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Molecular basis for treating endometriosis with aromatase inhibitors

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Although treatment of one unusually aggressive case of postmenopausal endometriosis with an aromatase inhibitor has been strikingly successful, large clinical trials are required to establish whether aromatase inhibitors will have a significant role in the medical management of endometriosis. Introduction of aromatase inhibitors into the treatment of endometriosis underscores the importance of basic research leading to the development of novel strategies in reproductive disorders. It was shown earlier that aromatase activity was not detectable in normal endometrium. Aromatase, however, is expressed inappropriately in endometriosis and stimulated by prostaglandin E_2. Aromatase activity gives rise to local biosynthesis of oestrogen, which, in turn, stimulates prostaglandin E_2 production, thus establishing a positive feedback cycle. This favours accumulation of oestrogen and prostaglandins in endometriosis, which is an inflammatory disorder dependent on oestrogen for growth.

Key words: aromatase/aromatase inhibitor/endometriosis/endometrium/oestrogen biosynthesis

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Background

Although current hormonal therapy of infertility associated with endometriosis is not of proven value, it is somewhat successful for pelvic pain associated with endometriosis (Olive and Schwartz, 1993). The relief, however, is relatively short-term (Waller and Shaw, 1993). Various agents used are comparable in terms of efficacy. Pelvic implants of endometriosis react histologically to steroid hormones in a manner similar to normal endometrium. For example, oestrogen stimulates growth of both endometriosis and eutopic endometrium. All medical treatments were designed to decrease oestrogen secretion by the ovaries [e.g. gonadotrophin releasing hormone (GnRH) agonists, oral contraceptives, Danazole and medroxyprogesterone acetate] or to antagonize the effects of oestrogen on endometriotic implants (e.g. oral contraceptives, Danazole and medroxyprogesterone acetate) (Table I). There is, however, a high incidence of recurrence after these medical therapies (Waller and Shaw, 1993). Eighteen months after completing a 6-month course of leuprolide acetate-depot, only 52% of patients had significant relief of pain (Waller and Shaw, 1993). The recurrence rate of pain in the rest of the patients was ~5–20% per year (reaching a cumulative average rate at 5 years as high as 53%). The recurrence rate at 5 years was as high as 75% in severe forms of endometriosis (Waller and Shaw, 1993). In women treated for pelvic pain, the symptoms usually return rather quickly after cessation of therapy. For a period of time after medical treatment, however, the intensity of symptoms is less severe. The recurrence rates after treatment with GnRH agonists are similar to those after Danazole, and both are similar to those obtained with surgical excision. Danazole is used less frequently due to its androgenic side-effects. A 6 month course of GnRH agonist treatment is currently the most popular regimen. The most serious side-effect of the GnRH agonist treatment for endometriosis is considered to be bone loss due to oestrogen deficiency, and oral oestrogen-progesterone preparations or bisphosphonates are usually 'added back' to minimize bone loss (Surrey *et al*., 1995).

As summarized above, we are still far from the cure of endometriosis, and current treatments are not satisfactory for effective control of pain. The radical treatment is the removal of both ovaries, and even this was not found to be effective in a

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number of cases of postmenopausal endometriosis (Metzger *et al*., 1991; Takayama *et al*., 1998). New strategies are needed to offer women with endometriosis a reasonable chance to live without suffering from chronic pelvic pain for decades. There are two important caveats, which are not addressed by the GnRH agonist treatment. Firstly, we have recently shown that extremely large quantities of oestrogen can be produced locally within the endometriotic cells, which represents an intracrine mechanism of oestrogen action, in contrast to ovarian secretion, which is an endocrine means of supplying this steroid to target tissues. Local oestrogen biosynthesis is not blocked by any of the currently used treatments for endometriosis. Secondly, oestradiol produced in peripheral tissue sites (e.g. adipose tissue and skin fibroblasts) may give rise to significant circulating levels of oestradiol in a number of women. Again, GnRH agonists do not inhibit peripheral oestrogen formation. Oestrogen production in these two extraovarian sources is probably an important reason for the high rate of treatment failures with GnRH agonists. Aromatase inhibitors are candidate therapeutic agents for endometriosis (Table I). Preliminary evidence suggests that aromatase inhibitors can eradicate unusually aggressive endometriotic lesions resistant to other therapy (Takayama *et al*., 1998).

