Human Reproduction Update 2000, Vol. 6, No. 5 pp. 413–418 © European Society of Human Reproduction and Embrology

Molecular basis for treating endometriosis with aromatase inhibitors

Serdar E.Bulun[1,*], Khaled M.Zeitoun[2], Kazuto Takayama[3] and Hironobu Sasano[3]

[1]Departments of Obstetrics and Gynecology and Molecular Genetics, University of Illinois at Chicago, IL 60612, [2]Department of Obstetrics and Gynecology, Columbia University College of Physicians and Surgeons, 622 W. 168th St, New York, NY 10032–3702 and [3]Departments Pathology and Obstetrics and Gynecology, Tohoku University School of Medicine, 2–1 Seiryo Machi, Sendai-Shi 980, Japan

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

TABLE OF CONTENTS

Background
Molecular aberrations in endometriosis
Origin of oestrogen in women
Aromatase expression in uterine tissues
Mechanisms responsible for aromatase expression in endometriosis
Aberrant expression of steroidogenic factor-1 (SF1) activates aromatase expression in endometriosis
Clinical relevance: treatment of endometriosis with aromatase inhibitors
Future considerations
Acknowledgements
References

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

*To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, University of Illinois at Chicago, 820 S. Wood St M/C 808, Chicago, IL 60612, USA. Tel: (312) 996-8197; Fax: (312) 996-4238; E-mail: sbulun@uic.edu

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

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

GnRH = gonadotrophin releasing hormone.

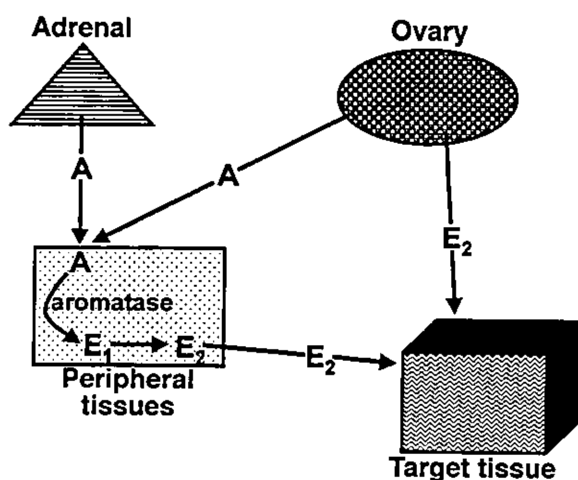

Figure 1. Origin of oestrogen in women. In ovulatory women, oestradiol (E_2) is secreted by the ovary in a cyclic manner. During treatment with a gonadotrophin releasing hormone analogue or postmenopausal period, however, peripheral tissues (e.g. adipose tissue and skin), represent the major source of 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

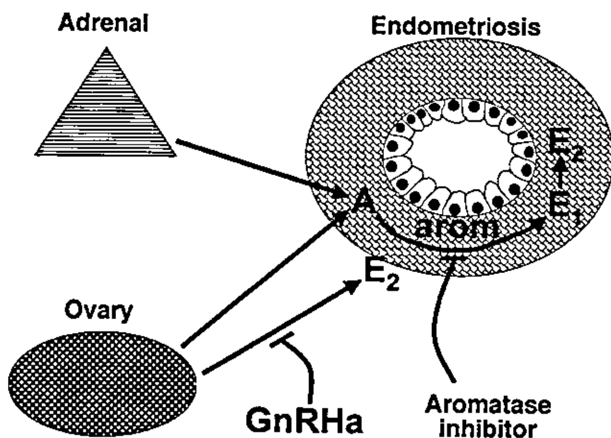

Figure 2. Aberrant aromatase expression in endometriosis. Androstenedione (A) of adrenal and ovarian origins become converted to oestrone (E_1) in endometriotic tissue. Oestrone itself is only weakly oestrogenic and should be converted to oestradiol (E_2) for full oestrogenic action. Endometriotic tissue 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.

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).

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).

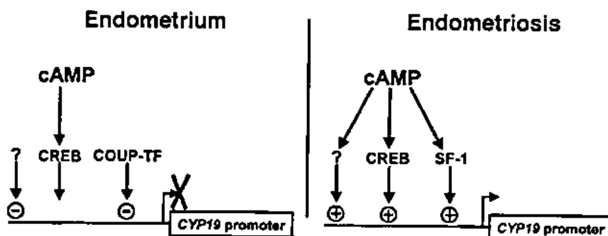

Figure 3. Regulation of *CYP19* (aromatase P450) gene expression via the ovarian-type promoter in stromal cells from the eutopic endometrium and endometriotic tissue (endometriosis). Aromatase P450 mRNA in the eutopic endometrium is absent or barely detectable. Thus, significant transcription of the *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.

