Abstract. Human endometrium expresses estrogen (ER) and progesterone (PR) receptors, which are related to autocrine and paracrine processes that respond to estrogen and progesterone. The ER and PR expression and distribution pattern may play an important role in endometrial function and pathogenesis. The aim of this study was to evaluate the distribution pattern of ER-α, ER-β and PR in normal (n=15) and malignant (n=11) human endometrial tissue. Commercially available monoclonal antibodies against ER-α, ER-β and PR were used. The distribution of the steroid receptors was evaluated using the IRS-score and the Mann-Whitney rank-sum test was used to compare the means. Correlation was assessed with the Spearman factor and linear regression analysis. ER-α, ER-β and PR declined significantly (p<0.05) in normal glandular epithelium from proliferative to late secretory phase, although the staining intensity of ER-β was lower than that of ER-α. ER-α, ER-β and PR were also expressed in malignant endometrial tissue. A significant correlation by regression analysis of ER-α and ER-β was demonstrated, showing a dependence in the expression of these steroid receptors. The ER-α/ER-β ratio decreased significantly from normal to malignant endometrial tissue (p<0.05), while the ER-β/ER-α ratio showed statistical differences within normal endometrial tissue. These results showed the presence of steroid receptors in normal and malignant human endometrium, indicating a significant role in endometrial physiology and malignant transformation.

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Endometrial cancer is becoming the most common gynecologic malignancy in the western world and occurs in reproductive and postmenopausal women (1, 2). It mostly appears in women with conditions such as estrogen-only hormone replacement therapy (HRT), obesity, polycystic ovary disease, nulliparity, estrogen-producing tumors and anovulation (1-3).

Estrogen (ER) and progesterone (PR) receptors belong to the nuclear steroid receptor superfamily. They are ligand-dependent transcriptional factors, which can bind to different DNA sites to initiate the expression of specific genes. In addition to this direct activation of target genes, indirect mechanisms through contacts with DNA-bound transcription factors (such as AP1 or NF-ÎκB) have been reported (3). The effect of the steroid hormones estrogen and progesterone are thought to be mediated through these receptors. ER and PR are closely related to the occurrence of autocrine and paracrine processes that respond to estrogen and progesterone (4). In several studies, both receptors were measured in paraffin-fixed human endometrium (4-8) and isolated glandular epithelial cells (9) using immunohistochemical assays. However, the exact mechanism for the proliferative effects of estrogens on the endometrium and their role in neoplasia still remain unknown. For several years, it was generally believed that just one single ER receptor existed. However, the discovery of a new nuclear receptor with specificity for estrogens has induced new insights in the estrogen signaling system (10). The novel receptor ER-β has a high (approx. 95%) homology in the DNA binding domain, but only 55% homology in the ligand binding domain compared to the classical ER (ER-α). ER-α can bind estradiol with a high affinity and react with a consensus estrogen response element (ERE), stimulating transcription of ER target genes (11).

The ER and PR expression and distribution patterns might play an important role in normal endometrial function and pathogenesis and the expression and relationship of the two distinct ER and PR could be of essential clinical implications.
The ER status is believed to provide prognostic information independent of tumor stage and grade in women with endometrial carcinoma (12-14). The ER-β/ER-α mRNA ratio was high in advanced invasive carcinoma. Additionally, Western blot analysis demonstrated that ER-β was highly expressed in comparison with ER-α in endometrial cancer with severe myometrial invasion, suggesting that ER-β is important in the progression of myometrial invasion (15). The intact synchronized expression of ER-β interacting with ER-α might be disrupted in neoplastic endometrium (16), playing an important role in endometrial pathogenesis.

The distribution patterns of ER-α, ER-β and PR using recently characterized monoclonal antibodies (8) and a commercially available polyclonal antibody (19) and a commercially available monoclonal antibody (9) for the experiments are listed in Table I. Sections of human breast cancer tissue and normal colon tissue samples were used for positive controls.


table

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Source</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-α</td>
<td>1D5</td>
<td>mouse IgG1</td>
<td>1:150</td>
<td>Immunotech, Hamburg, Germany</td>
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<tr>
<td>ER-β</td>
<td>PPG5/10</td>
<td>mouse IgG2a</td>
<td>1:50</td>
<td>Serotec, Oxford, United Kingdom</td>
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<tr>
<td>PR</td>
<td>10A9</td>
<td>mouse IgG2a</td>
<td>1:50</td>
<td>Immunotech, Hamburg, Germany</td>
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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, CA, USA). Mouse monoclonal antibodies used for the experiments are listed in Table I. Sections of human breast cancer tissue and normal colon tissue samples were used for positive controls.