Table I. Medical treatment of endometriosis

GnRH agonists
GnRH antagonists
Danazol (anabolic steroid)
Oral contraceptives
Progestins
Mifepristone (RU486; progesterone receptor antagonist)
Aromatase inhibitors

GnRH = gonadotrophin releasing hormone.

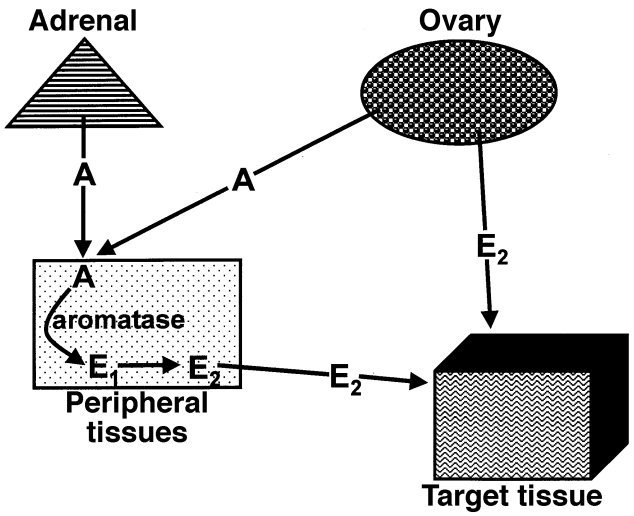

Figure 1. Origin of oestrogen in women. In ovulatory women, oestradiol (E_2) is secreted by the ovary in a cyclic manner. During treatment with a gonadotrophin releasing hormone analogue or postmenopausal period, however, peripheral tissues (e.g. adipose tissue and skin), represent the major source of oestradiol. Aromatase activity in these peripheral tissues gives rise to the conversion of androstenedione (A) of adrenal and ovarian origins to oestrone (E_1). Oestrone is further converted to the potent oestrogen oestradiol in peripheral tissues. Therefore, two important sources of circulating oestradiol in women are ovarian secretion and peripheral aromatization.

Molecular aberrations in endometriosis

The prevalence and genetics of endometriosis is somewhat similar to those of diabetes mellitus and asthma, in that endometriosis is a common chronic disorder inherited possibly in a polygenic fashion (Kennedy, 1999). Implantation of menstrual endometrium on peritoneal surfaces via retrograde menstruation is a widely accepted mechanism for the development of endometriosis (Sampson, 1927). On the other hand, since retrograde menstruation occurs in nearly all women in the reproductive age group, additional factors were postulated to contribute to the establishment of endometriotic implants in pelvic peritoneum. It was proposed that a defective immune system incapable of clearing peritoneal surfaces of menstrual debris might contribute to the development of endometriosis (Halme *et al*., 1988). Additionally, a number of molecular aberrations were found in endometriotic implants, which distinguish them from the eutopic endometrium, although both tissues appear to be histologically similar. As a further twist, these aberrations give rise to the gain or loss of various functions. The end result of these alterations is the enhancement of the growth and invasiveness of endometriotic implants. For example, impaired suppression of matrix metalloproteinases, which facilitate invasiveness, may contribute to establishment of ectopic lesions (Bruner *et al*., 1997; Sharpe-Timms *et al*., 1998). Overproduction of the cytokine RANTES by endometriotic implants may provide a mechanism for the recruitment of peritoneal leukocytes (Hornung *et al*., 1997). The abnormal presence of aromatase and absence of 17β-hydroxysteroid dehydrogenase type 2 in endometriotic implants in contrast to eutopic endometrium may give rise to excessive local production and impaired metabolism of oestradiol (Noble *et al*., 1996; Zeitoun *et al*., 1998). Consequently, elevated tissue levels of this mitogen will enhance the growth of endometriotic implants. In the following sections, we will discuss topics that are relevant to the use of aromatase inhibitors to treat endometriosis. We will initially review mechanisms of oestrogen production in humans. A discussion of the use of aromatase inhibitors in the treatment of endometriosis will follow.

Origin of oestrogen in women

Oestrogen is produced in several human tissues that contain the enzyme named aromatase, which catalyses the conversion of C_{19} steroids to oestrogens (Simpson *et al*., 1994). In premenopausal women, granulosa cells of the Graafian follicle in the ovary represent the primary site of aromatase expression. Therefore, ovarian secretion in a cyclic manner accounts for the largest portion of oestradiol production in women in the reproductive age group (Simpson *et al*., 1994). Aromatase expression in ovarian granulosa cells is under the control of FSH, which activates a signalling pathway involving cyclic adenosine monophosphate (cAMP), steroidogenic factor-1 (SF-1), and cAMP response

element binding protein (CREB). Binding of the two latter factors to the promoter of the *CYP19* (aromatase P450) gene gives rise to the events leading to the production of aromatase protein (Michael *et al*., 1995, 1997).

