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Aromatase expression in endometriotic tissues and cell cultures of patients with endometriosis

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Cytochrome *P*-450 aromatase is responsible for catalysing the conversion of androstendione into estrone, so its expression in endometriotic tissue could contribute to the development of endometriosis. The aims of this study were, on the one hand, to determine the presence of aromatase in eutopic and ectopic endometrium, healthy peritoneum, myometrium and leiomyomas from patients with (n = 61) and without endometriosis (n = 12) and, on the other hand, to determine the effect of peritoneal fluid (PF), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNFα) on aromatase activity from endometriotic stromal cells and subcutaneous adipocytes. After immunohistochemical analysis, aromatase expression was detected in the endometriotic tissue of 61% of patients, whereas the rest of the tissues, as well as those from disease-free women, were negative. Cell cultures were made to determine aromatase activity in endometriotic stromal cells and adipocytes. The addition of PF, TNFα and especially IL-6 ($P < 0.05$) stimulated the basal enzymatic activity observed in both cell types. Our findings confirm the presence of aromatase in endometriosis and probably the existence of a local estrogen production that may be stimulated by some factors such as cytokines present in the PF of these patients. Therefore, the use of aromatase inhibitors combined with immunomodulator agents could be a novel approach to be investigated in future clinical trials.

Key words: aromatase/endometriosis/IL-6/peritoneal fluid/TNFα

Introduction

Endometriosis is a common estrogen-dependent disease characterized by the presence of functional endometrium outside the uterus, and it is frequently associated with the apparition of endometriomata. Although the aetiology of the disease is not well established, several immunological and biochemical alterations are present in these patients (Bulun *et al*., 1999; Lebovic *et al*., 2001).

Abnormal levels of many different cytokines such as interleukin-6 (IL-6), IL-8 or tumour necrosis factor-alpha (TNFα) have been described in the serum and peritoneal fluid (PF) of women with endometriosis, thus involving these cytokines in the progression of the disease and its associated infertility (Iwabe *et al*., 2002). PF with a high cytokine level from patients with endometriosis has shown embryotoxic activity *in vitro* (Gómez-Torres *et al*., 2002).

Molecular anomalies such as the aberrant expression of cytochrome *P*-450 aromatase have been described in eutopic and ectopic endometrium of some women with endometriosis (Noble *et al*., 1996). This enzyme is responsible for catalysing the conversion of androstendione into estrone in certain human tissues such as the placenta, gonads, adipose tissue, skin and brain. Owing to the hormone-dependent character of the disease, the presence of aromatase and the consequent local estrogen production may promote the growth of endometriotic implants. The aims of this study were to determine (i) the presence of aromatase in eutopic and ectopic endometrium, apparently healthy peritoneum, myometrium and leiomyomas from patients with and without endometriosis and (ii) the aromatase activity of stromal cells derived from endometriotic tissue and adipocytes from subcutaneous adipose tissue in culture stimulated with PF, IL-6 and TNFα. Both cytokines seem to be involved in the regulation of aromatase activity of breast tumour-derived fibroblasts (Singh *et al*., 1997).

Materials and methods

Patients and tissue sampling

Participants were 61 women with endometriomata, who underwent conservative or total surgery for the treatment of moderate-to-severe endometriosis and had not received any medical or surgical treatment for at least 3 months before surgery. The non-endometriosis group included 12 women undergoing surgery for benign non-hormone-dependent ovarian tumours. Informed consent was obtained from each woman and the study approved by the Ethical Committee and Institutional Review Board of San Juan University Hospital.

During surgery, the first course of action in all participants was the aspiration of 5–10 ml of PF into a sterile container without haematic contamination. Then, endometriosis was evaluated and catalogued according to the revised classification criteria of the American Society for Reproductive Medicine (ASRM) (1996). Samples of eutopic endometrium (54), endometrioma (87 from the 61 patients), peritoneal endometriotic implants (10), apparently healthy peritoneum proximal to endometriotic lesions (61) and fat from the abdominal wall (61) were taken. Samples of endometrium, peritoneum and fat were also collected from all the control patients. If a hysterectomy was carried out, endometrium and myometrium (10 from the endometriosis and three from the non-endometriosis group) were obtained from the surgical piece. A myomectomy was also performed in six cases of endometriosis, where leiomyomas were also collected to be investigated. An attempt was made for surgery to be carried out in the second half of the cycle for all cases, although it was not always possible because of operating theatre availability. PF was centrifuged at

600 *g* for 10 min to remove cells and cellular debris before adding to cell cultures. The rest of the sample was frozen and stored at –20°C.