Briefly, paraffin-fixed tissue sections were dewaxed using xylol for 15 min, rehydrated in an alcohol raw, and subjected to antigen retrieval on a high setting for 10 min in a pressure cooker in sodium citrate buffer (pH 6.0), containing citrate acid 0.1M and sodium citrate 0.1M in distilled water. After cooling, the slides were washed twice in PBS. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (Merck) in methanol for 20 min. Non-specific binding of the primary antibodies was blocked by incubating the sections with "diluted normal serum" for 20 min at room temperature. Sections were then incubated at room temperature for 60 min with the primary antibodies. ER-α and PR were diluted in dilution medium (Dako, Glostrup, Denmark), while ER-β was diluted in PBS. After washing with PBS, the slides were incubated in "diluted biotinylated serum" for another 30 min at room temperature. After incubation with the avidin-biotin peroxidase complex (reagent ABC) for a further 30 min and a repeated washing step with PBS, visualization was performed with substrate and chromagen 3,3'-diaminobenzidine (DAB; Dako) for 8-10 min. The slides were further counterstained with Mayer’s acidic hematoxylin and washed in an alcohol raw (50-98%). After xylol treatment, the slides were covered. Negative controls were performed by replacing the primary antibody with normal mouse serum. Positive cells showed a brownish color and negative controls as well as unstained cells were blue.

Evaluation and statistical analysis. The intensity and distribution of the specific immunohistochemical staining reaction was evaluated using a semi-quantitative method (IRS-score), as previously described (25) and used in the evaluation of endometrial steroid receptor expression (9), as well as the expression of cathepsin D and late secretory phase (days 23-28, n=4), as previously described (7, 9). Additionally, endometrioid adenocarcinomas grade 1 (G1; n=3) and grade 2 (G2; n=8) were analyzed.

Materials and Methods

Tissue samples. Samples of normal human endometrium were obtained from 15 premenopausal, non-pregnant patients undergoing gynecological surgery either by D&C or hysterectomy for benign diseases. All women had had a normal and regular menstrual cycle with no hormonal treatment for the 3 months prior to surgery. Endometrial premenstrual samples were classified according to anamnetical and histological dating (23, 24) into proliferative (days 1-14, n=7), early secretory (days 15-22, n=4) and late secretory phase (days 23-28, n=4), as previously described (7, 9). Additionally, endometrioid adenocarcinomas grade 1 (G1; n=3) and grade 2 (G2; n=8) were analyzed.

Figures 1-8. Immunohistochemical expression of ER-α, ER-β and PR. During the proliferative phase, both glandular epithelial and stromal cells demonstrated intense nuclear immunoreactivity for ER-α (Figure 1, x125), while it decreased continuously till the late secretory phase. Endometrioid adenocarcinomas also expressed ER-α, but with a lower intensity (Figure 2, x400). The PR immunohistochemical reaction showed a similar declining pattern between proliferative phase (Figure 3, x400) and late secretory phase, while it was also expressed in stromal cells and endometrial adenocarcinomas (Figure 4, x400). ER-β immunostaining patterns were also expressed in endometrial glandular and stromal cells (with a minimal intensity) with a lower immunostaining intensity compared to the ER-α. The intensity was higher in the proliferative phase (Figure 5, x400), whereas in the late secretory phase, a minimal to no staining reaction was observed (Figure 6, x250). G1 carcinomas showed a low ER-β intensity (Figure 7, x400), in contrast to G2 carcinomas that showed minimal or no expression (Figure 8, x250).
(26) and CA-125 (27) in human endometrium. The IRS score was calculated as follows: IRS=SI x PP, 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 approximately 200 cells and it was defined as 0 = no staining, 1 = <10%, 2 = 11-50%, 3 = 51-80% and 4 = >81%. The samples were evaluated by two different observers and the mean of the results were used. The Mann-Whitney rank-sum test was used to compare the means of the different IRS scores (SPSS, Chigaco, IL, USA). Spearman-Rho factor and regression analysis was used to assess any correlation between the steroid receptors in endometrial cancer. The ratios of ER-α/ER-β and ER-β/ER-α were calculated and the means were compared using the Mann-Whitney rank-sum test. Significance was assumed at p<0.05.