Extraovarian tissues become the major source of oestrogen production after the cessation of ovarian function in postmenopausal women (Figure 1). In particular, markedly high levels of aromatase activity are present in adipose tissue and skin fibroblasts (Ackerman *et al*., 1981). Since adipose tissue comprises a significant portion of the human body, aromatase activity in this tissue probably accounts for the largest part of oestrogen production in postmenopausal women (MacDonald *et al*., 1978). Additionally, oestrogen produced locally by aromatase activity in breast adipose tissue promotes the development and growth of oestrogen-dependent breast malignancies (Bulun *et al*., 1993; Yue *et al*., 1998). Aromatase expression in adipose tissue is limited to undifferentiated fibroblasts and is not present in significant quantities in lipid-filled mature adipocytes (Ackerman *et al*., 1981; Price *et al*., 1992). Glucocorticoids together with members of the IL-6 cytokine family regulate aromatase expression in adipose fibroblasts via an alternative promoter that is located ~20 000 base pairs upstream of the ovarian promoter (Zhao *et al*., 1995). Androstenedione of adrenal origin is the major substrate for aromatase in extragonadal tissues (Simpson *et al*., 1994). Androstenedione is aromatized to become oestrone, which is further reduced to the potent oestrogen oestradiol in these tissues (MacDonald *et al*., 1978) (Figure 1).

Aromatase expression in uterine tissues

Both myometrium and endometrium undergo significant histological and biochemical changes under the influence of oestrogen. Oestrogen action in uterine tissues is of paramount physiological importance in terms of preparation for implantation. Oestrogen biosynthesis does not take place in healthy uterine tissues (Bulun *et al*., 1994a). On the other hand, we demonstrated *CYP19* gene expression and aromatase activity in uterine leiomyomas, endometrial cancer and endometriosis (Bulun *et al*., 1994a,b; Noble *et al*., 1996, 1997) (Figure 2). It is intriguing that all these pathological tissues use primarily the ovarian type promoter for aromatase expression. This promoter is stimulated via a cAMP-dependent signalling pathway in both leiomyomas and endometriosis (Bulun *et al*., 1994; Noble *et al*., 1996, 1997; Zeitoun *et al*., 1999). Thus, aromatase expression is inappropriately activated in oestrogen-dependent disorders of the uterus, whereas aromatase activity is absent in the disease-free counterparts of these tissues. In these particular instances, aberrant aromatase expression may be analogous to activation of an oncogene, since the end-product oestrogen is a potent mitogen for uterine leiomyomas, endometrial cancer and endometriosis (Figure 2).

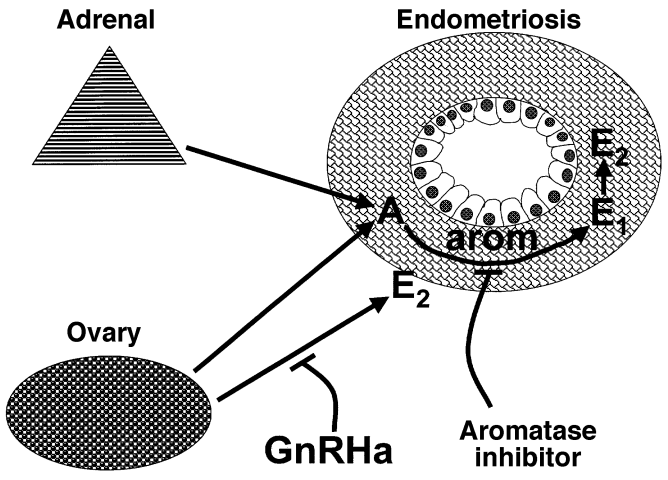

Figure 2. Aberrant aromatase expression in endometriosis. Androstenedione (A) of adrenal and ovarian origins become converted to oestrone (E_1) in endometriotic tissue. Oestrone itself is only weakly oestrogenic and should be converted to oestradiol (E_2) for full oestrogenic action. Endometriotic tissue expresses the enzyme, 17β-hydroxysteroid dehydrogenase type 1, which catalyses this conversion (Zeitoun *et al*., 1998). While gonadotrophin releasing hormone analogues (GnRHa) suppress oestradiol secretion from the ovary, only aromatase inhibitors are capable of eliminating oestrogen formation in endometriotic tissue in the presence of adrenal function.