Immunohistochemistry

Tissue samples were fixed with 10% paraformaldehyde solution, paraffin embedded and cut into 8-µm sections. To immunostain *P*-450arom, we deparaffinized the sections, hydrated in decreasing concentrations of ethanol, washed briefly in phosphate-buffered saline (PBS 0.01 M, pH 7.2) and incubated in hydrogen peroxide (3%, v/v) in methanol for 30 min to block endogenous peroxidase. After washing in PBS, sections were blocked in normal goat serum (Vector Laboratories, Burlingame, CA, USA) diluted 2% in PBS for 20 min at room temperature. Then, tissues were incubated with a mouse anti-aromatase monoclonal antibody (DPC Biermann, Bad Nauheim, Germany) diluted 1:50 in PBS overnight at 4°C. The characteristics and specificity of the antibody have been previously reported (Turner *et al*., 2002). A goat anti-mouse biotinylated secondary antibody (Vector) was diluted 1:200 in PBS and incubated with the tissue sections for 45 min at room temperature. Bound antibodies were visualized by incubating the sections with avidin–biotin–peroxidase–horse-radish peroxidase (ABC–HRP) complex (Vector) for 45 min followed by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA). Finally, sections were stained with Harris haematoxylin. Human placental sections were used as positive control for aromatase, whereas disease-free myometrium was considered as a negative control because this tissue lacks aromatase expression (Bulun *et al*., 1994; Noble *et al*., 1996).

Isolation and culture of stromal cells from endometriotic tissue

Fresh endometriotic tissue was collected in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12, 1:1, v/v; Sigma) for transport to the laboratory, where it was minced finely and digested with 2 mg/ml of collagenase (type IA; Sigma) in DMEM/F-12 at 37°C for 90 min on a shaking platform. At the end of the incubation, cell clumps were mechanically dispersed by repeated gentle pipetting. After enzymatic digestion, the cell suspension was filtered through a 250-µm nylon mesh filter to remove undigested tissue pieces.

The filtered fraction was suspended in 10 ml of fresh DMEM/F-12. Stromal cells were separated from clumps of epithelium by allowing the clumps to settle for a 30-min period at unit gravity. The top 8 ml of the medium (containing predominantly stromal cells) was removed, and the cells were collected by centrifugation at 300 *g* for 5 min. Then, the cells were washed in DMEM/F-12 at 300 *g* for 10 min and suspended in DMEM/F-12 supplemented with 100 IU/ml of penicillin G, 1 mg/ml of streptomycin, 2.5 mg/ml of amphotericin B, 1% glutamine and 10% fetal bovine serum. Then, the cells were dispensed into 24-well plates and were allowed to adhere to culture dishes for 20 h at 37°C in 5% CO_2 in air. The attached fibroblasts were washed carefully with DMEM/F-12 to remove non-adhering cells. Subsequently, complete medium (control) or complete medium supplemented with dexamethasone (200 nmol/l; Sigma), PF (10%, v/v), recombinant IL-6 (50 ng/ml; Sigma) or recombinant TNFα (10 ng/ml; Sigma) was added in duplicate (total volumes per well adjusted to 1 ml), and the cells were cultured for 48 h at 37°C in 5% CO_2.

Isolation and culture of adipocytes from subcutaneous tissue

Fresh abdominal subcutaneous adipose tissue was collected in DMEM/F-12, and then, it was minced finely and digested in DMEM/F-12 containing 2 mg/ml of collagenase type IA at 37°C for 60 min under shaking. After enzymatic digestion, the cell suspension was centrifuged at 360 *g* for 5 min, and the floating mature adipocytes were aspirated. After washing twice for 2 min at 360 *g*, adipocytes were dispensed into 24-well plates and incubated for 72 h at 37°C in 5% CO_2 in the same culture conditions as described above.

