Introduction

Endometriosis is a complex disorder that is characterized by the presence of endometrial tissue in ectopic sites outside the uterus and is linked to pelvic pain and infertility. The prevalence of endometriosis in women of reproductive age is estimated to be as high as 10% (Aral & Cates 1983, Wheeler 1989). Endometriosis is diagnosed in approximately 25% of women who undergo laparoscopy because of pelvic pain and in 20% of infertile women (Hasson 1976, Goldstein et al. 1980, Eskenazi & Warner 1997). It is a chronic and progressive disease that may give rise to a variety of severe and disabling symptoms including painful menses, painful intercourse, chronic pelvic pain, and infertility.

Endometriosis is probably inherited in a polygenic manner with an etiology of complex and multifactorial nature (Olive & Schwartz 1993). The most widely accepted mechanism for the pelvic disease is implantation of endometrial tissue on the peritoneum through retrograde menstruation, which was first proposed by Sampson (1927). Since retrograde menstruation occurs in at least 90% of all women, the presence of immunologic defects in women with endometriosis were hypothesized (Syrop & Halme 1987, Hill & Anderson 1989, Hill 1992, Olive &

Abstract

Estrogen is the most important known factor that stimulates the growth of endometriosis. Estrogen delivery to endometriotic implants was classically viewed to be only via the circulating blood in an endocrine fashion. We recently uncovered an autocrine positive feedback mechanism, which favored the continuous production of estrogen and prostaglandin (PG)E2 in the endometriotic stromal cells. The enzyme, aromatase, is aberrantly expressed in endometriotic stromal cells and catalyzes the conversion of C19 steroids to estrogens, which then stimulate cyclooxygenase-2 to increase the levels of PGE2. PGE2, in turn, is a potent inducer of aromatase activity in endometriotic stromal cells. Aromatase is not expressed in the eutopic endometrium. Aromatase expression in endometriosis and its inhibition in eutopic endometrium are controlled by the competitive binding of a stimulatory transcription factor, steroidogenic factor-1, and an inhibitory factor, chicken ovalbumin upstream promoter transcription factor to a regulatory element in the aromatase P450 gene promoter. In addition, we find that endometriotic tissue is deficient in 17β-hydroxysteroid dehydrogenase type 2, which is normally expressed in eutopic endometrial glandular cells and inactivates estradiol-17β to estrone. This deficiency is another aberration that favors higher levels of estradiol-17β in endometriotic tissues in comparison with the eutopic endometrium. The clinical relevance of local aromatase expression in endometriosis was exemplified by the successful treatment of an unusually aggressive form of recurrent endometriosis in a postmenopausal woman using an aromatase inhibitor.
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Schwartz 1993). These defects would presumably lead to impaired clearance of the menstrual debris on the peritoneal surfaces. On the other hand, data from other laboratories suggested that intrinsic molecular aberrations in the endometrium of women with endometriosis facilitated implantation of the endometrium on the pelvic peritoneum. The proposed intrinsic aberrations that were important in this hypothesis included deficient expression of an integrin (Lessey et al. 1994) and overexpression of complement 3 (Isaacsone et al. 1990) and certain cytokines (Ryan & Taylor 1997). Moreover, certain molecules such as tissue metalloproteinase inhibitor type 1 were shown to be expressed in endometriosis but not in the endometrium (Sharp et al. 1993, Sharpe-Timms et al. 1995). We recently demonstrated significant levels of aromatase activity and mRNA in the stromal cell component of endometriosis, whereas aromatase expression was either absent or barely detectable in the eutopic endometrium (Noble et al. 1996, 1997). We would like to clarify the terminology to be used here in reference to the tissues and cells that were studied: the terms ’endometriotic tissue’ and ‘endometriosis’ will refer to the pathological ectopic endometrium-like tissues in the pelvic peritoneum or ovaries. Extremely high levels of aromatase expression were found in the stromal cell component of endometriotic tissues. The term ‘endometrium’ refers to the eutopic or intrauterine endometrial tissue in its normal location. Aromatase expression is absent in the eutopic endometrium of women without endometriosis or any other uterine pathology and is barely detectable (only by reverse transcription (RT)-PCR) in the eutopic endometrium of women with endometriosis.

