ESTROGEN RECEPTOR B AGONIST INHIBITS PROLIFERATION OF ENDOMETRIAL STROMAL CELLS IN AN ESTROGEN-RECEPTOR INDEPENDENT MANNER

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Abstract - The finding that estrogen receptor ERβ agonists induce regression of ectopic endometrial implants in a rodent model has rekindled interest in the role of ERβ in the growth and maintenance of human endometriosis. We hypothesize that the ERβ agonist may have a direct effect on the human endometrium. We examined the mRNA and protein expression of ERβ in both eutopic and ectopic endometrium and performed a luciferase reporter assay for the presence of functional ERβ in human endometrial stromal cells. Diarylpropionitrile (DPN – a ERβ specific agonist) significantly inhibited endometrial stromal cell proliferation at 10 M by 25% but not at 10 nM. ERβ mRNA is present in both eutopic and ectopic endometrium; however, protein expression is absent in both stroma and epithelium. Our search for functional ERβ similarly showed an absence in endometrial stromal cells. These results suggest that the regression of endometriosis might be ERβ-independent.

Key words: Endometrium, neoplasm, pathology, estrogen, receptor

INTRODUCTION

Endometriosis is a complex gynecological disorder associated with pelvic pain and infertility. It affects between 5-15% of reproductive-age women (Goldman and Cramer 1990). The most important pathogenetic changes of endometriosis is its dependence on estrogen for growth, and the absence of a consistent response to progesterone or synthetic progestins (Bulun, Cheng et al. 2006). Current research has focused on specific aspects of the pathogenetic pathway of endometriosis. These include progesterone receptor (PR) modulators, tumor necrosis factor (TNF-α) inhibitors (Vigano 2003), angiogenesis inhibitors (Nap, Griffioen et al. 2004), matrix metalloproteinase inhibitors (D’Hooghe 2003), pentoxifylline (Szamatowicz, Laudanski et al. 2000), and most recently, estrogen receptor (ER) beta agonists (Harris, Albert et al. 2003).

In 1995, a second ER was cloned (Kuiper, Enmark et al. 1996); the former ER is now referred to as ERα and the latter as ERβ. Because endometriosis is an estrogen-dependent disease, the role of this estrogen receptor in endometriosis has been studied by several researchers. Controversy exists regarding the expression of specific ER proteins in the human endometrium. Analyses of the two ER isoforms indicate that eutopic normal endometrial stromal cells and endometrioma stromal cells both predominantly express ERα mRNA (Brandenberg-er, Lebovic et al. 1999). However, several researchers found markedly higher levels of ERβ and lower levels of ERα in human endometriotic tissues and pri-
mary stromal cells compared with eutopic endometrial tissues and cells (Brandenberger, Lebovic et al. 1999; Fujimoto, Hirose et al. 1999). The increased expression of ERβ in endometriosis may also in turn affect the levels of expression of ERα and PR (Bulun, Cheng et al. 2010). The models of action involving cooperation, as well as competition, between the two ER proteins have been proposed (Matthews and Gustafsson 2003). These findings suggest that additional research is needed to explore the role of ER in endometriosis.

Recently, the effects of an ERβ ligand were examined (Harris 2007). The effectiveness of a specific ERβ agonist in treating endometriosis in the rodent model has suggested that this compound may be a promising new treatment in combating the disease (Harris, Bruner-Tran et al. 2005). The current study tested the hypothesis that an ERβ agonist might have an inhibitory effect on endometriosis by acting directly on the ectopic endometrium.

MATERIALS AND METHODS

Patient recruitment, tissue acquisition and characterization

Healthy women with ovulatory menstrual cycles, who had not received hormones or GnRH agonist therapy for at least 6 months before surgery, were enrolled in this study. Subjects undergoing elective surgery for leiomyomata uteri or other benign uterine conditions were recruited for normal endometrial biopsies. Biopsies were obtained after the patients provided written informed consent, under a study protocol approved by the Committee on Human Research at the University of Wisconsin, Madison.

Human endometrial stromal cell cultures

Stromal cells were separated from glandular cells and debris by filtration through narrow-gauge sieves. Stromal cells were plated and subcultured to eliminate contamination by macrophages or other leukocytes. Extensive characterization of cell cultures confirmed that they were >95% pure and retained functional markers of their endometrial origin (Ryan, Schriock et al. 1994).