Acknowledgements

We thank Dee Alexander and Rosemary Bell for providing expert editorial assistance.

References

Ackerman, G.E., Smith, M.E., Mendelson, C.R. *et al.* (1981) Aromatization of androstenedione by human adipose tissue stromal cells in monolayer culture. *J. Clin. Endocrinol. Metab.*, **53**, 412–417.

Bruner, K.L., Matrisian, L.M., Rodgers, W.H. *et al.* (1997) Suppression of matrix metalloproteinases inhibits establishment of ectopic lesions by human endometrium in nude mice. *J. Clin. Invest.*, **99**, 2851–2857.

Bulun, S.E., Price, T.M., Mahendroo, M.S. *et al.* (1993) A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcription. *J. Clin. Endocrinol. Metab.*, **77**, 1622–1628.

Bulun, S.E., Economos, K., Miller, D. *et al.* (1994a) CYP19 (aromatase cytochrome P450) gene expression in human malignant endometrial tumors. *J. Clin. Endocrinol. Metab.*, **79**, 1831–1834.

Bulun, S.E., Simpson, E.R. and Word, R.A. (1994b) Expression of the CYP19 gene and its product aromatase cytochrome P450 in human leiomyoma tissues and cells in culture. *J. Clin. Endocrinol. Metab.*, **78**, 736–743.

Halme, J., White, C., Kauma, S. *et al.* (1988) Peritoneal macrophages from patients with endometriosis release growth factor activity *in vitro*. *J. Clin. Endocrinol. Metab.*, **66**, 1044–1049.

Hornung, D., Ryan, I.P., Chao, V.A. *et al.* (1997) Immunolocalization and regulation of the chemokine RANTES in human endometrial and endometriosis tissues. *J. Clin. Endocrinol. Metab.*, **82**, 1621–1628.

Huang, J.C., Dawood, M.Y. and Wu, K.K. (1996) Regulation of cyclooxygenase-2 gene in cultured endometrial stromal cells by sex steroids. *Proceedings of 52nd Annual Meeting of the American Society for Reproductive Medicine* 1:5 (Abstract).

Huang, J.-C., Liu, D.-Y., Yadollahi, S. *et al.* (1998) Interleukin-1β induces cyclooxygenase-2 gene expression in cultured endometrial stromal cells. *J. Clin. Endocrinol. Metab.*, **83**, 538–541.

Iveson, T.J., Smith, I.E., Ahern, J. *et al.* (1993) Phase I study of the oral non-

steroidal aromatase inhibitor CGS20267 in healthy post-menopausal women. *J. Clin. Endocrinol. Metab.*, **77**, 324–331.

Kennedy, S. (1999) The genetics of endometriosis. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **82**, 129–133.

MacDonald, P.C., Edman, C.D., Hemsell, D.L. *et al.* (1978) Effect of obesity on conversion of plasma androstenedione to estrone in postmenopausal women with and without endometrial cancer. *Am. J. Obstet. Gynecol.*, **130**, 448–455.

Metzger, D.A., Lessey, B.A., Soper, J.T. *et al.* (1991) Hormone-resistant endometriosis following total abdominal hysterectomy and bilateral salpingo-oophorectomy: correlation with histology and steroid receptor content. *Obstet. Gynecol.*, **78**, 946–950.

Michael, M.D., Kilgore, M.W., Morohashi, K.-I. *et al.* (1995) Ad4BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (CYP19) gene in the ovary. *J. Biol. Chem.*, **270**, 13561–13566.

Michael, M.D., Michael, L.F. and Simpson, E.R. (1997) A CRE-like sequence that binds CREB and contributes to cAMP-dependent regulation of the proximal promoter of the human aromatase P450 (CYP19) gene. *Mol. Cell. Endocrinol.*, **134**, 147–156.

Noble, L.S., Simpson, E.R., Johns, A. *et al.* (1996) Aromatase expression in endometriosis. *J. Clin. Endocrinol. Metab.*, **81**, 174–179.

Noble, L.S., Takayama, K., Putman, J.M. *et al.* (1997) Prostaglandin E2 stimulates aromatase expression in endometriosis-derived stromal cells. *J. Clin. Endocrinol. Metab.*, **82**, 600–606.

