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ORIGINAL PAPER

c-fos gene and protein expression in pelvic endometriosis: a local marker of estrogen action

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Abstract Endometriosis is an estrogen-dependent disease, causing pelvic pain and infertility. *c-fos* is an early transcription factor that has been reported to be related to estradiol-dependent cell proliferation. The aim of the present study was to assess the *c-fos* gene and protein expression in pelvic endometriotic implants in comparison to normal endometrium from infertile women. An open, prospective and controlled study included 15 infertile women with endometriosis and 19 control infertile women. Endometrial and endometriotic biopsies were performed at the follicular phase and the samples were processed for RT-PCR and immunohistochemistry. ERα mRNA levels were similar in the endometriotic implants/eutopic endometrium from women with endometriosis and in normal tissue ($P = 0.649$). The aromatase gene, however, was not expressed in the eutopic endometrium from either control or endometriosis groups, and was only expressed in 50% of endometriotic implants ($P = 0.044$). *c-fos* gene expression was higher in endometriotic implants (1.32 ± 0.13; $P = 0.011$) than in eutopic endometrium from patients with endometriosis (0.97 ± 0.11) or from the control group (0.91 ± 0.05). In addition, immunohistochemistry showed a more abundant distribution of c-Fos in the stroma of endometriotic tissue compared to eutopic endometrium. These data suggest that *c-fos* may play a role in the molecular mechanisms of estrogen action on the induction, promotion or progression of endometriosis.

Keywords Endometriosis · Estrogen · *c-fos* · Gene expression · Aromatase

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Introduction

Endometriosis is a common disorder in women of reproductive age that has been associated with pelvic pain and infertility. Endometriosis is characterized by the presence of endometrial tissue outside the uterus, mainly in the pelvic peritoneum. A prevalence of 6–10% in general female population and 35–50% in women with pain and/or infertility has been reported (Eskenazi and Warner 1997). While the etiopathogenesis of endometriosis is still not clearly established, the ectopic implantation from refluxed menstrual tissues, as reported by Sampson (1927) remains the most widely accepted theory. Immunologic alterations, genetic factors, and environmental factors also seem to play a role in the development of endometriosis (Yang et al. 2004). Moreover, intrinsic molecular defects in eutopic and/or ectopic endometrium such as aberrant expression of aromatase or an inappropriate expression of stimulatory transcription factors appear to be involved. Clinical observations and laboratory evidence suggest that estrogens play a paramount role in the establishment and maintenance of endometriosis (Giudice and Kao 2004; Othman et al. 2007).

Endometriosis is associated with increased secretion of pro-inflammatory cytokines, growth and angiogenic factors

which might be responsible for maintenance of the disorder and impairment of reproductive function. The *c-fos* and *c-jun* genes, members of the early gene family, are involved in the regulation of the transcription process by a complex of nuclear proteins known as AP-1. Recent studies suggest that AP-1 proteins control cell life and death through their ability to regulate the expression and function of cell cycle regulators (Shaulian and Karin 2001; Crowe et al. 2000). In addition, upon inflammatory conditions, a number of pro-inflammatory cytokines seems to be positively regulated by c-Fos (Asschert et al. 1999; Lee et al. 2004).

c-fos gene has also been reported to be related to estradiol-dependent cell proliferation (Crowe et al. 2000). *c-fos* codes a nuclear associated protein that amplifies the estrogen message by activating the transcription of other genes that control cell division. An estrogen-responsive element has been identified in the promoter region of the human *c-fos* gene and this finding supports the hypothesis that estrogen increases *c-fos* mRNA levels by directly stimulating *c-fos* gene transcription (Weisz and Rosales 1990). Moreover, Hennessy et al. (2005) have shown a rapid estradiol effect on the phosphorylation of CREB and ELK1 transcription factors and its subsequent binding to the *c-fos* promoter and transcription activation in T84 colonic carcinoma cells. In a previous study, we have shown that both *c-fos* gene and protein expression are induced in human endometrium during the proliferative phase of the human cycle and that *c-fos* gene expression presented a significant correlation with the circulating estradiol concentrations (Reis et al. 1999).

Therefore, the aim of the present study was to assess *c-fos* gene and protein expression in endometriotic implants in comparison to normal endometrium from infertile women. In order to better characterize the local estrogenic environment, we also measured two upstream components of the cascade, namely the estrogen receptor alpha (ERα) and aromatase, which is the enzyme involved in local estrogen synthesis.