Mechanisms responsible for aromatase expression in endometriosis

Upon demonstration of relatively high quantities of aromatase P450 (P450arom) transcripts in endometriosis (much higher than those found in the adipose tissue), we next used endometriosis-derived stromal cells in monolayer culture as a model system to study the regulation of aromatase (Noble *et al*., 1996, 1997). Endometriotic stromal cells cultured by this method have been previously characterized in terms of vimentin and cytokeratin expression and were reported to retain oestrogen receptors and oestrogen responsiveness (Ryan *et al*., 1994). We characterized these endometriotic stromal cells further by demonstrating prolactin mRNA expression in response to treatment with medroxyprogesterone acetate plus dibutyryl cAMP (our unpublished observations). This verifies the presence of endometrial-type cells in culture, which are responsive to hormonal treatment. Prolactin transcripts were also detected in cultured stromal cells from eutopic endometrium but not in ovarian granulosa and theca cells subjected to the same treatments (our unpublished observations). Baseline aromatase activity in endometriosis-derived stromal cells ranged from 0.65 to 6 pmol/mg protein/4 h. No significant stimulation of aromatase activity was observed by various cytokines [interleukin (IL)-1β, IL-2, IL-6, IL-11, oncostatin M, IL-15, tumour necrosis factor (TNF)-β] or steroids (oestradiol, progesterone agonist R5020, dexamethasone). Dibutyryl cAMP induced aromatase activity in these cells by 26-60-fold the baseline values, whereas the addition of phorbol acetate neither potentiated nor diminished this response (Noble *et al*., 1997). Because of the inflammatory nature of endometriosis, we treated these stromal cells with various prostanoids. Whereas treatments with prostaglandin (PG) I_2, $PGF_{2\alpha}$, PGJ_2 failed to elicit a response, PGE_2 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 (Noble *et al*., 1997). These changes in aromatase activity were accompanied by comparable changes in the amounts 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 the ovarian type promoter for aromatase expression in PGE_2- and dibutyryl cAMP-treated endometriotic cells.

In summary, PGE_2 induction of aromatase activity in endometrial stromal cells is mediated through increased intracellular levels of cAMP. The basis for markedly high levels of aromatase expression in endometriosis in contrast to 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 genetic predisposition to develop endometriosis may facilitate the implantation process and growth in pelvic peritoneum by increasing local oestradiol concentrations by the activities of aromatase and 17β-hydroxysteroid dehydrogenase (HSD) type 1 (Noble *et al*., 1996; Zeitoun *et al*., 1998). Oestradiol, in turn, will induce the activity of cyclo-oxygenase type 2 (COX-2), the rate-limiting enzyme for PGE_2 biosynthesis (Huang *et al*., 1996). The inflammatory process in endometriotic tissues giving rise to increased production of cytokines (e.g. IL-1β, TNFα) by monocytes and macrophages will also promote PGE_2 production in this tissue (Huang *et al*., 1998). Thus a positive feedback cycle is established, whereby local productions of oestrogen and PGE_2 are enhanced by complex molecular interactions.

Aberrant expression of steroidogenic factor-1 (SF-1) activates aromatase expression in endometriosis