Olive, D.L. and Schwartz, L.B. (1993) Endometriosis. *New Engl. J. Med.*, **328**, 1759–1769.

Price, T., Aitken, J., Head, J. *et al.* (1992) Determination of aromatase cytochrome P450 messenger RNA in human breast tissues by competitive polymerase chain reaction (PCR) amplification. *J. Clin. Endocrinol. Metab.*, **74**, 1247–1252.

Ryan, I., Schriock, E.D. and Taylor, R. (1994) Isolation, characterization, and comparison of human endometrial and endometriosis cells *in vitro*. *J. Clin. Endocrinol. Metab.*, **78**, 642–649.

Sampson, J.A. (1927) Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am. J. Obstet. Gynecol.*, **14**, 422–425.

Santen, R.J. (1991) Clinical use of aromatase inhibitors in human breast carcinoma. *J. Steroid Biochem. Mol. Biol.*, **40**, 247–253.

Santen, R.J. (1993) Editorial: estrogen synthesis inhibitors: from 'off the rack' to haute couture. *J. Clin. Endocrinol. Metab.*, **77**, 316–318.

Selvaraj, N., Bhatnagar, A.S. and Moudgal, N.R. (1995) Is there a role for estrogen in follicular maturation in the primate? *Endocrine*, **3**, 245–249.

Sharpe-Timms, K.L., Keisler, L.W., McIntush, E.W. *et al.* (1998) Tissue inhibitor of metalloproteinase-1 concentrations are attenuated in peritoneal fluid and sera of women with endometriosis and restored in sera by gonadotropin-releasing hormone agonist therapy. *Fertil. Steril.*, **69**, 1128–1134.

Simpson, E.R., Mahendroo, M.S., Means, G.D. *et al.* (1994) Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Rev.*, **15**, 342–355.

Sourdaine, P., Mullen, P., White, R. *et al.* (1996) Aromatase activity and CYP19 gene expression in breast cancers. *J. Steroid Biochem. Mol. Biol.*, **59**, 191–198.

Surrey, E.S., Voigt, B., Fournet, N. *et al.* (1995) Prolonged gonadotropin-releasing hormone agonist treatment of symptomatic endometriosis: the role of cyclic sodium etidronate and low-dose norethindrone 'add-back' therapy. *Fertil. Steril.*, **63**, 747–755.

Takayama, K., Zeitoun, K., Gunby, R.T. *et al.* (1998) Treatment of severe postmenopausal endometriosis with an aromatase inhibitor. *Fertil. Steril.*, **69**, 709–713.

Waller, K.G. and Shaw, R.W. (1993) Gonadotropin-releasing hormone analogues for the treatment of endometriosis: long-term follow-up. *Fertil. Steril.*, **59**, 511–515.

Yue, W., Wang, J.P., Hamilton, C.J. *et al.* (1998) In situ aromatization enhances breast tumor estradiol levels and cellular proliferation. *Cancer Res.*, **58**, 927–932.

Zhao, Y., Nichols, J.E., Bulun, S.E. *et al.* (1995) Aromatase P450 gene expression in human adipose tissue: Role of a Jak/STAT pathway in regulation of the adipose-specific promoter. *J. Biol. Chem.*, **270**, 16449–16457.

Zeitoun, K., Takayama, K., Sasano, H. *et al.* (1998) Deficient 17beta-hydroxysteroid dehydrogenase type 2 expression in endometriosis: failure to metabolize estradiol-17β. *J. Clin. Endocrinol. Metab.*, **83**, 4474–4480.

Zeitoun, K., Takayama, K., Michael, M.D. *et al.* (1999) Stimulation of aromatase P450 promoter (II) activity in endometriosis and its inhibition in endometrium are regulated by competitive binding of SF-1 and COUP-TF to the same *cis*-acting element. *Mol. Endocrinol.*, **13**, 239–253.

Received on January 25, 2000; accepted on June 19, 2000

REVIEW

Estrogen biosynthesis in endometriosis: molecular basis and clinical relevance

S E Bulun[1], K M Zeitoun[2], K Takayama[3] and H Sasano[3]

[1]Departments of Obstetrics and Gynecology and Molecular Genetics, University of Illinois at Chicago, 820 S. Wood St. M/C 808, Illinois 60612, USA
[2]Department of Obstetrics and Gynecology, Columbia University College of Physicians and Surgeons, 622 W. 168th St., New York, New York 10032-3702, USA
[3]Departments of Pathology and Obstetrics and Gynecology, Tohoku University School of Medicine, 2-1 Seiryo Machi, Sendai-Shi 980, Japan

