Estrogen Receptor-α and -β in Endometriosis and Normal Endometrium in Humans

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Purpose. Endometriosis is an estrogen-related disease. The biologic activity of estrogen is mediated by two high-affinity estrogen receptors, ER-α and ER-β. We aimed to evaluate the levels of expression of ER-α and -β in endometriotic lesions and normal endometrium.

Methods. Samples were divided into three groups: Group 1 comprised eutopic endometrium from normal controls (n = 10); Group 2 consisted of eutopic endometrium (n = 11) and Group 3 ectopic endometrium (n = 11) from endometriosis patients. ER-α and -β mRNA in each Group was surveyed with reverse transcriptase-polymerase chain reaction. Their protein levels were detected by Western blot analysis. ER-α and -β mRNA and protein expressions in each group were compared.

Results. The proportion of ER-α/-β mRNA expression was 67.15/89.48 in Group 3, 31.9/60.61 in Group 1, and 0/76.23% in Group 2. ER-α mRNA expression was highest in Group 1, moderate in Group 2, and lowest in Group 3 ($p < 0.05$). ER-β mRNA expression was highest in Group 1, moderate in Group 3, and lowest in Group 2 ($p < 0.05$). ER-α mRNA expression was significantly lower than ER-β mRNA in all groups. The proportion of ER-α/-β protein expression was 45.45/61.77 in Group 3, 47.21/60.52 in Group 1, and 37/48.21% in Group 2. ER protein expression in eutopic endometrium in Groups 3 and 1 did not differ significantly. ER protein expression in ectopic endometrium was significantly lower than in eutopic endometrium in Groups 3 and 1.

Conclusions. ER-β protein expression is higher than ER-α in normal endometrium and in endometritic tissue. ER-α mRNA and protein are suppressed in endometriotic tissue. The *ER* gene isoforms likely contribute to the pathogenesis of endometriosis. **(Mid Taiwan J Med 2005;10:65-72)**

Key words

endometriosis, estrogen receptor isoform, reverse transcription-polymerase chain reaction, Western blot analysis

INTRODUCTION

Endometriosis, a common gynecological disorder accounting for infertility and dysmenorrhea, occurs almost exclusively in menstruating women of reproductive age.

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Although the pathogenesis of endometriosis remains unclear, it is very likely that estrogen stimulates the growth of endometriotic tissue. Estrogen, a primarily reproductive hormone, influences the physiopathological changes of the uterus and mammary glands. Estrogen action upon the endometrium is very complex. Endometrium contains estrogen receptors (ER), which are involved in estrogen metabolism and endometrium growth. The binding of estrogen to nuclear estrogen receptors (ER) activates transcription of genes involved in cellular growth controls.

Estrogen is mediated within target cells by two ER subtypes, ER-alpha (ER-α) and beta (ER-β) [1,2]. The elucidation of the biological roles of the two ER subtypes might be useful in the development of therapeutic agents for endometriosis. Previous studies have demonstrated a differential characterization of the ER isoforms between the eutopic endometrium and ovarian endometriotic cysts [3-5]. ER-α and -β differ in their actions within different tissues [6]. The expression patterns of ER in endometriotic lesions are different from those in eutopic endometrium [7]. Different tissues show different levels of these receptor isoforms, which explain their different biological expression [8].

Few studies have investigated the expression levels of ER isoforms in endometriosis. Matsuzaki et al [9] demonstrated that the predominant expression of ER-α may be essential for the development of endometriosis. Furthermore, few authors have compared ER expression in eutopic and ectopic endometrium from the same individuals. In the present study, we aimed to investigate and compare the levels of messenger RNA (mRNA) and protein expression of ER-α and -β between ovarian endometriotic cysts and eutopic endometrium. This study might elucidate the relationship between ER isoforms and endometriosis development.

MATERIALS AND METHODS

Group 1 consisted of ectopic endometrium from normal controls (n = 10). Specimens from pre-menopausal Chinese women in Taiwan with surgically and histologically diagnosed endometriosis were divided into two groups; Group 2 consisted of eutopic endometrium (n = 11) and Group 3 comprised ectopic endometrium (n = 11). The specimens of eutopic endometrium in Group 1 and ovarian endometrial cyst in Group 2 were from the same patients. All 11 patients had severe endometriosis and underwent laparoscopy cystectomy as well as dilation and curettage. Control samples were obtained from normal eutopic endometrial tissue of 10 patients with cervical intraepithelial neoplasma (moderated and severe dysplasia) undergoing conization. Sonography confirmed no endometriosis. All endometrium specimens were obtained during the late proliferative phase.

All patients had regular menstrual cycles. None of them had been undergoing hormonal treatments, such as gonadotrophin-releasing hormone analogue (GnRHa) or sex steroids for 6-months before surgery. All operations were performed by the same surgeon (Lin WC). The study was approved by the ethical committee and institutional review board of the China Medical University Hospital. Informed consent was signed by all women who donated their specimens.