**Results**

All three steroid receptors were immunohistochemically expressed in normal and malignant endometrial tissue. However, adenocarcinomas with grade 1 and grade 2 showed a lower staining reaction than normal endometrium. During the proliferative phase, both glandular epithelial and stromal cells demonstrated nuclear immunoreactivity for ER-α, ER-β and PR, confirming previous results (4-8, 19). While the staining reaction of ER-α in biopsies in the proliferative phase was intense (Figure 1), it decreased continuously till the late secretory phase. Additionally, the ER-α expression was low in adenocarcinomas grade 2 (Figure 2). The PR immunohistochemical reaction showed in normal endometrium a similar declining pattern between the proliferative phase (Figure 3) and late secretory phase, with lower intensity in endometroid adenocarcinomas (Figure 4). The ER-β immunostaining patterns were also demonstrated in endometrial glandular and stromal cells. However, ER-β immunostaining intensity was lower compared to the ER-α staining reaction. The intensity of positive epithelial cells was higher in the proliferative phase (Figure 5) than in the late secretory phase (Figure 6). Adenocarcinomas grade 1 also showed a low intensity (Figure 7), in contrast to grade 2 carcinomas that showed minimal or no expression (Figure 8).

The IRS score for ER-α declined significantly (p<0.05) in the glandular epithelium from the proliferative to early secretory phase (Figure 9), reaching the lowest expression in the late secretory phase (p<0.05). Additionally, a significantly lower expression was demonstrated between proliferative and early secretory phases compared to adenocarcinomas grade 2 (p<0.05). The IRS score for ER-β also declined significantly (p<0.05) in the glandular epithelium from the proliferative to early and late secretory phases (Figure 10). Adenocarcinomas grade 2 also showed a significantly lower immunohistochemical reaction compared to the proliferative phase (p<0.05). The PR expression decreased significantly (p<0.05) between the proliferative and late secretory phase (Figure 11), with no statistical differences between normal and malignant endometrial tissue.

A correlation was demonstrated only for ER-α and ER-β in endometrial carcinomas (p<0.05). Regression analysis revealed a positive correlation between ER-α and ER-β (r²=0.800, p<0.001), while no correlation was observed between ER’s and PR (p=0.057). The ratio of ER-α/ER-β was high during the proliferative phase, decreasing significantly (p<0.05) as the endometrial neoplastic transformation developed. Interestingly, the ratio between ER-β/ER-α showed significant differences in normal human endometrial tissue (p<0.05) between the proliferative and early as well as late secretory phases (Figure 12).
Discussion

The endometrium is one principal target tissue of the pituitary-gonadal axis, but has also been recognized as an endocrine organ. Endometrial carcinomas can arise from hyperplastic precursor lesions, probably due to unopposed stimulation by estrogen (2). The estrogen-dependent type of endometrial carcinomas consist of histopathologically well-differentiated endometroid carcinomas (G1-G2), although some types of endometrial cancer might be estrogen-independent (2). There is a clinical need for a simple and efficient marker of the activity of this tissue, especially with regard to endometrial pathogenesis and carcinogenesis.

Several studies have measured ER and PR in normal and malignant human endometrium using immunohistochemical assays. Our results confirm the cyclical variations of ER and PR in normal endometrial tissue, as described by several authors (4-9). These immunohistochemical results show the presence of steroid receptors in human epithelium, indicating that these cells respond to estrogen and progesterone (4). It is generally accepted that estradiol up-regulates ER and PR, while progesterone down-regulates both receptors (5). Recently, a significant decline of ER-α expression was demonstrated in glandular epithelial cells after stimulation with tamoxifen (an antiestrogen), and the phytoestrogen genistein, whereas PR increased significantly after stimulation with both substances (28), suggesting a functional association between these two steroid receptors.