Considerable circumstantial and laboratory evidence suggests that endometriosis is an estrogen-dependent disease (Dizerga et al. 1980). For example, the usefulness of gonadotropin-releasing hormone agonists in suppressing ovarian steroidogenesis and progestins (which act to inhibit estrogen action) in the management of endometriosis is well recognized. Moreover, we recently reported the successful treatment of an unusually aggressive type of recurrent postmenopausal endometriosis using an aromatase inhibitor (Takayama et al. 1998). The responsiveness of endometriosis to estrogen and progesterone is also evident from hormone-dependent histological changes in this tissue similar to those in eutopic endometrium. In addition, the expression of estrogen and progesterone receptors has been demonstrated in endometriotic tissue (Lessey et al. 1989).

The delivery of estrogen to endometriotic implants has been assumed by many to be only via the circulating blood in an endocrine fashion. We, and others, however, have recently demonstrated markedly high levels of aromatase P450 mRNA and activity in pelvic endometriotic implants (Noble et al. 1996, 1997, Kitawaki et al. 1997). Moreover, prostaglandin (PG)E2, which is produced in very high levels in endometriotic tissues, was found to be the most potent inducer of aromatase activity in endometriosis-derived stromal cells (Badawy et al. 1984, De Leon et al. 1988, Karck et al. 1996, Noble et al. 1996, 1997). The production of PGE2 in eutopic endometrial stromal cells, in turn, was demonstrated to be greatly stimulated by cytokines and estradiol-17β via enhancement of cyclooxygenase-2 (COX-2) expression (Ishihara et al. 1995, Kennard et al. 1995, Huang et al. 1996). Finally, the expression of 17β-hydroxysteroid dehydrogenase (17β-HSD), the enzyme that is induced by progesterone and inactivates estradiol-17β (by conversion to estrone) in eutopic endometrium, was recently shown to be deficient in endometriotic tissues biopsied during the mid-secretory phase of the cycle (Zeitoun et al. 1998). Collectively, these data support the model in which alterations in the expression of aromatase, COX-2, and 17β-HSD type 2 in endometriosis may lead to increased local concentrations of estradiol-17β by enhancing its production and diminishing its metabolism (Fig. 1). In fact, higher concentrations of estradiol-17β have been detected in the peritoneal fluid of women with endometriosis than normal controls (DeLeon et al. 1986).

Mechanisms of estrogen biosynthesis and metabolism in endometriosis

Estrogen biosynthesis and metabolism in humans

Aromatase P450 (P450arom) catalyzes the conversion of androstenedione to estrone, and testosterone to estradiol-17β in a number of human cells, including placental syncytiotrophoblast, ovarian granulosa cells, and adipose and skin fibroblasts (Simpson et al. 1994). In the human, aromatase expression is regulated by usage of alternative and partially tissue-specific promoters in the placenta (promoter I.1), adipose tissue (promoters I.4, I.3 and II), and ovary (promoter II). Activation of these promoters, and thus aromatase expression, in these tissues is controlled by various hormones. In ovarian granulosa cells, follicle-stimulating hormone stimulates the activation of promoter II via a cAMP-dependent signaling pathway. In adipose fibroblasts, glucocorticoids and members of the interleukin (IL)-6 cytokine family give rise to activation of promoter I.4, whereas treatment with cAMP analogs or PGE2 switches the promoter use to I.3 and II in these cells. Estrogen biosynthesis in peripheral tissues (adipose tissue, skin, and endometriosis) is dependent for substrate on circulating androstenedione, which is produced by the adrenal cortex. Importantly, the product of aromatase activity in these tissues, namely estrone, is only very weakly estrogenic, and must therefore be converted to estradiol-17β in tissue sites of...
action. Evidence from several laboratories indicates that 17β-HSD type 1, which is present in these peripheral tissues, catalyzes this conversion. We recently demonstrated the expression of 17β-HSD type 1 in endometriotic tissues (Zeitoun et al. 1998). Another 17β-HSD isozyme, 17β-HSD type 2, catalyzes the conversions of estradiol-17β to estrone, and testosterone to androstenedione, in a number of human tissues, including the placenta and liver (Andersson & Moghrabi 1997). In addition, very high levels of 17β-HSD type 2 transcripts have been demonstrated in the glandular epithelial cell fraction of the human endometrium during the secretory phase, suggesting that progesterone stimulates this enzyme (Casey et al. 1994, Mustonen et al. 1998). In fact, estradiol dehydrogenase activity (oxidation of estradiol-17β to estrone) in endometrial tissues and isolated glandular epithelial component has been shown to be stimulated by progesterone in earlier reports (Tseng & Gurpide 1974, 1975, Satyaswaroop et al. 1979). The inactivation of estradiol-17β to estrone by the secretory phase endometrium has been viewed as an important protective mechanism in this estrogen-responsive tissue.