Materials and methods

Patients

Thirty-four infertile patients undergoing complete diagnostic workup for couple infertility were selected for this study on a consecutive basis. Inclusion criteria were (1) the need of diagnostic laparoscopy and (2) no use of hormonal medication in the previous 3 months. They were allocated in two groups according to the histological and laparoscopic findings: pelvic endometriosis ($n = 15$) and control ($n = 19$). The study did not include any patient with uterine fibroids, adenomyosis, or active pelvic inflammatory disease.

Endometriosis was defined as the ectopic presence of endometrial glands and/or stroma and it was confirmed by histology in all patients with suspected lesions at laparoscopy. All patients had stage III or IV, according to the revised classification of the American Society of Reproductive Medicine (ASRM 1997). The size of the lesions ranged from 1 to 7 cm (median = 3 cm) and the localization included ovarian ($n = 8$) and peritoneal ($n = 7$) implants.

Laparoscopy with biopsy of the endometriotic implants and a concomitant aspirative biopsy of the eutopic endometrium were performed on the first half of the menstrual cycle. A single sample of endometriotic tissue was obtained from the largest lesion. The laparoscopic evaluation was always done by the same surgical team, composed by three experienced surgeons, including one of the study authors (M.M.C.).

Samples were fractioned and one portion was immediately frozen in liquid nitrogen and stored at −80°C until mRNA extraction, while the other was fixed in 10% buffered formalin and embedded in paraffin for subsequent histological diagnosis and immunohistochemistry. The study protocol was approved by the local Ethics Committee, and informed consent was obtained from every subject.

Study protocol and assays

Body mass index (BMI = current measured weight in kg divided by height in m^2) and waist-to-hip ratio (waist in cm divided by hip in cm) were obtained. Blood samples were drawn on the first half of the menstrual cycle for determination of sex hormone binding globulin (SHBG). SHBG concentrations were measured by chemiluminescent enzyme immunoassay (CLEIA) (DPV, Los Angeles, CA). The SHBG assay had a detection limit of 0.7 mIU/ml and intra- and inter-assay CV was 5.2 and 8.0%, respectively.

RT-PCR protocol

Total RNA extraction and cDNA synthesis were carried out as previously described (Oliveira et al. 2003). Total RNA was extracted by Trizol (Gibco BRL, Gaithersburg, MD) and the first strand cDNA was synthesized from 5 μg total RNA, using the SuperScript Preamplification System (Invitrogen®, Carlsbad, CA) according the manufacturer's instructions. PCR was carried out in a final volume of 50 μl. One tenth of the first strand cDNA synthesis product was denatured at 94°C for 1 min in the presence of 20 mM Tris–HCl pH 8.4 plus 50 mM KCl and 1.5 mM $MgCl_2$. Then, 1.25 U of Taq DNA polymerase was added together with the same Tris–HCl buffer, 1.5 mM $MgCl_2$, 0.4 μM sense and antisense primers and 0.2 mM dNTP mix. A 442 base pair (bp) fragment of the *c-fos* (Rossi et al. 1996),

a 133 bp fragment of the aromatase and a 483 bp portion of the ERα cDNA sequence were amplified using primers designed to span intron-exon borders so as to prevent amplification of any contaminating genomic DNA. Amplification of a 623 bp cDNA fragment corresponding to the ubiquitously expressed protein β2-microglobulin (β2-m) was performed to adjust the cDNA amounts in each sample (Gussow et al. 1987). The cDNA sequences of the *c-fos*, aromatase and ERα are those found at GeneBank accession nos. V01512, NM 000103 and NM 000125, respectively. The standardization of PCR reactions was done by testing a number of cycles (20–45) and the amplification was performed in the linear range. Final PCR conditions were as follows: 30 cycles (2 min 94°C, 30 s 94°C, 45 s 54°C, 90 s 72°C, 5 min 72°C) for *c-fos*, 35 cycles (2 min 94°C, 30 s 94°C, 45 s 56°C, 30 s 72°C) for aromatase, 35 cycles (2 min 94°C, 1 min 94°C, 30 s 65°C, 1 min 72°C, 5 min 72°C) for ERα and 30 cycles (2 min 94°C, 1 min 94°C, 1 min 55°C, 1 min 72°C, 5 min 72°C) for β_2-m. cDNA from placenta was used as a positive control for all PCR reactions. The product of a first strand reaction performed without reverse transcriptase was also amplified to serve as negative control. A sample of the PCR was size-fractionated on a 1.5% agarose gel stained with ethidium bromide, run at 100 V and visualized under UV light. The expected bands were quantified by densitometric analysis using an image-processing system (ImageMaster VDS, Pharmacia Biotech, and Uppsala, Sweden).