The ER status is believed to provide prognostic information independent of tumor stage and grade in women with endometrial carcinoma (12-14). Alteration in estrogen signaling pathways may occur during endometrial tumorigenesis, and provide evidence that ER-α expression may play an important role in the regulation of PR in normal and malignant endometrium (20). PR has also been implicated in the development of endometrial cancer, exerting its effects by its two receptors PR-A and PR-B, and a decrease in PR-B has been observed in poorly-differentiated endometrial cancer cell lines (29). These observations suggest an association between malignancy and disturbances in the relative abundance of ER and PR subtypes. It is thought that PR-A in the endometrium downregulates estrogen action by preventing ER-α from transactivation. In contrast, the activated PR-B isoform acts as an endometrial estrogen-agonist (30). However, PR, in contrast to ER, is suggested to be more predictive of disease-free survival (31, 32). The ER and PR expression was correlated with the nuclear grade, being higher in G1 tumors (33). There was a correlation between receptor concentration and grade, with a significant difference between grades 1 and 2 versus grade 3 (34). We demonstrated significant differences of ER-α and ER-β between normal human endometrial cells in the proliferative phase compared to adenocarcinomas grade 2, suggesting a substantially lower expression of steroid receptors in endometrial cancer. Therefore, cancer cells might transform to more aggressive cells types during advancement of endometrial cancer and lose their sex steroidal dependency. Interestingly, a high level of ER-α has been demonstrated to be beneficial in the control of female cancers due to the observed inhibitory effect on angiogenic pathways (35). It is not known if this is true for endometrial carcinomas.

Studies in normal and pathological human endometrium have reported mostly on the ER-β mRNA expression (15-22). Recently, the expression of ER-β by immunohistochemical means in normal human endometrium was demonstrated using a polyclonal (19) and a monoclonal (8) antibody. Using a commercially available monoclonal antibody against ER-β antigen, we demonstrated the immunolocalization of ER-β in malignant human endometrial tissue. The presence and distribution pattern of ER-β in glandular epithelial cells is in agreement with previous reports (15, 17-22). Our results indicate that glandular ER-β expression predominantly occurs during the proliferative phase, declining as the menstrual phase continues, as demonstrated for the ER-α expression (8). Differences in tissue distribution of ER-α and ER-β during the menstrual cycle suggest a substantial role in the modulation and function of estrogen activity in human endometrial tissue. However, the intensity of the ER-β staining reaction was lower compared to ER-α and PR, as demonstrated through the combination of a semi-quantitative analysis and statistical regression procedure (8).

Since the ratio of ER-α/ER-β is uniform without statistical differences in normal endometrial tissue, several regulatory functions between both ER's may be involved. ER-β could have a different action and activity compared to ER-α, since it exerts opposite transcriptional effects after binding to estrogens and antiestrogens than its ER-α counterpart (36). Differences in the ER-α/ER-β ratio could have important functional implications, probably as a result of the different binding characteristics (11). The immunohistochemical ratio ER-β/ER-α demonstrated statistically significantly differences during menstrual phases in normal human endometrium, suggesting an important function. Additionally, the significant differences of the immunohistochemical ratio ER-α/ER-β between normal and neoplastic endometrial tissue suggests an important role in malignant transformation. Therefore, a synchronized expression of ER-α and ER-β seems to be essential for estrogen-related transcriptional activity in target organs. A disrupted expression of ER-α and ER-β might play an important role in endometrial pathogenesis and carcinogenesis (16). We demonstrated a significant correlation in the expression pattern of ER-α and ER-β, suggesting an important role in maintaining the functional status of the ER's. Interestingly, ER-α showed a significant decline with tamoxifen, an antiestrogen and genistein, a...
phytoestrogen, whereas PR expression increased significantly (28). Whether ER-β expression is influenced by PR and/or progesterone remains to be elucidated.

In conclusion, we demonstrated ER-α, ER-β and PR expressions in normal and neoplastic human endometrium using immunohistochemical means. A cyclical variation was observed during the menstrual cycle, while endometrioid adenocarcinomas expressed all three steroid receptors but with lower intensity than proliferative endometrium. A significant correlation by regression analysis of ER-α and ER-β was also demonstrated in malignant endometrial tissue, showing dependence in the expression of all these steroid receptors. These results show the presence of steroid receptors in human epithelium, indicating that these cells respond to estrogen and progesterone, playing a significant role in endometrial physiology and tumorigenesis.

References


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