All tissues were frozen immediately after surgical resection and stored in liquid nitrogen until extraction of mRNA. Total RNA was extracted from 0.1 g of endometrium by TRIzol reagent (Life Technologies, USA) according to the manufacture's protocol. The RNA was stored either as a pellet in ethanol or solubilized in RNase-free water and kept at −70°C. Reverse transcription was performed as previously described [10]. Briefly, 3 μg of total RNA was reverse transcribed using 2.5 μM oligo dT primers (Promega, USA), 1 mM of dNTP,

Table 1. The primer sequences and RT-PCR conditions for ER-α, ER-β and β-actin

CR primer sequences (5'→3')*		RT conditions (˚C/min)	PCR conditions (˚C/sec)		
			Denature	Annealing	Extension
ER-α	F-TGCCAAGGAGACTCGCTA (bases 766-783)	39˚C/60 min	94˚C/30 sec	60˚C/45 sec	72˚C/45 sec
	R-TCA ACATTCTCCCTC CTC (base 1028-1011)				
ER-β	F-TTCCCAGCAATGTCACTAACT (base 33-53)				
	R-CTCTTTGAACCTGGACCAGTA (base 291-271)				
β-actin	F-CTCTTCCAGCCTTCCTTCCTG (bases 822-842)				
	R-GAAGCATTTGCGGTGGACGAT (bases 1166-1146)				

*F and R indicate forward and reverse primers.

20 U human ribonuclease inhibitor (HT Biotechnology), and 5 U super reverse transcriptase (RT) (HT Biotechnology) in RT buffer (25 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM DTT, and 5 mM $MgCl_2$) in a total reaction volume of 20 μL at 39˚C for 60 min (Perkin-Elmer/Cetus).

In each PCR reaction, 3 μL of RT product was added to a final volume of 50 μL containing 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Triton X-100, 0.001% gelatin, 200 μM of each dNTP, 0.5 unit of *Taq* DNA polymerase (HT Biotechnology), and 0.2 μM of each primer pair. The β-actin was used as an internal control for normalization of ER mRNA. The PCR primer sequences of ER-α, ER-β and β-actin are listed in Table 1.

After RT-PCR, DNA products amplified by the ER-α, ER-β and β-actin primers sets were resolved on a 2% agarose gel with ethidium bromide. Relative abundance of the PCR products was determined by gel electrophoresis followed by densitometer analysis (Molecular Dynamics, Model: PD486-010, USA). The relative abundances of ER-α, ER-β and β-actin were scanned with a laser densitometer (Molecular Dynamics) and integrated with the Image Quant™ densitometer software. The ratios of ER-α/β-actin and ER-β/β-actin in each group were measured as a relative quantification of ER-α and ER-β mRNA

Endometrium samples were diluted with deionized water (1:100) and mixed with sample buffer (5:1). Samples containing 9 mg of protein (approximately 20 mL) and a prestained protein marker were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (7.5% stacking gel and 12% separating gel), and the proteins were transferred to nitrocellulose filters. These filters were blocked overnight at 4˚C in 3% nonfat powdered milk in Trisbuffered saline and Tween 20 (TBST). After blocking, the nitrocellulose filters were washed twice in TBST for 10 minutes. They were incubated overnight at 4˚C with monoclonal antibodies against human ER-α and ER-β (Affinity BioReagents PA1-308, USA).

After incubation, the nitrocellulose filters were washed three times for 10 minutes in TBST, and the primary antibodies were complexed with a rabbit anti-mouse IgG alkaline phosphatase conjugate (1:1000) for 60 minutes at room temperature. After washing the filters three times with TBST, the blot was developed by adding the alkaline phosphatase substrate solution (Promega, Madison, WI). As soon as the bands developed to the desired intensity, the reaction was stopped by washing the blot in a solution consisting of 20 mM Tris-HCL and 5 mM ethylenediaminete-traacetic acid (EDTA), pH 8.0. The blots were air dried at room temperature. The hydropholoic polyvinylidene difluoride membranes (PVDF, Life Science, USA) were then washed three times.

The proteoglycans were finally visualized by a secondary peroxidase-conjugated anti-rabbit

Table 2. The proportions of estrogen receptor-α and -β mRNA expressions in endometrium and endometrioma

mRNA	Estrogen receptor-α (%)*‡	Estrogen receptor-β (%)†‡
Group 1. Eutopic endometrium from normal controls†	67.15 ± 6.82	89.48 ± 27.03
Group 2. Eutopic endometrium from endometriosis patients†	31.9 ± 8.2	60.61 ± 9.34
Group 3. Ectopic endometrium from endometriosis patients‡	0	76.23 ± 7.3

*ER-α expression was highest in Group1, moderate in Group 2, and lowest in Group3 (t test, Wilcoxon rank-sum test, $p < 0.05$). †ER-β expression was highest in Group 1, moderate in Group 3, and lowest in Group 2 (ANOVA, Kruskal-Wallis test, $p < 0.05$). ‡ER-α expression was lower than ER-β in all groups (t test, Wilcoxon rank-sum test, $p < 0.05$).