Immunohistochemistry

Immunohistochemistry was performed using the avidin-biotin-peroxidase method, as previously described (Reis et al. 1999). Briefly, antigen retrieval was enhanced by boiling the slides for 5 min in 0.01 M citrate buffer, pH 6.0, followed by incubation at room temperature and PBS washing. After exposure to 1% H_2O_2 in methanol to block endogenous peroxidase, sections were treated with normal goat serum for 30 min to suppress nonspecific binding. Rabbit anti-human phosphorylated c-Fos (phospho T232, purchased from Abcam, Cambridge, UK) was diluted 1:80 and applied for 48 h at 4°C. All samples were run together, including negative controls in which the primary antibody was replaced by nonimmune rabbit serum (Calbiochem, Merck Biosciences) at the same dilution. The sections were then counterstained with hematoxylin, dehydrated and mounted.

The intensity of immunostaining was graded in absent, mild, moderate or strong and converted to H-score (% of cells stained mild + 2× % of cells stained moderate + 3× % of cells stained strong) at two random fields at 400× magnification (McClelland et al. 1990).

Statistical analysis

Data are described as means and standard error of the mean (±SEM). Differences between group means were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's test. Differences were considered statistically significant at $P < 0.05$. All analyses were performed using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA).

Results

All biopsy samples were available for endometrial dating and 27 samples were also suitable for RT-PCR, 11 of which belonged to the control group and 14 to the endometriosis group (eight samples of endometriotic tissue and six of eutopic endometrium). Those were the samples of sufficient size to allow good quality RNA extraction along with histological and immunohistochemical preparations.

Patients from both groups of infertile control women and infertile women with endometriosis presented similar ages (32.9 ± 0.9 years and 31.7 ± 1.5 years, control and endometriosis groups, respectively, $P = 0.47$), BMI (24 ± 0.9 and 22 ± 0.7 kg/m^2, $P = 0.08$) and WHR (0.85 ± 0.04 and 0.76 ± 0.02, $P = 0.10$). SHBG level did not differ between groups (47.6 ± 5.5 and 42.8 ± 4.2 nmol/l, $P = 0.50$), and estradiol concentrations were all in the normal range.

Figure 1 shows the gene expression of ERα and aromatase. ERα mRNA levels were similar in the endometriotic implants/eutopic endometrium from women with endometriosis and in normal tissue ($P = 0.649$). The aromatase gene, however, was not expressed in the endometrium from either control or endometriosis groups, and was only expressed in 50% of endometriotic implants ($P = 0.044$); (Fig. 1).

Figure 2 shows that the *c-fos* gene expression was higher in endometriotic implants (1.32 ± 0.13; $P = 0.011$) in comparison with both the endometrium from control group (0.91 ± 0.05) and eutopic endometrium from the endometriosis group (0.97 ± 0.11).

Figure 3 shows the immunohistochemical localization of phosphorylated c-Fos in representative biopsies of endometrium and endometriosis. Nuclear staining was observed at both stromal and epithelial compartments, with scattered distribution in normal endometrium and in eutopic endometrium from women with endometriosis. In the endometriotic tissue, however, c-Fos immunostaining was more abundant, especially in the stroma and in the vascular endothelium. The median H-scores of phyosphorylated c-Fos immunostaining in the stroma were 134, 156, and 260 in the control, eutopic and ectopic endometrium, respectively. Negative controls did not stain at all.

