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Changes in glandular and stromal estrogen and progesterone receptor isoform expression in eutopic and ectopic endometrium following treatment with the levonorgestrel-releasing intrauterine system

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ABSTRACT

Objectives: The levonorgestrel (LNG) intrauterine system (LNG-IUS) has been shown to improve symptoms in women with minimal to moderate endometriosis. The precise mechanism for this is unknown. We hypothesized that this involves alteration in the expression of estrogen receptors (ER) and progesterone receptors (PR).
Study design: A prospective study of tissues obtained prospectively from 28 women with laparoscopically confirmed minimal to moderate endometriosis treated with LNG-IUS for 6 months. Endometrial and endometriotic biopsies obtained before and 6 months after treatment were processed and stained for ER-α, ER-β and PR expression by immunohistochemistry. Photographs were obtained and the receptors quantified.
Results: The mean (±SD) age of the 28 women was 31 ± 7.2 (range 18–42) years. Eight of them at initial biopsy were in the proliferative phase and 20 in the secretory phase. ER-α, ER-β and PR expression decreased significantly in the glandular ($P < 0.0001$) and stromal ($P < 0.0001$) compartments of the eutopic endometrium after treatment with LNG-IUS. Similarly, ER-α, ER-β and PR were significantly decreased in the stromal compartment of ectopic endometrium ($P < 0.0001$), and significantly decreased in the ectopic glands of ER-α ($P < 0.0001$), ER-β ($P = 0.0002$) and PR ($P = 0.0064$) expression.
Conclusion: The ameliorative effect of LNG-IUS on the symptoms of minimal to moderate endometriosis is likely modulated through a decrease in the expression of glandular and stromal ER-α, ER-β and PR in the ectopic endometrium.

1. Introduction

Endometriosis, which affects 5–10% of women of reproductive age, is characterised by the presence of viable and hormone responsive endometrial glands and stroma outside the uterus [1]. It is commonly referred to as a condition of theories with regard to its pathogenesis. No single theory can explain its varied locations. Transplantation, implantation and growth of viable endometrial cells found in retrograde menstrual debris is however the most widely accepted theory [2,3].

The gold standard medical treatment option for minimal to moderate endometriosis is gonadotropin-releasing hormone (GnRH) analogues [4]. Hypo-estrogenic adverse effects, however, including osteopenia and vasomotor symptoms, limit their acceptability and duration of administration. Consequently, treatment with GnRH analogues alone is often limited to 6 months, although longer duration of treatment with add-back hormone therapy is acceptable [4]. While systemic progestogens administered parentally are effective, high circulating levels of these progestogens are commonly associated with side effects such as weight gain, oily skin, depression and breast tenderness – factors which significantly affect compliance and thus continuation rates [5,6].

The levonorgestrel intrauterine system (LNG-IUS), which delivers the progestin levonorgestrel (LNG) into the uterine cavity at a steady rate of 20 μg/day, has been shown in several pilot studies to significantly improve symptoms of endometriosis [7–9]. The LNG-IUS has several advantages over other treatment options including duration of action (5 years), fewer progestogenic side effects and independence from regular intake by the individual, when inserted in women with menorrhagia [7–10].

The released levonorgestrel has been shown to suppress estrogen-induced changes in eutopic endometrium by directly

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decreasing the number of estrogen receptors (ER), leading to an inactive epithelium [11], glandular atrophy and pseudo-decidualisation of the stroma within 1–3 months of insertion [10,12]. Whether this mechanism of action is similar in the ectopic endometrial tissue in women with endometriosis is uncertain. Eutopic and ectopic endometrium are known to contain ER and progesterone receptors (PR), and significant levels of LNG have been demonstrated in the serum and peritoneal fluid of women on LNG-IUS used as treatment for symptomatic endometriosis [13]. We therefore hypothesized that the LNG from this device is effective in women with endometriosis by its action on these receptors.

The aim of this study was therefore to examine changes in ER-α, ER-β and PR expression in eutopic and ectopic endometrial tissue from women who were on the LNG-IUS for the symptomatic treatment of minimal to moderate endometriosis.

2. Material and methods

This was a study of prospectively collected tissues from women attending the gynaecology outpatient department of the University Hospitals of Leicester NHS Trust, from April 2000 to March 2002, with suspected symptoms of endometriosis. Those who met the inclusion criteria were recruited into the study after giving a written signed informed consent. These criteria included laparoscopically confirmed minimal to moderate endometriosis (American Fertility Society, 1985), not on any form of contraception or hormonal treatment for the preceding 3 months, not planning a pregnancy for the following 6 months, willingness to undergo a second laparoscopy after 6 months of treatment, no contra-indications to an intrauterine device, BMI < 27 kg/M^2 and between 18 and 45 years of age. The exclusion criteria included infertility as a symptom, a clinical history of pelvic inflammatory disease (PID) and contra-indications to the use of the levonorgestrel-releasing intrauterine system (LNG-IUS or Mirena® Bayer, Newbury, Berkshire, United Kingdom). The study was approved by the Leicestershire, Northamptonshire and Rutland (LNR) Research Ethics Committee.