Table 3. The estrogen receptor-α and -β protein expression in eutopic and ectopic endometrium

Western blotting	Estrogen receptor-α (%)*	Estrogen receptor-β (%)*
Group 1. Eutopic endometrium from normal controls†	45.45 ± 3.99	61.77 ± 10.68
Group 2. Eutopic endometrium from endometriosis patients†	47.21 ± 3.79	60.52 ± 5.25
Group 3. Ectopic endometrium from endometriosis patients‡	37 ± 3.53	48.21 ± 10.06

*ER-β expressions are higher than those of ER-α in all groups (t test, $p < 0.05$). †ER expression between both groups was not significantly different (t test). ‡ER expression in ectopic endometrium was lower than that in eutopic endometrium from normal and endometriosis individuals (t test, $p < 0.05$).

antibody according to the manufacturer's instructions (Sigma A-9169, USA). Proteins that reacted positively with monoclonal ER-α and ER-β were determined by electrophoresis using standard molecular weight markers. The results were analyzed by densitometer software (Gel-Pro Analyzer V. 3.0). Each group of ER-mRNA and ER protein were compared also. The SASS system with t test, Wilcoxon rank-sum test, ANOVA, and Kruskal-Wallis test were utilized for statistical analyses (Tables 2, 3). A $p < 0.05$ was defined as statistically significant.

RESULTS

RT-PCR

The proportions of ER-α/-β mRNA expression in each group were: 1) 67.15 ± 6.82/89.48 ± 27.03%; 2) 31.9 ± 8.2/60.61 ± 9.34%; 3) 0/76.23 ± 7.3% (Table 2). ER-α expression was highest in Group 3, moderate in Group 1, and lowest in Group 2 ($p < 0.05$). ER-α expression was highest in Group 3, moderate in Group 2, and lowest in Group 1 ($p < 0.05$). ER-α expression was lower than ER-β in all groups ($p < 0.05$).

Western blotting

The proportions of ER-α/-β protein expression in each group were: 1) 45.45 ± 3.99/61.77 ± 10.68%; 2) 47.21 ± 3.79/60.52 ± 5.25%; 3) 37 ± 3.53/48.21 ± 10.06% (Table 3). ER-β expression was higher than ER-α expression in all groups ($p < 0.05$). There was no significant difference between ER expression in eutopic endometrium specimens and endometriosis specimens. ER expression in ectopic endometrium was lower than that in eutopic endometrium from normal and endometriosis individuals ($p < 0.05$).

DISCUSSION

Endometriosis is related to changes in levels of several cytokines in serum and peritoneal fluid [11]. Endometriosis involves a complex interaction between hormone and cytokine activation, immunoinflammatory processes, and genetic factors [12]. Estrogens play a central role in reproductive physiology. Endometriosis develops mostly in women of reproductive age and regresses after menopause or ovariectomy, which suggests its estrogen-dependent growth. Estrogen secreted by the ovaries is necessary for the development of endometriosis. GnRHa is the most effective treatment. The extent of endometriosis regression by GnRHa may be partly dependent on the low concentrations of ER found in endometriosis

patients [13].

There are at least two ER isoforms (ER-α and -β) which mediate the complex conjunction with other coactivators. Differential transcript processing, ligand specificity and biological actions of the ER isoforms may influence differential growth responses in normal and ectopic endometrium [3]. ER could directly or indirectly interact with other cytokine/steroid receptor superfamilies. Both ER-α and β have nearly identical DNA-binding domains but show much greater variation with respect to the ligand-binding domains.

Compounds with preferential affinity for one receptor do exist [6]. Different tissues harbor different regulator proteins, which allow contact with the target gene control region [14]. Variations in the expression of these ER isoforms in the same tissue or organ and different physiologic responses are indicators that there is a variable target tissue response to the same estrogen ligand. Furthermore, the ligand/receptor complex is not recognized in the same cells, owing in part to the pattern of active genes and to steroid receptor co-regulators which modulate the ER regulation of gene expression [6,15]. These findings explain how different ER ligands manifest different responses in the same cell types and how the same ligand causes different responses in different cell types.