An intriguing observation during the previous studies was the unresponsiveness of eutopic endometrial stromal cells to cAMP analogues in contrast to drastic cAMP induction of aromatase expression in endometriosis-derived cells (Figure 3). Thus, we decided to determine whether differential binding of transcription factors to the *CYP19* (aromatase P450) promoter in response to cAMP is a mechanism involved in this process. Deletion mutants of the 5′-flanking region of this promoter fused to Luciferase reporter gene were transfected into endometriotic stromal cells. Two critical regulatory regions for cAMP induction of promoter activity were identified: a –214/-100 bp proximal region responsible for a 3.7-fold induction and a –517/-214 distal region responsible for potentiation of cAMP response up to 13-fold. In the proximal region, we studied eutopic endometrial and endometriotic nuclear protein binding to a nuclear receptor half-site and an imperfect cAMP response element (CRE). CRE-binding activity in nuclear proteins from both endometriotic and eutopic endometrial cells gave rise to formation of identical DNA–protein complexes (Figure 3). The nuclear receptor half-site probe, on the other hand, formed a distinct complex with nuclear proteins from endometriotic cells, which migrated at a much faster rate compared with the complex formed with nuclear proteins from eutopic endometrial cells. Employing recombinant proteins and antibodies against SF-1 and chicken ovalbumin upstream promoter-transcription factor (COUP-TF), we demonstrated that COUP-TF, but not SF-1, bound to nuclear receptor half-site in eutopic endometrial cells, whereas SF-1 was the primary nuclear receptor half-site-binding protein in endometriotic cells (Figure 3). In fact, COUP-TF mRNA was present in both eutopic endometrial and endometriotic tissues, whereas SF-1 mRNA was detected in all endometriotic tissues, but in only three out of 15 eutopic endometrial tissues. Moreover, we demonstrated a dose-dependent direct competition between SF-1 and COUP-TF for occupancy of the nuclear receptor half-site, to which SF-1 bound with a higher affinity. Finally, ectopic expression of SF-1 in eutopic endometrial and endometriotic cells strikingly potentiated baseline and cAMP-induced activities of the –517 promoter construct, whereas ectopic expression of COUP-TF almost completely abolished these activities. In conclusion, COUP-TF is a factor responsible for the inhibition of aromatase expression in eutopic endometrial stromal cells, which lack SF-1 expression in the majority of the samples; whereas aberrant SF-1 expression in endometriotic stromal cells can override 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 (Zeitoun *et al*., 1999) (Figure 3).

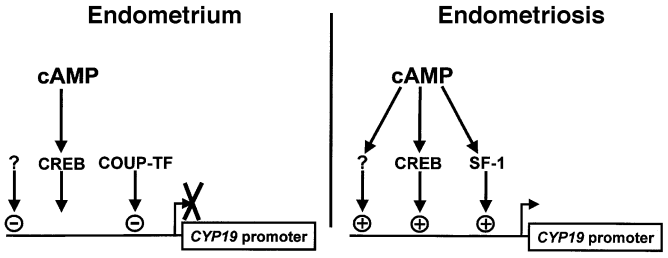

Figure 3. Regulation of *CYP19* (aromatase P450) gene expression via the ovarian-type promoter in stromal cells from the eutopic endometrium and endometriotic tissue (endometriosis). Aromatase P450 mRNA in the eutopic endometrium is absent or barely detectable. Thus, significant transcription of the *CYP19* gene is blocked in this tissue. In endometriosis-derived stromal cells, on the other hand, levels of aromatase P450 mRNA can be stimulated to extremely high levels by cAMP analogues. Cyclic AMP response element binding protein (CREB) binds to this promoter in both cell types but does not determine by itself the absence or presence of promoter activity. The inhibition of aromatase expression in eutopic endometrium is regulated by binding of the inhibitory transcription factor, chicken ovalbumin upstream promoter-transcription factor (COUP-TF) to the *CYP19* gene promoter. The stimulatory transcription factor, steroidogenic factor-1 (SF-1), is aberrantly expressed in endometriotic tissue and competes with COUP-TF to occupy the same response element. Binding of SF-1 to the *CYP19* gene promoter together with CREB gives rise to the activation of aromatase expression.

Clinical relevance: treatment of endometriosis with aromatase inhibitors

Aromatase inhibitors have been widely used to treat postmenopausal breast cancer (Santen, 1991). The therapeutic potential of aromatase inhibitors in breast cancer is comparable to that of tamoxifen (Santen, 1993). Two possible effects of these medications in breast cancer were postulated: Aromatase inhibitors suppress oestrogen production in peripheral tissues such as fat and decrease circulating oestrogen levels considerably (Iveson *et al*., 1993). Additionally, it was proposed that inhibition of local aromatase activity in breast tissue proximal to malignant cells is a key mechanism responsible for the therapeutic effects of these inhibitors (Sourdaine *et al*., 1996; Yue *et al*., 1998). Very little is known about possible hormonal and reproductive alterations caused by aromatase inhibitors in women in the reproductive age group. One study on adult female bonnet monkeys revealed that ovulation continued to occur despite markedly reduced levels of oestradiol (Selvaraj *et al*., 1995). Nonetheless, the successful use of aromatase inhibitors in the

treatment of postmenopausal breast cancer, which is an oestrogen-dependent disease, and aberrant expression of aromatase in endometriotic implants encouraged us to use these medications to treat endometriosis.