The trial protocol has already been published [14]. Briefly, this consisted of an initial laparoscopy to confirm the diagnosis of endometriosis after which biopsies of the eutopic and ectopic endometrium were performed followed by the insertion of the LNG-IUS. Six months later, a second-look laparoscopy was performed and the biopsies repeated. Thirty-four women were recruited and 28 had the second-look laparoscopy.

Paraffin embedded tissue blocks (eutopic and ectopic endometrium) were identified from stored tissues collected from the women who had participated in the prospective study already published [14]. Four tissue blocks in total were identified from each woman, two taken before and two taken 6 months after insertion of the LNG-IUS. Breast tissues, obtained from women undergoing breast reduction surgery, were used as positive controls. The negative controls were generated by omitting the ER-α, ER-β or PR antibodies in these tissues.

2.1. Immunohistochemistry

All tissues were fixed in 10% neutral-buffered formalin, and paraffin embedded blocks made using routine methods. Sections cut at 4 μm thickness were mounted on silane coated slides and dried thoroughly before immunohistochemistry. Briefly, slides were deparaffinised in xylene and then hydrated through a graded series of ethanol solutions. The antigens were unmasked by immersing the sections in sodium citrate (Sigma–Aldrich, United Kingdom) buffer solution (10 mM solution, pH 6), and by heating them at 800 W in a microwave for 30 min. Endogenous peroxidase activity was blocked with 6% hydrogen peroxide solution for 10 min and slides were briefly washed in running tap water for 5 min at room temperature. The slides were then allowed to cool for 20 min and washed in running tap water for 5 min. Non-specific binding sites were blocked first in Tris-buffered saline supplemented with 0.1% bovine serum albumin (TBS/0.1% BSA), pH 7.6 with a stirrer. This was followed by the addition of a 1:10 dilution of normal rabbit serum [(NRS) Dako, Glostrup, Denmark] in TBS/0.1% BSA. After 30 min, slides were drained and endogenous biotin sites blocked with avidin–biotin blocking solutions according to the manufacturer's instructions (Vector Laboratories, Burlingame, California, USA). The slides were next washed in TBS/0.1% BSA for 5 min. The sections were then incubated with PR primary monoclonal antibody NCL-PGR-AB; 1:40 dilution (Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom) or ER-α (NCL-ER-6F11) 1:50 dilution (Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom) or ER-β (clone 14C8) 1:200 dilution (Abcam, Cambridge, United Kingdom) overnight at 4 °C, in a humid chamber. Sections were next washed in TBS/0.1% BSA for 20 min with stirring followed by incubation with biotinylated rabbit anti-mouse immunoglobulin (Dakocytomation, Glostrup, Denmark) diluted to 1:400 in TBS/0.1% BSA at room temperature for 30 min, before being washed in TBS/0.1% BSA for 20 min. Secondary antibodies were amplified with horse-radish peroxidase conjugated avidin–biotin complexes for 30 min according to the manufacturer's instructions (ABC Elite Vector Laboratories, Burlingame, California, USA). Antibody binding was visualised using 3,3′-diaminobenzidine tetrahydrochloride (DAB) for 5 min according to the manufacturer's instructions (Sigma, Poole, United Kingdom). Finally, sections were lightly counterstained with Mayer's haematoxylin (Sigma–Aldrich, United Kingdom) for 40 s, dehydrated through a series of ethanol solutions, cleared through xylene and mounted in Permount mounting medium (DPX, Fluka, Buchs, Switzerland).

2.2. Histomorphometric analysis

Images of immunoreactive sections were captured on an Axioplan compound microscope equipped with a Sony analogue colour video camera (Model DXC-151P, Sony; Japan) and Axiovision version 4 image capture and analysis software (Zeiss, Milton Keynes, Bucks, United Kingdom) at 40× magnification. Ten random views were taken from each slide and all the nuclei counted using a 10×25 graticule. The glandular and stromal cell compartments were analysed separately. Positive cells were identified by the presence of a brown nuclear stain of ER-α, ER-β or PR and the negative cells by the presence of a strong blue stained nucleus. Nerve cells and blood vessels were excluded from the analyses. The number of positive cells was obtained by counting the brown nuclei and the number of negative cells by counting the blue nuclei and the percentage positivity assessed from the total. Expression level was then determined by dividing the stained nuclei by the total stained and unstained nuclei and multiplying by 100 to generate a percentage.

2.3. Statistical analysis

The results are expressed as mean and standard errors of the mean (SEM). Normality of the data was determined using the Shapiro-Wilk test and because the data were not normally distributed, non-parametric statistical analysis (Mann–Whitney *U*-test) was performed to determine differences in the percentages of positive ER-α, ER-β and PR expression in eutopic and ectopic endometrium before and 6 months after treatment with LNG-IUS. Statistical analyses were performed with the aid of Graphpad