**Aromatase expression in Müllerian-derived tissues**

Müllerian-derived tissues are targets of estrogen action. Because aromatase is expressed in extraglandular tissues, we have investigated the regulation of expression of this gene in estrogen-dependent neoplasia or disorders that involve müllerian-derived tissues. First, using an [3H]water assay and quantitative RT-PCR, we were unable to detect aromatase activity or mRNA in disease-free endometrium, myometrium, or endometrial stromal cells in culture (derived from eutopic endometrium from disease-free women) (Bulun et al. 1993). On the other hand, aromatase expression was demonstrable in the disease states of these tissues. For example, in endometrial cancer, aromatase transcripts are readily demonstrable by RT-PCR, and aromatase expression was found to be regulated by promoter II in this malignant tissue (Bulun et al. 1994). Next, extremely high levels of aromatase transcripts were found in uterine leiomyoma tissues from 32 of 35 women and in apparently normal myometrial tissues adjacent to leiomyomata (18 of 24 evaluated) but
not in normal myometrial tissues from disease-free uteri (Bulun et al. 1994a). In leiomyoma-derived smooth muscle cells maintained in primary culture, treatment with dibutyryl (Bt2cAMP) acted to increase aromatase activity. Addition of phorbol diacetate potentiated this stimulatory effect of Bt2cAMP. Again, promoter II was found to be primarily responsible for aromatase expression in leiomyoma tissues and cells. These findings led us to investigate the expression of aromatase in endometriosis, another estrogen-dependent disorder of a Müllerian tissue. In an initial study, we found high levels of P450arom transcripts in all 17 endometriotic tissues from extraovarian pelvic sites evaluated (Noble et al. 1996, 1997). The levels of P450arom transcripts (normalized to total RNA) in endometriotic tissues were 3.2 times those in adipose tissue. Eutopic endometrium (obtained by endometrial curettage) from these patients also contained P450arom transcripts, albeit in quantities barely detectable by RT-PCR (Noble et al. 1996, 1997). P450arom transcripts could not be detected in the disease-free pelvic peritoneum proximal to endometriotic implants or in the intrauterine endometrial curettings from disease-free women. Thus, we hypothesize that estrogen-responsive Müllerian-derived neoplasia and endometriosis are disorders with aberrant aromatase expression that may give rise to an increase in the concentration of bioactive estrogen in situ (Bulun et al. 1994b). Moreover, a common cAMP-dependent signaling pathway seems to be responsible for activating P450arom promoter II in these disorders (Bulun et al. 1997).