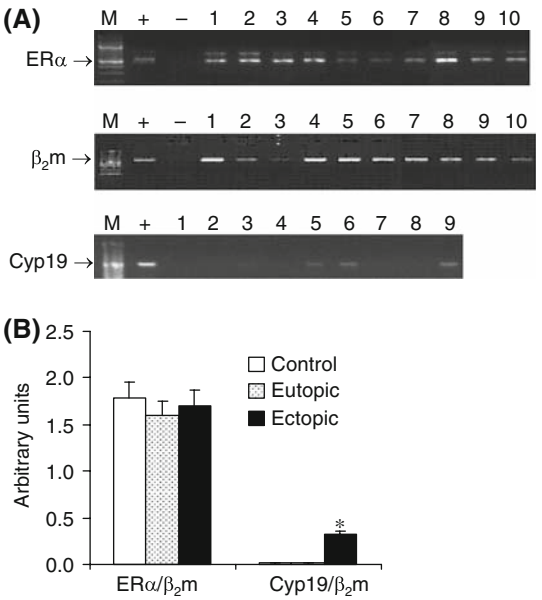

Fig. 1 **a** Representative gel showing ERα and aromatase mRNA levels determined by RT-PCR from normal endometrium and endometriosis. 483, 133 and 623 bp fragments corresponding, respectively, to ER-α, aromatase and β2-microglobulin (β2-m). RT-PCR products are visualized in agarose gel stained with ethidium bromide. The number across the top of the gel refers to the patients. ER-α: *Lines 1–4* control (*C*) *Lines 5–7* eutopic endometrium (*EU*) *Lines 8–10* Ectopic endometrium (*ECT*); aromatase (cyp19) *Lines 1–2* = C *lines 3–4* = EU *lines 5–9* = ECT. **b** ER-α and aromatase mRNA semi-quantitative results in relation to β2-m optical density (arbitrary units) of bands (mean ± SEM). $^*P = 0.044$ aromatase in ECT vs. C and EU (one-way ANOVA followed by Duncan's test)

Discussion

Estrogens are among the main hormonal regulators of human endometrium, and are associated with clinical diseases such as endometriosis. However, the mechanisms involved in the development of endometriosis and their associations with estrogen-dependent specific molecular defects are still unclear. In the present study, we focused on molecular aspects related to the estrogen action and metabolism in eutopic and ectopic endometrial samples from infertile women with and without endometriosis.

Both groups were similar in terms of age and anthropometric variables related to adiposity and corporal composition that might influence circulating estrogen concentrations. Moreover, serum SHBG levels, a biological marker of systemic estrogen action were also similar in both groups. Therefore, these data suggest that circulating estrogen levels do not seem to be related to the molecular results obtained in the present study.

It has been hypothesized that estrogen acts via a single receptor, ERα, in the formation and maintenance of endometriotic lesions (Matsuzaki et al. 2001; Dotzlaw et al. 1997). In the present study, ERα gene expression did not differ in ectopic and eutopic endometrium from patients with endometriosis or in infertile control women. These results confirm those from other authors who, in addition, did not observe differences in ER distribution during the menstrual cycle in endometriotic tissue (Fujishita et al. 1997; Matsuzaki et al. 2000). While aromatase is the main enzyme responsible

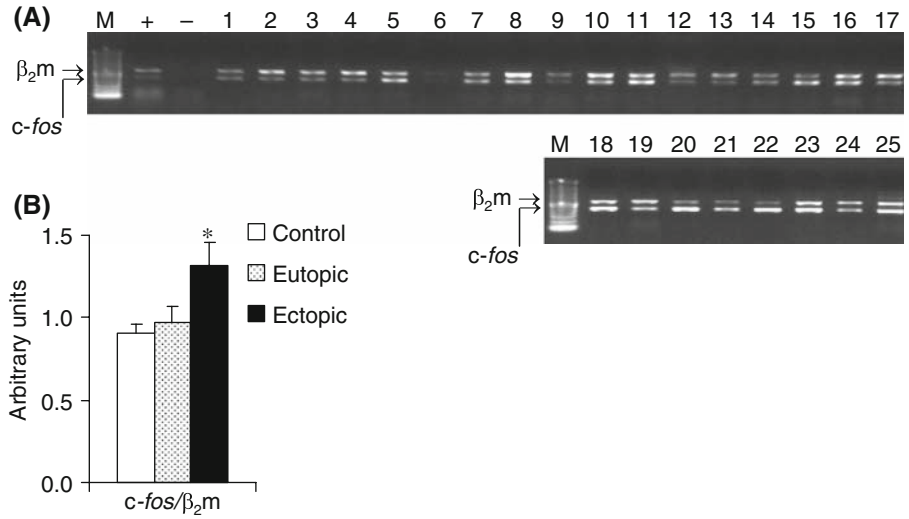

Fig. 2 **a** Representative gel showing *c-fos* mRNA levels determined by RT-PCR from normal endometrium and endometriosis. 442 and 623 bp fragments corresponding, respectively, to *c-fos* and β2-microglobulin (β2-m) RT-PCR products are visualized in agarose gel stained with ethidium bromide. The number across the top of the gel refers to the patients. *Lines 1–11* control (*C*) *lines* 12–17 eutopic endometrium (*EU*) lines 18–25 = ectopic endometrium (*ECT*). **b** *c-fos* mRNA semi-quantitative results in relation to β2-m optical density (arbitrary units) of bands (mean ± SEM). $^*P = 0.011$ *c-fos* in ECT vs. EU and C (one-way ANOVA followed by Duncan's test)