Aromatase activity assay

Aromatase activity was measured using a tritiated water-release (^3H_2O) assay by incubating intact fibroblasts and adipocytes in serum-free medium for 20 h with 20 pmol [1β-^{3}H]-androst-4-ene-3,17-dione (specific activity 15–30 Ci/mmol; PerkinElmer, Boston, MA, USA) at 37°C in 5% CO_2 with shaking. Activity is expressed as mean ± SEM femtomoles of product produced per milligram protein per incubation time (fmol ^3H_2O/mg protein/20 h), as reported previously (Lephart and Simpson, 1991).

Statistical analysis

All statistical calculations were performed using the Statistics Package for Social Sciences (SPSS) Windows Release 13.0 software package. The chi-square test was used to relate aromatase expression to the menstrual cycle phase or severity of patients. The non-parametric Wilcoxon test was used to compare the different stimulation conditions, whereas the non-parametric Mann–Whitney *U*-test was used to compare groups. $P < 0.05$ was considered statistically significant.

Results

Aromatase expression in endometriotic tissue

We observed positive immunohistochemical expression for aromatase in the stroma of endometriotic tissue from 37 of 61 patients included in the study (61%) (Figure 1A–C). In nine of them, the positive expression was only observed in endometrioma from one ovary

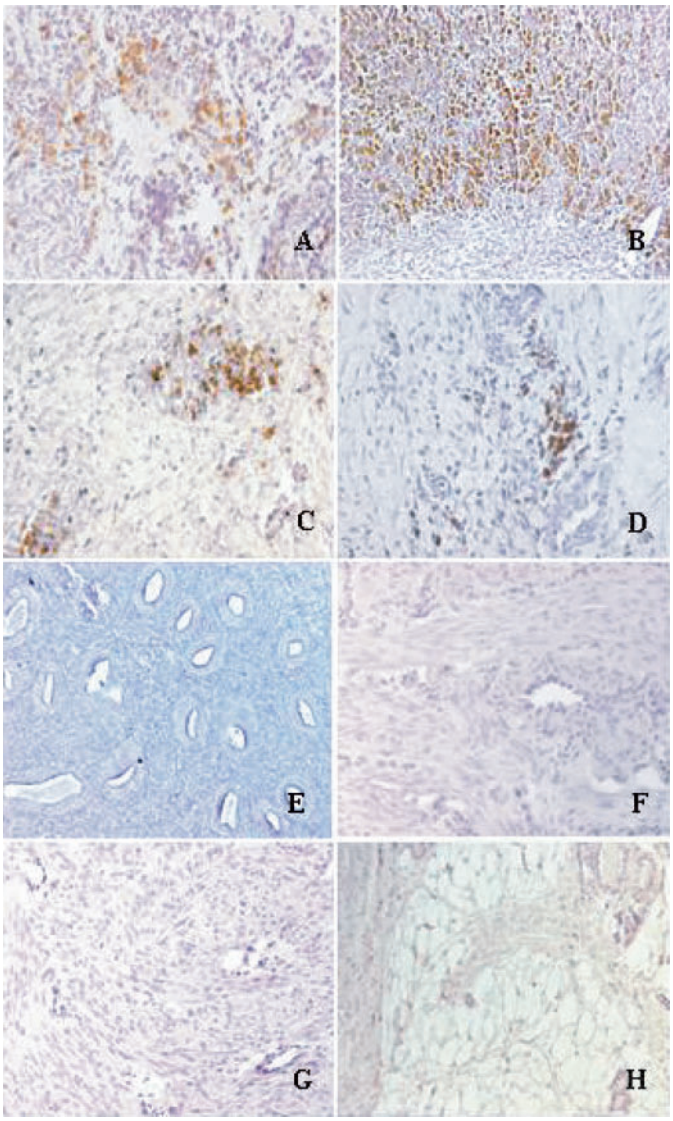

Figure 1. Immunostaining for aromatase in endometrioma (**A**–**C**), peritoneal endometriotic implant (**D**), eutopic endometrium (**E**), myometrium (**F**), leiomyoma (**G**) and healthy peritoneum (**H**) from women with endometriosis. Aromatase is immunolocalized in endometriotic stromal cells. No staining was observed in the rest of the tissues tested from patients with endometriosis as well as in all samples from disease-free women. Original magnification: **A**, **C**, **D**, ×200; **B**, **E**–**H**, ×100.