**Regulation of aromatase expression in endometriotic stromal cells (Noble et al.1997)**

Upon demonstration of relatively high quantities of P450arom transcripts in endometriosis (much higher than those found in the adipose tissue), we next used endometriotic stromal cells in monolayer culture as a model system to study the regulation of aromatase expression (Noble et al.1997). Glands and stromal cells of ovarian endometriomas and eutopic endometrium were separated by the method of Satyaswaroop et al. (1979) and the stromal cells were cultured using a previously reported protocol (Satyaswaroop et al. 1979, Ryan et al. 1994). These cultured stromal cells were reported to retain estrogen receptors and estrogen responsiveness (Ryan et al. 1994). The endometriotic stromal cells cultured by this method were also characterized in terms of vimentin and cytokeratin expression (Ryan et al. 1994). Baseline aromatase activity in endometriotic stromal cells ranged from 0.65 to 6 pmol/4 h per mg protein. No significant stimulation of aromatase activity was observed by various cytokines (IL-1β, IL-2, IL-6, IL-11, oncostatin M, IL-15, tumour necrosis factor) or steroids (estradiol-17β, progesterone agonist R5020, dexamethasone). Bt2cAMP induced aromatase activity in these cells by 26 to 60 times the baseline values (Fig. 2), whereas the addition of phorbol acetate neither potentiated nor diminished this response. Because of the inflammatory nature of endometriosis, we treated these stromal cells with various prostanoids. Whereas treatments with PGI2, PGF2α, or PGF2 failed to elicit a response, PGE2 treatment gave rise to a dose-dependent induction of aromatase activity by up to 19- to 44-fold in endometriosis-derived cells from different patients (Fig. 2) (Noble et al.1997). These changes in aromatase activity were accompanied by comparable changes in the levels of P450arom mRNA. A modified rapid amplification of 5'-cDNA ends (5'-RACE)/ Southern hybridization of the promoter-specific sequences in P450arom transcripts revealed almost exclusive use of promoter II for aromatase expression in PGE2- or Bt2cAMP-treated endometriotic cells.

The summary of our findings thus far is as follows. PGE2 induction of aromatase activity in endometriotic stromal cells is mediated possibly through increased intracellular levels of cAMP. The basis for markedly high levels of aromatase expression in endometriosis in contrast with absent or barely detectable quantities in the eutopic endometrium may be due to the transformation of endometrial stromal cells after implantation in the pelvic peritoneum and ovary in response to locally produced...
paracrine factors. The potential aromatization capability of eutopic endometrial cells from women with the genetic predisposition to develop endometriosis may facilitate the implantation process and growth in pelvic peritoneum by increasing local estradiol-17β concentrations by the activities of aromatase and 17β-HSD type 1 (Noble et al. 1996, 1997, Zeitoun et al. 1998). Estradiol-17β, in turn, will induce the activity of COX-2, the rate-limiting enzyme for PGE₂ biosynthesis (Huang et al. 1996). The inflammatory process in endometriotic tissues giving rise to increased production of cytokines (e.g. IL-1β, tumour necrosis factor α) by monocytes and macrophages will also promote PGE₂ production in this tissue (Guan et al. 1997). Thus a positive feedback cycle is established, whereby local production of estrogen and PGE₂ is enhanced by complex molecular interactions (Fig. 3).

Stimulation of aromatase P450 promoter (II) activity in endometriosis and its inhibition in endometrium are regulated by competitive binding of steroidogenic factor-1 (SF-1) and COUP-TF to the same cis-acting element (Zeitoun et al. 1999)