(Requests for offprints should be addressed to S E Bulun; Email: sbulun@uic.edu)

ABSTRACT

Conversion of C_{19} steroids to estrogens is catalyzed by aromatase in human ovary, placenta and extraglandular tissues such as adipose tissue, skin and the brain. Aromatase activity is not detectable in normal endometrium. In contrast, aromatase is expressed aberrantly in endometriosis and is stimulated by prostaglandin E_2 (PGE_2). This results in local production of estrogen, which induces PGE_2 formation and establishes a positive feedback cycle. Another abnormality in endometriosis, i.e. deficient hydroxysteroid dehydrogenase (17β-HSD) type 2 expression, impairs the inactivation of estradiol to estrone. These molecular aberrations collectively favor accumulation of increasing quantities of estradiol and PGE_2 in endometriosis. The clinical relevance of these findings was exemplified by the successful treatment of an unusually aggressive case of postmenopausal endometriosis using an aromatase inhibitor.
Journal of Molecular Endocrinology (2000) **25**, 35–42

INTRODUCTION

Endometriosis is a chronic disease manifested by pelvic pain and infertility and defined as the presence of endometrial glands and stroma within the pelvic peritoneum and other extra-uterine sites. It is estimated to affect 2–10% of women in the reproductive age group (Vessey *et al.* 1993, Kjerulff *et al.* 1996). Endometriosis is viewed to be a polygenically inherited disease of complex multifactorial etiology (Olive & Schwartz 1993). Sampson's theory of transplantation of endometrial tissue on the pelvic peritoneum via retrograde menstruation is the most widely accepted explanation for the development of pelvic endometriosis because of convincing circumstantial and experimental evidence (Sampson 1927). Since retrograde menstruation is observed in almost all cycling women, endometriosis is postulated to develop as a result of the coexistence of a defect in clearance of the menstrual efflux from pelvic peritoneal surfaces, possibly involving the immune system (Halme *et al.* 1988). Alternatively, intrinsic molecular aberrations in pelvic endometriotic implants were proposed to contribute significantly to development of endometriosis. Aberrant expression of aromatase, certain cytokines and tissue metalloproteinases, deficiency of 17β-hydroxysteroid dehydrogenase (17β-HSD) type 2 and resistance to the protective action of progesterone are some of these molecular abnormalities (Khorram *et al.* 1993, Sharpe-Timms *et al.* 1995, Noble *et al.* 1996, Osteen *et al.* 1996, Bruner *et al.* 1997, Zeitoun *et al.* 1998, 1999). Since endometriosis is an estrogen-dependent disorder, aromatase expression and 17β-HSD type 2 deficiency are of paramount importance in the pathophysiology of endometriosis. In this article, aberrant mechanisms of estrogen biosynthesis and metabolism in women with endometriosis are reviewed, with emphasis on identifying targets for new treatment strategies.

0952–5041/00/025–035 © 2000 Society for Endocrinology *Printed in Great Britain*
Online version via http://www.endocrinology.org

DISCUSSION

Estrogen biosynthesis and metabolism in humans

The conversion of androstenedione and testosterone to estrone and estradiol is catalyzed by aromatase, which is expressed in a number of human tissues and cells such as ovarian granulosa cells, placental syncytiotrophoblast, adipose tissue and skin fibroblasts, and the brain. In the reproductive-age woman, the ovary is the most important site of estrogen biosynthesis, and this takes place in a cyclic fashion. Upon binding of follicle-stimulating hormone (FSH) to its G-protein-coupled receptor in the granulosa cell membrane, intracellular cAMP levels rise and enhance binding of two critical transcription factors, i.e. steroidogenic factor-1 (SF-1) and cAMP response element binding protein (CREB), to the classically located proximal promoter II of the aromatase gene (Michael *et al.* 1995, 1997). This, in turn, activates aromatase expression and consequently estrogen secretion from the pre-ovulatory follicle (Simpson *et al.* 1994, Michael *et al.* 1995).

On the other hand, in postmenopausal women, estrogen formation takes place in extra-ovarian tissues such as the adipose tissue and skin (MacDonald *et al.* 1967, 1978, Ackerman *et al.* 1981) (Fig. 1). In contrast to cAMP regulation of aromatase expression in the ovary, this is controlled primarily by cytokines (IL-6, IL-11, TNFα) and glucocorticoids via the alternative use of promoter I.4 in adipose tissue and skin fibroblasts (Simpson *et al.* 1994). The major substrate for aromatase in adipose tissue and skin is androstenedione of adrenal origin. In postmenopausal women, approximately 2% of circulating androstenedione is converted to estrone, which is further converted to estradiol in these extra-ovarian tissues. This may give rise to significant serum levels of estradiol capable of causing endometrial hyperplasia or even carcinoma (MacDonald *et al.* 1967, 1978).