despite existing bilateral ovarian endometriomata. Peritoneal endometriotic implants were also collected in 10 cases of endometriosis. The implants showed positive staining in six patients who expressed aromatase in at least one endometrioma (Figure 1D). In the other four patients, peritoneal lesions, as well as ovarian endometriosis, were negative. Aromatase was not detected in endometrium (Figure 1E), myometrium (Figure 1F), leiomyoma (Figure 1G) or healthy peritoneum (Figure 1H) from women with endometriosis or in all tissues collected from women without endometriosis.

The presence of aromatase was related to the menstrual cycle phase in which the tissues were obtained and to the stage of endometriosis (Table I). At the time of intervention, the menstrual cycle day of five endometriosis patients was unknown. Aromatase expression was observed in nine of 21 patients who were in the first half of the cycle compared with 25 of 35 who were in the second phase; these differences were statistically significant ($P < 0.05$). However, no relation was observed between the presence of aromatase and moderate or severe endometriosis observed in our patients.

Aromatase activity in cell cultures

The determination of aromatase activity in endometriotic stromal cells is represented in Figure 2A as mean values ± SEM, obtained from all cell cultures. Some of the samples were used to optimize the methods, so we considered the results obtained from cultures of 29 endometriomata (21 positive and eight negative for aromatase). The addition of dexamethasone demonstrated the presence of aromatase activity in cells derived from the tissues that showed immunopositive expression ($P < 0.001$ versus basal level). IL-6 significantly stimulated aromatase activity in these cells ($P < 0.05$ versus basal), reaching values similar to those observed with dexamethasone. PF and TNFα also increased this activity, although differences were not statistically significant.

Similar effects were observed in adipocyte cultures (Figure 2B), where aromatase activity was lower than that detected in endometriotic fibroblasts. Twenty-six cultures (17 endometriosis and nine non-endometriosis patients) were successfully performed. Although no significant differences were found between endometriosis and the control group, it seems that adipocytes from women suffering from the disease presented a higher level of aromatase activity, especially after stimulation with PF or IL-6 ($P < 0.05$).

Discussion

One of the standard treatments for endometriosis is the reduction of steroid hormones with GnRH analogues. Unfortunately, it can have serious implications for fertility, and long-term treatment strategies cannot be applied because of side effects. Also, recurrence rates after cessation of this therapy are high (Waller and Shaw, 1993). The existence of adipose tissue and endometriotic foci as extraovarian sources of estrogen is probably an important reason for the high rate of failure of hormonal treatments.

Table I. Aromatase expression in endometriotic tissue in relation to the menstrual cycle phase and the stage of endometriosis

	Aromatase expression		Total (%)
	Positive (%)	Negative (%)	
Menstrual cycle phase			
Unknown	3 (60)	2 (40)	5 (100)
First half	9 (43)	12 (57)	21 (100)
Second half	25 (71)*	10 (29)	35 (100)
Stage of endometriosis			
Mild	0	2 (100)	2 (100)
Moderate	12 (63)	7 (37)	19 (100)
Severe	25 (63)	15 (37)	40 (100)

Results are the number of patients with percentages in parentheses. *$P < 0.05$ (chi-square test).