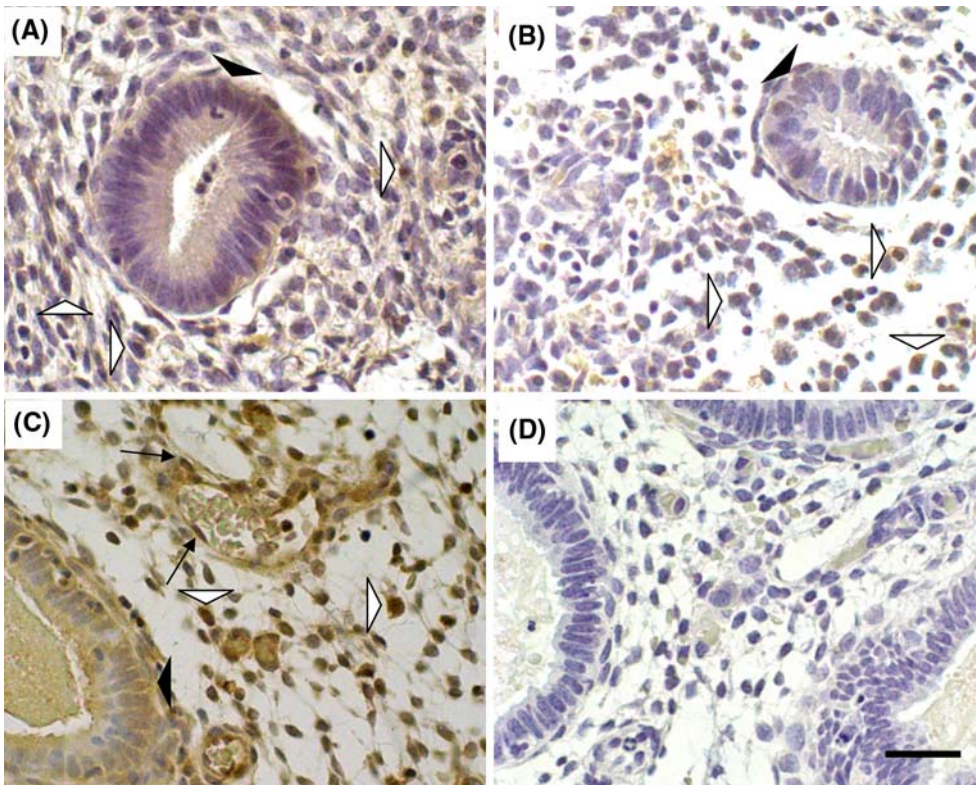

Fig. 3 Immunohistochemical localization of phosphorylated c-Fos. **a** Normal proliferative endometrium **b** eutopic endometrium from women with endometriosis **c** endometriotic tissue **d** negative control. Examples of positive nuclear staining are indicated in endometrial stromal cells (*white arrowheads*), glandular epithelial cells (*black arrowheads*) and vascular endothelium (*black arrows*). *Scale bar* 25 μm

for the local synthesis of estrogens, normal endometrial tissue does not express aromatase gene (Noble et al. 1996). Our data showing absence of aromatase transcripts in the eutopic endometrium from either control or endometriosis groups are in agreement with these studies. Aromatase gene was expressed, however, in around 50% of endometriotic implants. Aromatase could increase estrogen levels in the local ectopic tissues, thus promoting the growth and implantation of endometriosis, mediated by the ERα. Huang et al. (2005) showed a positive correlation between aromatase and ER proteins in different types of endometriosis including ovarian, vagina-rectum, peritoneum and uterine serous endometriosis tissues.

There is a dearth of studies reporting changes on *c-fos* gene expression in endometriosis. Luo et al. (2004), using an in vitro model of human endometrial cells, showed that GnRH agonists and TGFβ1 alter the endometrial expression of fibronectin, by means of a differential regulation of *c-fos* and *c-jun* transcriptional activation. Our results using RT-PCR and immunohistochemistry show that *c-fos* expression was higher in endometriotic implants in comparison with the eutopic endometrium from both control and endometriosis groups.

These changes in *c-fos* mRNA and phosphorylated protein levels might be related to an altered molecular mechanism in endometriotic tissue associated to higher local estrogen levels or disturbed estrogen metabolism or both. To our knowledge, this is the first description of *c-fos* mRNA and phosphorylated protein expression levels in endometriosis. However, an independent study published recently partially confirms our findings, by showing increased immunostaining of c-Fos protein in endometriotic lesions, although without specific assessment of the phosphorylated isoform (Pan et al. 2008).

In conclusion, data from the present study suggest that *c-fos* may play a role in the molecular mechanisms of estrogen action on the induction, promotion or progression of endometriosis. Further studies are needed to assess if *c-fos* gene expression may be a marker of the efficacy of suppressive treatment of endometriosis.

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