RT-PCR

Total RNA was extracted from endometrium and endometriosis lesions using the TRIzol reagent kit (GIBCO BRL, Gaithersburg, MD). After reverse transcription of 5 µg of total RNA using oligo (dT) primers and a Moloney Murine Leukemia Virus Transcriptase kit (Promega, USA), the resulting single-stranded cDNA was subjected to PCR. The primers were designed using GeneFisher software. The forward and reverse primers used were: ERβ forward, 5’- TTC CTC CTA TGT AGA CAG CCA CCA T -3’; ERβ reverse, 5’- TAC CAA CTC CTT GTC GGC CAA CT -3’. cyclophilin forward, 5’ –CCG AGG AAA ACC GGG TAC TAT –3’, cyclophilin reverse, 5’ –AGA TTC TAG GAT ACT GCG AGC A–3’. The expected final PCR products from human ERβ were 800 bases long; the PCR products from human cyclophilin were 600 bases long. The PCR programs used were: 35 cycles of 1 min at 94°C, 1 min at 59°C, 2 min at 72°C. Final products were extended to full length by incubation for 5 min at 72°C. Reaction products were separated on 1% agarose gels.

Treatment of stromal cells with ER agonists

Steroid hormones were added in 0.1% ethanol directly to the cells growing in MEM-α media with 5% charcoal stripped serum. The concentrations of ligands are listed as follows: 17β Estradiol 10⁻⁸ M, 10⁻⁹ M (Sigma, St. Louis, MO), DPN 10⁻⁸ M, 10⁻⁵ M (Tocris Cookson, Ellisville, MO).

Gene promoters and luciferase assays

The synthetic strogen response element (ERE) construct and ERβ expression vector were gifts from Dr. Jyoti Watters (University of Wisconsin-Madison). The ERβ expression vector was a gift from Dr. Elaine Alarid (University of Wisconsin-Madison). All constructs were sequenced by the University of Wisconsin Madison Biotechnology Center to verify that the correct sequences were present.
Transient transfections were performed in human endometrial stromal cells grown in MEM-α with 5% charcoal stripped serum and antibiotics in 12-well plates at ~50% confluence. 1 µg of ERE promoter (firefly luciferase, experimental reporter) was added to each well using QIAGEN Polyfect® reagent (Valencia, CA, USA). The ERE transfection efficiencies were normalized to an independent control plasmid (0.5µg β-galactosidase expression vector) that was co-transfected simultaneously. In some experiments, ERα and ERβ were over-expressed by co-transfection of an expression vector (0.5µg/well). A non-specific vector was added to normalize total transfected DNA in each well. The results are presented as luciferase activity after correcting for transfection efficiency. The reporter vector was assayed in at least three independent cultures. The same amount of empty vector was analyzed in pilot experiments as a control and revealed low basal activity.

**Immunohistochemistry**

Paraffin sections (5 µm) were mounted, deparaffinized in xylene, and hydrated gradually through decreasing concentrations of ethanol followed by a wash in tap water. The slides were then placed in citrate buffer (pH 6.0), microwaved to the point of boiling and then left in hot buffer for an additional 30 min for antigen retrieval. Endogenous peroxidase was quenched with methanolic hydrogen peroxide (3%) for 10 min, followed by running tap water rinse to clear. The slides were rinsed three times in PBS (pH 7.4) and incubated with horse serum (Sigma, St. Louis, MO). Subsequently, they were incubated with mouse anti-human ERβ antibodies (Serotec MCA1974T, Oxford, UK) in PBS at 25°C for 1 h, followed by three rinses with PBS. The slides were then incubated with biotinylated horse anti-mouse antibodies (Vector Lab, Burlingame, CA) for 30 min, followed by three rinses with PBS. Chromogen was developed using Novared hydrogen peroxide substrate (Vector Lab, Burlingame, CA) for 5-8 min and after washing in tap water counterstained in Harris hematoxylin for 5 min. Negative control sections were incubated with control isotype IgG. Photography was performed using a microscope (Leica, Wetzlar, Germany) with Magnafire photomicrographic equipment (Optronics, Goleta, CA).