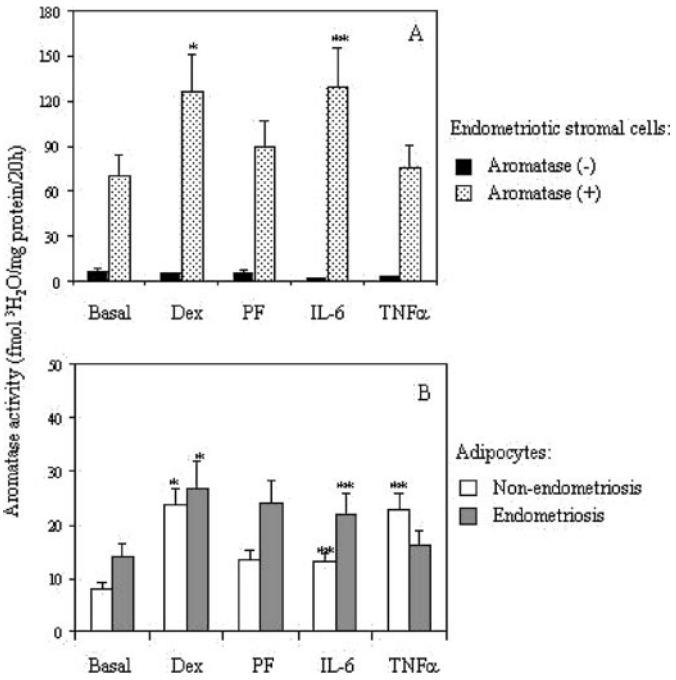

Figure 2. Effect of dexamethasone (Dex, 100 nmol/l), peritoneal fluid (PF, 10%, v/v), interleukin-6 (IL-6, 50 ng/ml) and tumour necrosis factor-alpha (TNFα, 10 ng/ml) on aromatase activity in (**A**) stromal cells derived from positive ($n = 21$) or negative ($n = 8$) endometriotic tissue for aromatase; (**B**) adipocytes of abdominal subcutaneous tissue from patients with ($n = 17$) and without endometriosis ($n = 9$). Dexamethasone demonstrated the presence of aromatase activity in stromal cells from immunopositive tissues; PF, TNFα and especially IL-6 increased such activity. As for adipocytes, endometriosis patients presented higher values of aromatase activity than disease-free women, especially after stimulation with PF or IL-6. Activity is expressed as mean ± SEM fmol/mg protein/20 h. *$P < 0.001$ and **$P < 0.05$ versus basal activity (Wilcoxon and Mann–Whitney *U*-test).

The expression of *P*-450arom in endometriosis was first reported by Noble *et al*. (1996), suggesting a possible local production of estrogen that, acting in an autocrine or paracrine fashion, may promote the growth of the implants (Bulun *et al*., 1999). The product of aromatase reaction, estrone, is converted to the potent 17β-estradiol (E_2) by 17β-hydroxysteroid dehydrogenase (17βHSD) type 1 in both eutopic endometrium and endometriosis. However, the inactivation of E_2 is impaired in endometriotic tissues because of deficient expression of 17βHSD type 2 (Zeitoun *et al*., 1998), an important pathophysiological complement which can give rise to elevated local levels of estrogen for prolonged periods.

Several authors have localized aromatase in endometriotic stromal cells (Noble *et al*., 1997; Gurates *et al*., 2002), whereas others found it in epithelial glandular cells (Kitawaki *et al*., 1997). In another report (Kusuki *et al*., 2001), this group described aromatase in the endometriosis epithelial lining of an ovarian cystoadenocarcinoma arising from ovarian endometriosis. After revising this report, we found that positive staining was mainly in the stroma; the epithelial staining was unclear and could be due to the diaminobenzidine.

On Western blot analysis, the monoclonal antibody used in this study detects a single protein band of ~55 kDa in microsomal extracts of the placenta and ovary consistent with the reported size of the protein (Mendelson *et al*., 1985). By use of the antibody, immunohistochemical localization of *P*-450arom was demonstrated in placenta, ovary and

testes from human, rat and marmoset (Turner *et al*., 2002). After the analysis of endometriotic samples, we found aromatase in 61% of our patients. The tissues showed marked heterogeneity in the level of immunostaining, which was localized in the stroma and not in the epithelium of the lesions. These findings were supported by the significant aromatase activity that we also observed in cultured stromal cells derived from these tissues, as previously described.

The lack of cyclicity of ectopic endometrial tissue has been reported in several studies. Hormonal receptor expression (Nisolle *et al*., 1997) or distribution of leukocyte subpopulations (Bulmer *et al*., 1998) is different compared with eutopic endometrium, and no changes were observed throughout the menstrual cycle. In our study, aromatase expression was preferentially detected in the second half of the cycle and does not seem to be related to the degree of severity of endometriosis observed in our patients. Only two cases were diagnosed as mild endometriosis and both were immunonegative.

