted epithelial cells was higher in the proliferative phase (Fig. 1e) decreasing in early (Fig. 1f) and in late secretory phase (Fig. 1g) during the menstrual cycle. ERβ was also expressed in atrophic endometrium with the weakest immunolabelling intensity compared to premenopausal endometrial tissue (Fig. 1h). Interestingly, stromal cells expressed immunohistochemically both ER with a variable intensity.

The PR-A immunohistochemical reaction showed a similar declining immunolabelling between proliferative (Fig. 1i) and early secretory phase (Fig. 1j) compared to late secretory phase (Fig. 1k), while it was also expressed in stromal cells. Atrophic endometrium showed also a PR-A immunolabelling being more intense compared to late secretory phase (Fig. 1l). PR-B was also demonstrated in both premenopausal and postmenopausal endometrial tissue. While the immunolabelling was intense during proliferative (Fig. 1m) and early secretory phase (Fig. 1n), decreasing during late secretory phase (Fig. 1o) with the ongoing of the menstrual cycle, reaching a weak immunostaining in atrophic endometrium (Fig. 1p). Interestingly, stromal cells also expressed the PR-B, but with a variable pattern. The immunolabelling intensity for both PR receptors was, in contrast to ER, similar.

The IRS score for the ERαx declined significantly (p<0.001) in the glandular epithelium from proliferative to late secretory phase. The intensity score for ERβ also declined in the glandular epithelium from proliferative to early (p<0.05) and late secretory phase (p<0.05) (Fig. 2). The PR-A expression also decreased significantly between proliferative and late secretory phase (p<0.001) and early secretory and late secretory phase (p<0.05). PR-B showed a similar decline between proliferative and early secretory compared to late secretory phase (p<0.05 each) (Fig. 2).

Atrophic tissue demonstrated the lowest immunolabelling expression for ERβ and PR-B compared to premenopausal endometrium. The immunostaining for ERα was similar to late secretory phase. Interestingly, the PR-A expression was significantly higher in atrophic endometrium compared to late secretory phase (p<0.05). The IRS score showed a significant decline from proliferative phase to atrophic endometrium for ERα (p<0.05), ERβ (p<0.01) and PR-B (p<0.05). Additionally, PR-B was also significantly

Fig. 1a-p. Immunohistochemical expression of ERα, ERβ and PR-A, PR-B. While the staining reaction of ERα in the proliferative phase was intense (a), it decreased continuously from early (b) till late secretory phase (c). Atrophic endometrial tissue expressed ERα with a lower intensity than proliferative endometrial tissue (d). The intensity of positive reacted epithelial cells against ERβ antibody was higher in the proliferative phase (e) decreasing in early (f) and in late secretory phase (g) during the menstrual cycle. ERβ was also expressed in atrophic endometrium with the weakest immunolabelling intensity compared to premenopausal endometrial tissue (h). The PR-A immunohistochemical reaction showed a similar declining immunolabelling between proliferative (i) and early secretory phase (j) compared to late secretory phase (k), while it was also expressed in stromal cells. Atrophic endometrium also showed also a PR-A immunolabelling being more intense compared to late secretory phase (l). While the immunolabelling of PR-B was intense during the proliferative (m) and early secretory phase (n), it decreased during late secretory phase (o), reaching a weak immunostaining in atrophic endometrium (p). Interestingly, stromal cells also expressed the PR-B, but with a variable pattern. a-p, x 250
higher during early secretory phase than atrophic tissue (p<0.05) (Fig. 2).

Discussion

The endometrium is one principal target tissue of the pituitary-gonadal axis, but has also been recognized as an endocrine organ itself. The knowledge of the distribution pattern of steroid receptors might contribute to the understanding of steroid hormone function in human endometrium. Additionally, the newly detected steroid receptors ERß and PR-B and their characterisation in human endometrial tissue might elucidate several “paradox” findings that cannot be explained with the classical two receptor explanation. As a characteristic example of such contradictory findings of the antiestrogenic and partially estrogenic effect of tamoxifen (an antiestrogen widely used in the treatment of breast cancer) should be mentioned. Meanwhile, it is believed that tamoxifen exerts its antiestrogenic effects through the ERα, while its estrogenic potential is mediated through ERß (Kuiper et al., 1997; Peach et al., 1997; Mylonas et al., 2003a).