An intriguing observation made during the previous studies was the lack of aromatase expression in eutopic endometrial stromal cells in contrast with significant levels of aromatase mRNA and activity in endometriotic stromal cells, which can be strikingly induced by cAMP analogs. Thus, we sought to determine whether differential binding of transcription factors to the P450arom promoter in response to cAMP is a mechanism involved in this process. First, we demonstrated by 5'-RACE that P450arom expression in pelvic endometriotic lesions is regulated almost exclusively via the alternative promoter II. Then, luciferase reporter plasmids containing deletion mutations of the 5'-flanking region of promoter II were transfected into endometriotic stromal cells. We identified two critical regulatory regions for cAMP induction of promoter II activity: (i) –214/–100 bp proximal region responsible for a 3.7-fold induction, and (ii) –517/–214 bp distal region responsible for potentiation of cAMP response up to 13-fold. In the –214/–100 bp region, we studied eutopic endometrial and endometriotic nuclear protein binding to a nuclear receptor half-site (NRHS) (AGGTCA) and an imperfect cAMP-responsive element (CRE) (TGCACGTCA). Using an electro-phoretic mobility-shift assay, CRE-binding activity in nuclear proteins from both endometriotic and eutopic endometrial cells was found to give rise to formation of identical DNA-protein complexes, which led us to conclude that CRE did not account for differential aromatase expression. The NRHS probe, on the other hand, formed a distinct complex with nuclear proteins from endometriotic cells, which migrated at a much faster rate than the complex formed with nuclear proteins from eutopic endometrial cells. Employing recombinant proteins and antibodies against SF-1 and COUP-TF, we demonstrated that COUP-TF but not SF-1 bound with a higher affinity. Finally, overexpression of COUP-TF for occupancy of the NRHS, to which SF-1 binds with a lower affinity in eutopic endometrium are regulated by competitive binding of steroidogenic factor-1 (SF-1) and COUP-TF to the same cis-acting element (Zeitoun et al. 1999)
construct, whereas overexpression of COUP-TF almost completely abolished these activities. In conclusion, COUP-TF is responsible for the inhibition of P450arom expression in eutopic endometrial stromal cells, which lack SF-1 expression in the majority (80%) of the samples, whereas aberrant SF-1 expression in endometriotic stromal cells overrides this inhibition by competing for the same DNA-binding site, which is likely to account for high levels of baseline and cAMP-induced aromatase activity (Fig. 4).

**Deficient expression of 17β-HSD type 2 in endometriosis in contrast with eutopic endometrium (Zeitoun et al. 1998)**

Interconversions of estradiol-17β ↔ estrone are catalyzed by two enzymes encoded by two separate genes (Penning
1997). 17\(\beta\)-HSD type 1 favors the formation of estradiol-17\(\beta\), whereas type 2 inactivates estradiol-17\(\beta\) by converting it to estrone. We recently demonstrated by Northern-blot analysis the presence of transcripts of 17\(\beta\)-HSD type 1, which catalyzes the conversion of estrone to estradiol-17\(\beta\), in both eutopic endometrium and endometriosis. Thus, it follows that the product of the aromatase reaction, namely estrone, which is weakly estrogenic can be converted to the potent estrogen, estradiol-17\(\beta\), in endometriotic tissues.

It was previously demonstrated that progesterone stimulates the inactivation of estradiol-17\(\beta\) through conversion to estrone in eutopic endometrial epithelial cells. Subsequently, 17\(\beta\)-HSD type 2 was shown to catalyze this reaction, and its transcripts were detected in the epithelial cell component of eutopic endometrium in secretory phase. Because estradiol-17\(\beta\) plays a critical role in the development and growth of endometriosis, we studied 17\(\beta\)-HSD type 2 expression in endometriotic tissues and eutopic endometrium. We demonstrated by Northern-blot analysis the presence of 17\(\beta\)-HSD type 2 transcripts in all RNA samples of secretory eutopic endometrium (\(n=12\)) but not in samples of secretory endometriotic lesions (\(n=10\)), including paired samples of secretory eutopic endometrium and endometriosis obtained simultaneously from four patients. These transcripts were not detectable in any paired samples of proliferative eutopic endometrium or endometriosis (\(n=4\)), as expected. Next, we confirmed these findings by demonstration of immunoreactive 17\(\beta\)-HSD type 2 in epithelial cells of secretory eutopic endometrium in 11 out of 13 samples employing a monoclonal antibody against 17\(\beta\)-HSD type 2, whereas 17\(\beta\)-HSD type 2 was absent from paired secretory endometriotic tissues (\(n=4\)). Proliferative eutopic endometrial (\(n=8\)) and endometriotic (\(n=4\)) tissues were both negative for immunoreactive 17\(\beta\)-HSD type 2 except for barely detectable levels in one eutopic endometrial sample. Finally, we sought to determine whether deficient 17\(\beta\)-HSD type 2 expression in endometriotic tissues is due to impaired progesterone action in endometriosis. We determined by immuno-histochemistry the expression of progesterone and estrogen receptors in these paired samples of secretory (\(n=4\)) and proliferative (\(n=4\)) eutopic endometrium and endometriosis, and no differences could be demonstrated. In conclusion, inactivation of estradiol-17\(\beta\) is impaired in endometriotic tissues as the result of deficient expression of 17\(\beta\)-HSD type 2, which is normally expressed in eutopic endometrium in response to progesterone. The lack of 17\(\beta\)-HSD type 2 expression in endometriosis is not due to alterations in the levels of immuno-reactive progesterone or estrogen receptors in this tissue and may be related to an inhibitory aberration in the signaling pathway that regulates 17\(\beta\)-HSD type 2 expression.