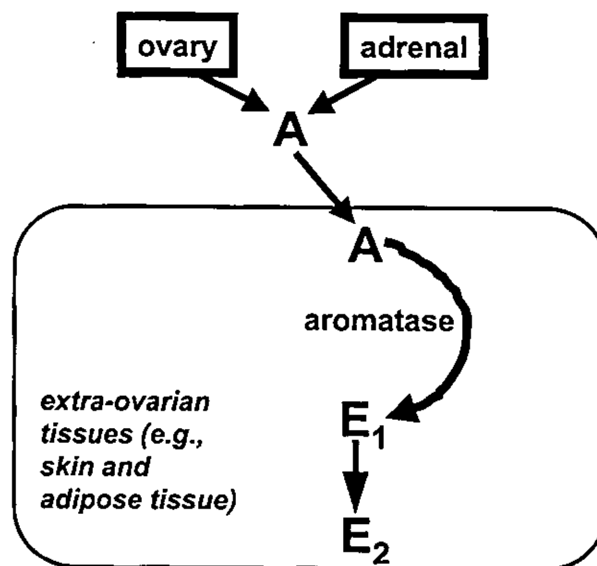

FIGURE 1. Extra-ovarian estrogen formation in women. Estradiol (E_2) in women is either directly secreted by the ovary or produced in extra-ovarian sites (adipose tissue and skin). The principal substrate for extra-ovarian aromatase activity in women is androstenedione (A) of adrenal and ovarian origins. Androstenedione is converted by aromatase to estrone (E_1) in adipose tissue and skin fibroblasts. Estrone is further converted to E_2 by 17β-HSD type 1 activity in these peripheral tissues. Thus, extra-ovarian aromatization is the major source for circulating E_2 in the postmenopausal period or during ovarian suppression.

Aromatase expression in Müllerian-derived tissues

Müllerian tissues are known targets of estrogen action. Until recently, estrogen action has been classically viewed to occur only via an 'endocrine' mechanism: in other words, it was thought that only circulating estradiol, whether secreted by the ovary or formed in the adipose tissue, could exert an estrogenic effect after delivery to target tissues via the bloodstream. Studies on aromatase expression in breast cancer demonstrated that paracrine mechanisms play an important role in estrogen action in this tissue (Bulun *et al.* 1993*a*). Estrogen produced by aromatase activity in breast adipose tissue fibroblasts was demonstrated to promote the growth of adjacent malignant breast epithelial cells (Yue *et al.* 1998). Finally, we demonstrated an 'intracrine' effect of estrogen in uterine leiomyomas and endometriosis: estrogen produced by aromatase activity in the cytoplasm of leiomyoma smooth muscle cells or endometriotic stromal cells can exert its effects by readily binding to its nuclear receptor within the same cell (Bulun *et al.* 1994, Noble *et al.* 1996, 1997). Disease-free endometrium and myometrium, on the other hand, lack aromatase expression (Bulun *et al.* 1993*b*, Noble *et al.* 1997).

The significance of aromatase expression in endometriosis

Among estrogen-responsive pelvic disorders, aromatase expression was studied in greatest detail in endometriosis (Bulun *et al.* 1993*b*, Noble *et al.* 1996, 1997, Zeitoun *et al.* 1999). Firstly, extremely high