Aromatase has also been described in eutopic endometrium from patients with endometriosis, although this expression is weak and barely detectable. (Noble *et al*., 1996, 1997), as well as in adenomyotic tissue, leiomyomas and eutopic endometrium from patients with adenomyosis or leiomyomas. This enzyme is also present in adenomyotic tissue, however, it is absent in the eutopic endometrium (Kitawaki *et al*., 1997, 1999) and myometrium (Bulun *et al*., 1994; Noble *et al*., 1996) of disease-free women. These findings are partially supported by our data. Aromatase was not detected in eutopic endometrium of any patient, both with and without endometriosis. It was present neither in myometrium and leiomyomas nor in apparently healthy peritoneum, as previously described (Noble *et al*., 1996). These discrepancies may be due to the methodology used in this study. Since aromatase expression is always lower in endometrium or leiomyoma than in endometriotic lesions, and is described controversially for the eutopic endometrium, qRT–PCR would be very helpful to confirm these results. Studies are underway to address this question.

Normal and malignant breast tissues also express the aromatase enzyme complex, so estrogen formation *in situ* could produce a mytogenic effect on the cancer cells. Cytokines such as IL-6 and TNFα have been identified as important factors in regulating aromatase activity in fibroblasts derived from these tissues (Macdiarmid *et al*., 1994; Purohit *et al*., 1995). Also, the conditioned medium collected from macrophages and lymphocytes is able to stimulate such activity in normal breast tissue (Singh *et al*., 1997). We observed that TNFα and especially IL-6 significantly stimulated the aromatase activity of endometriotic stromal cells and subcutaneous adipocytes. The results suggest a possible genetic mechanism of increased enzyme activity in adipose tissue from women with endometriosis compared with that from disease-free women, although no significant differences were found. A greater number of experiments would be necessary to confirm these results.

Endometriosis is also a local pelvic inflammatory process, with an altered function of peritoneal immune-related cells unable to eliminate the ectopic tissue (Wilson *et al*., 1994). Peritoneal macrophages increase in number and activation status in these patients, and consequently, there is a higher release of macrophage-derived products, such as growth factors and cytokines, which could be involved in the pathophysiology of the disease (Halme *et al*., 1984; Dunselman *et al*., 1988). These cytokines include IL-6 and TNFα, and they have been described to be elevated in PF from patients with endometriosis (Iwabe *et al*., 2002), so they could be involved in the regulation of aromatase expression. The increase in such activity observed in our cultures after the addition of PF suggests that it contains some factors able to stimulate this enzyme.

The use of immunomodulatory agents to restore the immunological deregulation associated with endometriosis may be an ideal treatment to control its development without affecting fertility. Recently, it has been described as having a beneficial effect produced by a combined treatment with intracystic recombinant IL-2 and GnRH analogues in women with endometriosis after transvaginal puncture and aspiration of endometriomata. These patients showed a decrease in the serum cytokine production (Velasco *et al*., 2005), and the time until the recurrence of the disease was significantly longer (Acién *et al*., 2003). In a similar clinical trial, patients treated with two doses of IL-2 had better clinical evolution than patients who received only one (Acién *et al*., 2005).

Thus, a local estrogen biosynthesis in endometriosis, perhaps in concert with macrophage-derived cytokines, could contribute to the development and maintenance of the disease independently of peripheral levels. Recently, molecular basis for the use of aromatase inhibitors in endometriosis has been discussed (Bulun *et al*., 1999, 2005). Its therapeutic potential has been reported by the successful treatment of recurrent severe endometriosis first in a post-menopausal woman (Takayama *et al*., 1998) and recently in a young ovariectomized woman (Razzi *et al*., 2004). In recent clinical trials, aromatase inhibitors combined with GnRH analogues (Soysal *et al*., 2004) or oral contraceptives (Amsterdam *et al*., 2005) seem to decrease endometriosis symptoms, and no significant side effects were noted.

In conclusion, this is a confirmatory report of aromatase expression in endometriosis, which may be regulated by a complex interaction of macrophage-derived products present in PF. Further studies are necessary to find this connection and to establish therapeutic modalities using aromatase inhibitors because of their side effects. To avoid these inconveniences, it would be interesting to investigate their local administration into endometriomata, alone or together with immunomodulatory agents, as a novel approach to the treatment of endometriosis.

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