The first reported use of an aromatase inhibitor to treat endometriosis (Takayama 1998)

Aromatase inhibitors have been widely used to treat breast cancer (Brodie 1991). We recently evaluated a 57-year-old woman, who presented with recurrent severe endo-metriosis after hysterectomy and bilateral salpingo-oophorectomy. Two additional laparotomies were performed because of severe pelvic pain and bilateral ureteral obstruction giving rise to left renal atrophy and right hydronephrosis. Recently, recurrent pelvic endometriosis, evident from a 30 mm vaginal lesion visible on speculum examination, did not respond to oral megestrol acetate treatment for 4 months. We administered anastrozole (an aromatase inhibitor) orally, 1 mg/day, and elemental calcium, 1.5 g/day, for 9 months. Alendronate (a non-estrogenic inhibitor of bone resorption), 10 mg/day, was added to this regimen. The vaginal lesion was biopsied before and 6 months after the onset of treatment. The circulating levels of estradiol-17\(\beta\) were reduced to approximately 50% of the baseline value after treatment with anastrozole. Pain rapidly decreased and completely disappeared after the second month of treatment. The 30x30x20 mm bright-red polypoid vaginal lesion was reduced to a 3 mm area of gray tissue by the end of 9 months of treatment. Markedly high pretreatment levels of P450arom mRNA in the endometriotic tissue became undetectable in a rebiopsy specimen after 6 months of treatment. Bone density of the lumbar spine had decreased by 6.2% after 9 months of treatment. No other side effects were noted. This is the first description of the use of an aromatase inhibitor in the treatment of endometriosis. The short-term results were extraordinarily successful, with elimination of pain and near-complete eradication of implants associated with severe endometriosis not responsive to other therapy. The occurrence of significant bone loss despite the addition of alendronate to the treatment regimen in this particular case should be studied further in large clinical trials. Besides the expected inhibition of aromatase enzyme activity by anastrozole, the disappearance of aromatase mRNA expression in the lesion may be explained by denial of estrogen which is known to stimulate local biosynthesis of PGE\(_2\), which in turn, stimulates aromatase expression (Fig. 5). We conclude that the recently developed potent aromatase inhibitors are candidate drugs in the treatment of endometriosis that is resistant to standard regimens.

Conclusions

The development and growth of endometriosis is estrogen-dependent. Several molecular aberrations were found to be present in endometriotic tissues (in contrast with the eutopic endometrium), which favor increased local levels of estradiol-17\(\beta\). In fact, we uncovered a
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positive feedback mechanism that is responsible for continuous formation of estradiol-17β and PGE₂ through upregulation of aromatase and COX-2 in endometriotic stromal cells. Levels of estradiol-17β in endometriotic tissue are further increased by impaired inactivation of this steroid because of deficient 17β-HSD type 2 expression in endometriotic epithelial cells. aberrant regulation of steroidogenic enzymes in endometriotic tissues giving rise to elevated estradiol-17β levels is possibly one of many metabolic abnormalities that promote the development and growth of this tissue. These studies have already led us to successfully use an aromatase inhibitor to treat endometriosis. We believe that determination of such molecular aberrations in endometriosis will give rise to identification of other molecular targets for potential treatments.

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