We demonstrated the expression of ERα, ERß, PR-A and PR-B in normal human endometrial tissue. However, ERß immunolabelling is less intense than that of ERα. There have been several studies measuring ER and PR in the human endometrium using immunohistochemical assays. Our results confirm the cyclical variations of ER and PR as described by several authors (Garcia et al., 1988; Snijders et al., 1992; Jones et al., 1995; Mylonas et al., 2004a, 2005). These immunohistochemical results show the presence of steroid receptors in human epithelium, indicating that these cells respond to estrogen and progesterone (Snijders et al., 1992). ER concentrations are maximal in the proliferative phase and decline in both the glandular and stromal compartment in the secretory phase of the cycle (Fujishita et al., 1997). The ER expression and distribution pattern might play an important role in normal endometrial function and pathogenesis and the expression and relationship of these steroid receptors could be of essential clinical implications. The presence and distribution pattern of ERß in endometrial epithelial cells confirms several previous reports (Rey et al., 1998; Matsuzaki et al., 1999; Lecce et al., 2001; Mylonas et al., 2004a, 2005) but not with the results of some other research groups (Critchley et al., 2002; Taylor et al., 2005). Our results indicate that glandular ERß expression predominantly occurs during the proliferative phase, declining as the menstrual phase continues. Differences in tissue distribution of ERα and ERß during the menstrual cycle suggest a substantial role in the modulation and function of estrogen activity in human endometrial tissue. ERß could have a different action and activity compared to ERα, since it exerts opposite transcriptional effects after binding to estrogens and antiestrogens than its ERα counterpart (Paech et al., 1997). Therefore, a synchronized expression of ERα and ERß seems to be essential to estrogen-related transcriptional activity in target organs. Differences in the ERα/ERß ratio could have important functional implications, probably as a result of the different binding characteristics (Kuiper et al., 1997). In addition, a disrupted expression of ERα and β might play important roles in endometrial pathogenesis and carcinogenesis.

**Fig. 2.** IRS-score for ERa, ERß, PR-A and PR-B. The IRS score for ERa and ERß declined significantly (p<0.001 and p<0.05 respectively) in the glandular epithelium from proliferative to late secretory phase (one asterisk). Additionally, ERß expression was higher during proliferative compared to early secretory phase (p<0.05) (one asterisk). PR-A and PR-B reached the lowest significant expression in late secretory phase compared to samples from proliferative phase (p<0.001 and p<0.05 respectively, one asterisk) and early secretory compared to late secretory phase (p<0.05 each, one asterisk). ERα and ERß immunolabelling was similar to secretory endometrium, but significantly lower compared to samples from the proliferative phase (p<0.05 and p<0.01 respectively). Atrophic endometrium showed a similar PR-A expression with proliferative endometrial tissue, being significantly higher than late secretory endometrium (p<0.05) (two asterisks). The PR-B expression also decreased significantly between proliferative and early secretory phase compared to atrophic endometrium (p<0.05 each) (two asterisks).
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(Fujimoto et al., 2002). Whether the ERß expression is influenced by PR-A or PR-B and/or progesterone remains to be elucidated.

During the proliferative phase, endometrial estradiol concentrations are high with a high tissue to plasma ratio (Alsbach et al., 1983). The high steroid receptor expression in glandular epithelium during the proliferative phase probably results from increased local estradiol concentration (Snijders et al., 1992). The significant decrease of ER in glandular epithelium is consistent with a drop in endometrial concentrations (Alsbach et al., 1983). Therefore, cyclical sex steroid hormone expressions are associated with the known changes of their receptors in endometrium. Additionally, since the transcription of the PR gene is induced by estrogens and inhibited by progesterone, the expression of ER and PR are thought to be closely associated (Savouret et al., 1994a,b). Recently, a significant decline of ERß compared to proliferative showed a significant decrease with tamoxifen, an antiestrogen and genistein, a phytoestrogen, whereas PR expression increased significantly (Mylonas et al., 2003a). Therefore, we speculate that normal postmenopausal endometrial tissue is marked by a down-regulation of ERα, ERβ, PR-A and PR-B, but with a lower intensity compared to proliferative endometria. In atrophic endometrial tissue we observed, as expected, a down-regulation of ERα and ERß compared to proliferative endometrium, probably due to the decreased production of estradiol in the postmenopause. While PR-B decrease significantly in atrophic endometrial tissue, an up-regulation of PR-A was observed compared to late secretory endometrial tissue. Interestingly, ERß showed a significant decline with tamoxifen, an antiestrogen and genistein, a phytoestrogen, whereas PR expression increased significantly (Mylonas et al., 2003a). Therefore, we speculate that normal postmenopausal endometrial tissue is marked by a down-regulation of ERα, ERß and PR-B but with a subsequent higher expression of PR-A. Thus, the PR-A/PR-B ratio might play an important role in endometrial transission and subsequently influence endometrial pathogenesis.

In conclusion, we demonstrated ERα, ERß and PR-A and PR-B expression in normal human endometrium with a cyclical variation regarding all four steroid receptors during the menstrual cycle. These results show the presence of steroid receptors in human epithelium, indicating that these cells respond to estrogen and progesterone, mediating a significant role in endometrial physiology.

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References


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beta-A (-betaA) and beta-B (-betaB) are differentially localised in normal, hyperplastic and malignant human endometrial tissue. Acta Histochem. 108, 1-11.


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