ERs are related to numerous cancers, including cervical cancer [16], ovarian cancer [17], breast cancer [18] and endometrial cancer [19]. Fujimoto et al [4] demonstrated that the ER-α mRNA was higher than the ER-β mRNA in normal endometrium. Matsuzaki et al [9] observed that both eutopic endometrium and endometriotic tissues showed predominantly higher levels of ER-α than ER-β mRNA. The relative ratio of ER-α to ER-β mRNA in red peritoneal lesions was significantly higher than in black lesions and ovarian endometriotic cysts [9]. There was no significant difference in ER-α/ER-β between proliferative eutopic endometrium and red peritoneal lesions. Matsuzaki et al [20] suggested that the principal and regulatory effects of estrogens are mediated mainly via ER-α rather than ER-β in both eutopic endometrium and endometriotic cysts.

However, conflicting results have also been reported. Some investigators have demonstrated no association between ER and ovarian tumor [21] or with recurrent spontaneous abortion [22]. Others have reported that expression of ER-α mRNA in endometriosis did not respond to endocrinological alterations during the menstrual cycle, suggesting damage or suppression of ER-α cascade in endometriosis tissue [4]. Fazleabas et al [23] demonstrated in a baboon model that ER-β was only expressed in ectopic endometriotic lesions. The over-expression of ER-β in endometriosis lesions might be related to a unique estrogen-dependent growth and spreading of ovarian endometriomata [4]. ER-β might regulate, modulate or inhibit directly or indirectly the gene expression of ER-α [24].

Fujimoto et al reported that ER-β mRNA expression in endometriosis was significantly higher than in normal endometrium, while ER-α mRNA expression was relatively lower and more random [4]. In this study, we found similar results in ER-β, but no change in ER-α. Our results revealed that the ER-α mRNA was lower than the ER-β mRNA in endometriotic tissue and in normal endometrium, which was incompatible with the reports by Fujimoto et al [4] and Matsuzaki et al [9]. This discrepancy might be due to the racial difference, case variation, different disease stage and menstrual period, or method modification between the studies. In fact, the ER-α/β ratio might be influenced by the menstrual cycle. In normal uterine endometrium, ER-β mRNA was expressed at a much lower level than ER-α mRNA [4]. During the menstrual period, the expression pattern of ER-β mRNA was similar to that of ER-α mRNA [4].

Furthermore, other substances (eg, growth factors, hormones, or cytokines) might also be involved in the complex processes.

In conclusion, we found that ER-β protein expression was higher than ER-α protein expression in normal endometrium and endometriotic tissue. ER-α mRNA and protein were suppressed in ectopic and eutopic endometrium from endometriosis individuals. ER-β might fail to act with ER-α for normal estrogen dependency. The different expression of ER subunits might contribute to the pathogenesis of endometriosis. This finding could provide a database for further surveys of ER expression in normal endometrium and endometriosis tissues. However, the real role ER isoforms play in the development of endometriosis remains to be clarified.

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雌激素α與β接受體在子宮內膜異位症與正常子宮內膜組織之表現

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目的　子宮內膜異位症之發生過程與雌激素有關，雌激素可經由α與β接受體結合作用引發進一步之生理作用機轉。本篇研究將計畫評估比較子宮內膜α與β接受體在子宮內膜異位症與正常子宮內膜組織之表現差異。

方法　所有實驗材料區分為三組：1)子宮內膜組織(取自子宮內膜異位症女性，n = 11)；2)子宮內膜異位瘤組織(取自子宮內膜異位症女性，n = 11)；3)子宮內膜組織(取自正常女性，n = 10)。三組之雌激素α與β接受體之信使核糖酸(mRNA)以反轉錄聚合酶鏈反應(RT-PCR)進一步偵測，以西方點漬法(Western blotting)偵測其蛋白質表現，並進一步比較各組mRNA與蛋白質之表現差異。

結果　各組雌激素α與β接受體之mRNA表現比例分別為：1) 31.9/60.61；2) 0/76.23；3) 67.15/89.48%。α接受體之表現於第一組織表現最高，其次為第二組，最低為第三組。於各組中亦發現雌激素α之表現均比β接受體低。各組雌激素α與β接受體之蛋白質表現比例分別為：1) 47.21/60.52；2) 37/48.21；3) 45.45/61.77%。第一組與第二組之子宮內膜組織雌激素接受體蛋白質表現並無明顯差異。第三組子宮內膜異位瘤之激素接受體蛋白質明顯比第一組與第二組之蛋白質表現為低。

結論　雌激素β接受體之mRNA與蛋白質表現均比雌激素α接受體為高，相對而言，於子宮內膜異位瘤之雌激素α接受體之mRNA與蛋白質表現明顯受到抑制。雌激素接受體之同型接受體基因可能與子宮內膜異位症之致病機轉有關。（中台灣醫誌 2005;10:65-72）

關鍵詞

子宮內膜異位，雌激素接受體，反轉錄聚合酶鏈反應，西方點漬法

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