The woman in the first published report was referred to us due to vaginal cuff endometriosis resistant to all existing treatments including bilateral oophorectomy followed by multiple laparotomies for resection of lesions (Takayama *et al*., 1998). We followed the size of this vaginal lesion by direct visualization. Briefly, this 57 year old woman weighing 217 lbs underwent hysterectomy and bilateral oophorectomy 20 years prior to our evaluation. After surgical menopause, endometriosis recurred twice causing bilateral blockage of ureters and complete loss of left kidney function. She had two laparotomies for resection of retroperitoneal endometriosis and infiltrated segments of ureters followed by bilateral ureteral reimplantation. A year before the initiation of treatment with an aromatase inhibitor, endometriosis recurred for the third time at her vaginal cuff and did not respond to treatment with megesterol acetate for 4 months. At this point, she was taking large doses of hydrocodone, methadone and non-steroidal anti-inflammatory medications. Her serum FSH level was in the postmenopausal range (61 IU/l), whereas oestradiol level was higher than expected (46 pg/ml). High oestradiol level might be explicable in terms of obesity, which is known be associated with increased oestrogen formation (MacDonald *et al*., 1978). High levels of aromatase P450 mRNA were detected in a biopsy of the vaginal endometriotic implant. After the aromatase inhibitor anastrozole, 1 mg/day (plus alendronate 10 mg/day and calcium supplement) was initiated, pelvic pain rapidly decreased and disappeared within 2 months, and she discontinued all pain medications. Oestradiol level was reduced to 27 pg/ml. Endometriotic implant at the vaginal apex decreased from a 30 mm red polypoid mass to a 3 mm scar tissue within 9 months. A bone loss of 6.2% was detected in the lumbar spine over this period (Takayama *et al*., 1998). Interestingly, no aromatase P450 mRNA was detectable in a repeat biopsy of the vaginal implant during the sixth month of therapy. One explanation for this finding is that denial of oestrogen to endometriotic tissue (treated with the aromatase inhibitor) caused a decrease in the formation of PGE_2, and thus, prevented the induction of aromatase by PGE_2. Hence, we postulate that the benefit of treatment with an aromatase inhibitor is 2-fold: the inhibitor blocks aromatase activity directly in the endometriotic tissue; and, the lowering of oestrogen levels in the endometriotic tissue suppresses COX-2 expression and, in turn, PGE_2 formation, thus interrupting the positive feedback loop (Takayama, Zeitoun *et al*., 1998).

Recurrent postmenopausal endometriosis possibly represents a subset of this disease that is resistant to treatment with progestins (Metzger *et al*., 1991; Takayama *et al*., 1998). Aromatase inhibitors may be the only available medical treatment for these types of lesions. Since our patient had a significant bone loss during the treatment with an aromatase inhibitor despite adding back a bisphophonate, this requires further investigation.

Future considerations

The aetiology of endometriosis appears to be extremely complex. One aspect involves aberrant activation or inhibition of certain genes in endometriotic implants, which lead to elevated local concentrations of oestradiol, a known mitogen for endometriosis. Aberrant expression of the transcription factor, SF-1 in endometriotic tissue gives rise to the inappropriate presence of aromatase expression leading to local oestrogen biosynthesis. This may play a significant role in the aetiology of postmenopausal endometriosis as exemplified by a remarkable response of this disorder to treatment with an aromatase inhibitor. Although both SF-1 and aromatase are expressed in endometriotic implants from premenopausal women, the clinical significance of these findings in this group is yet to be determined. It is tempting to postulate that addition of aromatase inhibitors to GnRH analogues may increase the disease-free interval significantly. It is further tempting to postulate that the use of an aromatase inhibitor as a single agent may suppress endometriosis, while permitting a woman to ovulate, since aromatase inhibitors at therapeutic doses for breast cancer and endometriosis do not block ovulation (Selvaraj *et al*., 1995). Thus, aromatase inhibitors may potentially be used in the treatment of infertility associated with endometriosis. These issues are yet to be clarified by future studies. Finally, the most serious side-effect of aromatase inhibitors appears to be bone loss (Takayama *et al*., 1998). There are, however, no large-scale studies to assess the magnitude of this potential side-effect. Since we are entering an era of use of aromatase inhibitors to treat non-malignant disorders such as endometriosis, these will be extremely important questions. The roles of various add-back regimens to prevent this potential side-effect remain to be seen.

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