**Proliferation assay**

The BrdU labeling and detection kit measures cell proliferation by quantitating BrdU incorporated into the newly synthesized DNA of replicating cells. The incorporated BrdU can be detected by a quantitative cellular enzyme immunoassay (Biotrak, Amersham Pharmacia Biotech Ltd, UK) using mAbs directed against BrdU. Briefly, the endometrial stromal cells were cultured in 96-well microtiter plates (10^4 cell/well). Cells are allowed to attach for 3 h and then cultured with 5% charcoal stripped serum media overnight. After a 12 h pre-incubation period without serum, the respective cells were treated with E2 10^-9 M DPN 10^-8 and 10^-5 M in a serum-free medium and incubated for an additional 24 h. After that, the cells were labeled with 10 µmol/l BrdU (100 µl/well) and incubated for 4 h at 37°C. The cells were fixed and genomic DNA was denatured by adding 200 µl/well of blocking reagent (1:10) for 30 min at room temperature. Peroxidase-labeled anti-BrdU antibody (1:100) was added (100 µl/well) and incubated for 90 min at room temperature. After washing three times, TMB (3,3',5,5'-tetramethylbenzidine) substrate solution was added (100 µl/well) and incubated for 15 min at room temperature for color appearance, and finally optical density was measured using a microplate reader at an absorbance of 450 nm. The absorbance values correlated directly to the amount of DNA synthesis and thereby to the number of proliferating cells in culture.

**Data presentation and statistical analysis**

ERE luciferase assay data are expressed as mean± standard deviation (SD) for at least three independent experiments. The number of experiments is represented as “n” in the Results section below. As normalized data were expressed as a percentage of controls, the results were compared with non-parametric Mann-Whitney or Kruskal-Wallis tests using Minitab software. Two tailed tests with P<0.05 were considered significant.
RESULTS

ERβ mRNA expression in human endometrium:

The results of RT-PCR analyses of ERβ expression in the endometrium of women with endometriosis or without endometriosis are summarized in Table 1 (E stands for endometrium, O stands for endometriosis lesion). ERβ mRNA is expressed in 7 out of the 8 endometrial samples. The two endometriosis lesions also express ERβ mRNA. Fig. 1 is a photograph of a representative gel.

Immunohistochemistry

For detection of ERβ protein expression in human endometrium and endometriosis lesions, immunohistochemistry was performed. Human ovary was used as a positive control (Saunders, Millar et al. 2000) and isotype-matched mouse IgG was used as a negative control in all staining experiments. Fig. 2A shows the ovary stained with ERβ antibody; positive granulosar cells are indicated with an arrow. Nuclear staining is found in granulosar cells and theca cells. Fig. 2B shows the results of normal cycling human endometrium; the positive endothelial cells are indicated with an arrow. Fig. 2C shows an endometriosis lesion with ERβ staining. As can be seen, neither endometrial stromal cells nor epithelial cells are positive for ERβ; positive staining is found in some endothelial cells.

ERE reporter assay: (N=4)

Since the immunohistochemical study showed no expression of ERβ protein in normal or pathological endometriosis, we designed an additional study to determine whether there is functional ERβ in human endometrium. Primary cultures of human endometrial stromal cells were transfected with plasmid containing the luciferase reporter gene under the control of ERE. After transfection, cells were treated with 17β estradiol or a specific ERβ agonist (DPN), with or without co-transfection of ERβ or ERβ genes. β-galactosidase plasmid served as a transfection control. An ERE construct without any treatment served as negative control; co-transfection of ERα and ERβ expression vectors served as positive control. DPN plus ERE luciferase were to test whether there is any functional ERβ in endometrial stromal cells. The results are summarized in Table 2, and show that there is no functional ERβ in endometrial stromal cells.

BrdU incorporation assay: (n=5)

Treatment of endometrial stromal cells with 17β estradiol at 1 nM significantly increased BrdU incorporation by 33±56%, DPN at 10 µM significantly inhibited BrdU incorporation by 24±25%, while at the concentration of 1 nM and 10 nM, BrdU incorporation was 102±6% and 121±32%, respectively, and there was no significant difference compared with vehicle control (Fig. 3).

DISCUSSION

Endometriosis is a debilitating disease that is not adequately treated by currently available medical therapies. The effectiveness of a specific ERβ ago-
Fig. 2. Immunohistological staining of human endometrium with ERβ antibody. (A) Normal cycling human ovary served as positive control; the arrow indicates positive granulosar cells and theca cells. (B) Normal human endometrium stained with monoclonal ERβ antibody; positive endothelial cells are indicated with an arrow. (C) An ovarian endometriosis lesion stained with ERβ antibody. (D) Negative control stained with isotype control IgG.

Fig. 3. Effects of 17β estradiol or the ERβ agonist DPN on the cell proliferation ability of human endometrial stromal cells. Human endometrial stromal cells were treated with 1 nM 17β Estradiol or DPN (10 µM, 1 nM and10 nM, separately) for 24 h and the content of BrdU incorporation was detected with the BrdU labeling and detection kit. The shaded bars and error bars represent the means and standard deviations, respectively, determined from at least three independent assessments of 100 cells each in a single experiment; similar results were obtained in three independent experiments. Control-untreated group, 1-1 nM 17β estradiol-treated group, 2-10 µM DPN-treated group, 3-1 nM DPN-treated group, 4-10 nM DPN-treated group; # indicated P<0.05 compared with control groups.
nist in treating endometriosis in the rodent model has suggested that this compound may be a promising new treatment in combating the disease (Harris, Bruner-Tran et al. 2005). The ERβ agonist is effective not only in intact mice, but also in nude mice and ovariectomized mice, which suggests that the possible mechanism that caused the regression of endometriosis is not mediated by the immune or endocrine systems. The current study tested the hypothesis that an ERβ agonist might have an inhibitory effect on endometriosis by acting directly on the ectopic endometrium.

In this study, the mRNA expression of ERβ in human endometrium is in agreement with previous investigations (Brandenberger et al., 1999; Critchley et al., 2002). However, immunohistochemistry failed to demonstrate that ERβ protein was expressed in ectopic endometrium, aside from its presence in endothelial cells. This absence of ERβ is in agreement with the findings of some authors (Fazleabas et al., 2003), but in conflict with others (Critchley et al., 2002). Recently, Xue et al. found that the ERβ mRNA was strikingly higher (~34-fold) in endometriotic stromal cells, whereas it was much lower or nearly absent in endometrial stromal cells (Xue et al., 2007). The presence of ERβ in endometrial endothelium is in agreement with Critchley et al. (2001). The discrepancies of ERβ expression may be relevant for tissues from different sources, the detection methods and the detection levels.

In an attempt to determine which of these explanations is correct, we used an ERE reporter construct to test for functional ERβ in endometrial stromal cells. This approach was expected to be more sensitive than immunohistochemistry for detection of ERβ protein expression. Although the experiment showed endogenous ERβ expression, as well as a strong and consistent response of the ERE construct to co-transfection of ERα and ERβ, no endogenous ERβ activation of the ERE was identified in response to the ERβ specific ligand. Our results indicate that the ERβ protein is not present in human endometrial stromal cells and epithelial cells, although the mRNA is transcribed. Eukaryotic cells possess a variety of means by which they are able to regulate the expression of the genes they contain. These mechanisms include control at the level of transcription, differential splicing, regulation of the nuclear export of transcripts, modulation of mRNA stability and changes in the rates of translation of specific mRNAs. Although transcriptional regulation is obviously crucial, the control of gene expression also relies heavily on translational selectivity and the mechanism of protein synthesis provides the cell with a variety of sophisticated and subtle means to modulate the rates of production of key proteins, independently of events in the nucleus. We hypothesize that either the ERβ protein is not translated or the expression of the ERβ protein is not detectable by immunostaining.

Recently, some researchers suggested that ERβ agonists might be useful as a novel therapeutic approach to improve ovarian function in sub- or infertile women (Hegele-Hartung, Siebel et al. 2004; Zhao, Dahlman-Wright et al. 2008). Another study showed that an ERβ-selective agonist is effective against inflammatory pain and this may contribute to the endometriosis-related pelvic pain (Leventhal, Brandt et al. 2006). In our study, the ERβ agonist DPN inhibited BrdU incorporation at 10 µM but not at 10 nM, while the EC50 values for E ERβ was 66 nM, and there was no detectable ERβ protein in the endometrial stromal cells. These results indicate that the significant inhibition of proliferation with 10 µM DPN might not mediated by ERβ.

Previously we reported that endometrial stromal cells express an aryl hydrocarbon receptor (AhR) (Zhao, Pritts et al. 2002). AhR is known to cause G1 arrest and proliferation inhibition in other cell types (Laiosa, Wyman et al. 2003; Jin, Jung et al. 2004). Further studies need to be performed in order to investigate whether the proliferation inhibition and regression of endometriosis caused by an ERβ agonist is also mediated by AhR.

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