levels of aromatase mRNA were found in extra-ovarian endometriotic implants and endometriomas. Secondly, endometriosis-derived stromal cells in culture incubated with a cAMP analog displayed extraordinarily high levels of aromatase activity comparable to that in placental syncytiotrophoblast (Noble *et al.* 1997). These exciting findings led us to test a battery of growth factors, cytokines and other substances that might induce aromatase activity via a cAMP-dependent pathway in endometriosis. Prostaglandin E_2 (PGE_2) was found to be the most potent known inducer of aromatase activity in endometriotic stromal cells (Noble *et al.* 1997). In fact, this PGE_2 effect was found to be mediated via the cAMP-inducing EP_2 receptor subtype. Moreover, estrogen was reported to increase PGE_2 formation by stimulating cyclo-oxygenase type 2 (COX-2) enzyme in endometrial stromal cells in culture (Huang *et al.* 1996). Thus, a positive feedback loop for continuous local productions of estrogen and PGs is established, favoring the proliferative and inflammatory characteristics of endometriosis (Fig. 2). Additionally, aromatase mRNA was also detected in the eutopic endometrial samples of women with moderate to severe endometriosis (but not in those of disease-free women) albeit in much smaller quantities compared with endometriotic implants (Noble *et al.* 1996). This may be suggestive of a genetic defect in women with endometriosis, which is manifested by this subtle finding in the eutopic endometrium. We propose that when defective endometrium with low levels of aberrant aromatase expression reaches the pelvic peritoneum by retrograde menstruation, it causes an inflammatory reaction that exponentially increases local aromatase activity, i.e. estrogen formation, induced directly or indirectly by PGs and cytokines (Noble *et al.* 1997). It would be rather naive to propose that aberrant aromatase expression is the only important molecular mechanism in the development and growth of pelvic endometriosis. There may be many other molecular mechanisms that favor the development of endometriosis: abnormal expression of proteinase type enzymes that remodel tissues or their inhibitors (matrix metalloproteinases, tissue inhibitor of metalloproteinase-1), certain cytokines (IL-6, RANTES) and growth factors (EGF) represent some of mechanisms (Khorram *et al.* 1993, Sharpe-Timms *et al.* 1995, Osteen *et al.* 1996, Bruner *et al.* 1997). Alternatively, a defective immune system that fails to clear peritoneal surfaces of the retrograde menstrual efflux has been proposed in the development of endometriosis (Halme *et al.* 1988, Hill 1992). The development of endometriosis in an individual woman probably requires the coexistence of a threshold number of these aberrations. Nonetheless, aberrant aromatase expression is clinically relevant, since aromatase inhibitors suppress postmenopausal endometriosis (Takayama *et al.* 1998).

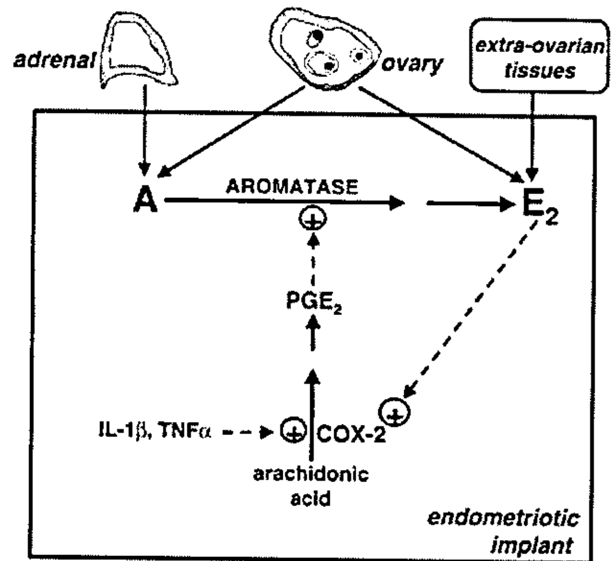

FIGURE 2. Origin of estrogen in endometriotic lesions: estradiol (E_2) that affects an endometriotic lesion arises from several body sites. In an ovulatory woman, E_2 is secreted directly from the ovary in a cyclic fashion. In the early follicular phase and after menopause, extra-ovarian tissues (adipose and skin) are the most important sources to account for the circulating E_2. Estradiol is also produced locally in the endometriotic implant itself in both ovulatory and postmenopausal women. The most important precursor, androstenedione (A) of adrenal and ovarian origins, becomes converted to estrone (E_1) that is in turn reduced to E_2 in these tissues and endometriotic implants. We demonstrated significant levels of 17β-hydroxysteroid dehydrogenase type 1 expression in endometriosis, which catalyzes the conversion of E_1 to E_2 (Zeitoun *et al.* 1998). Estradiol and cytokines (IL-lα, TNFβ), which are increased in endometriosis, induce cyclo-oxygenase-2 (COX-2) giving rise to elevated concentrations of PGE_2 in this tissue (Huang *et al.*). PGE_2 in turn, is the most potent known stimulator of aromatase in endometriotic stromal cells (Noble *et al.* 1997). This establishes a positive feedback loop in favor of continuous estrogen formation in endometriosis.

Regulation of aromatase expression in endometriotic stromal cells

As emphasized earlier, PGE_2 was found to be the most potent known inducer of aromatase activity by increasing cAMP levels via cell surface EP_2 receptors in endometriotic stromal cells (Noble

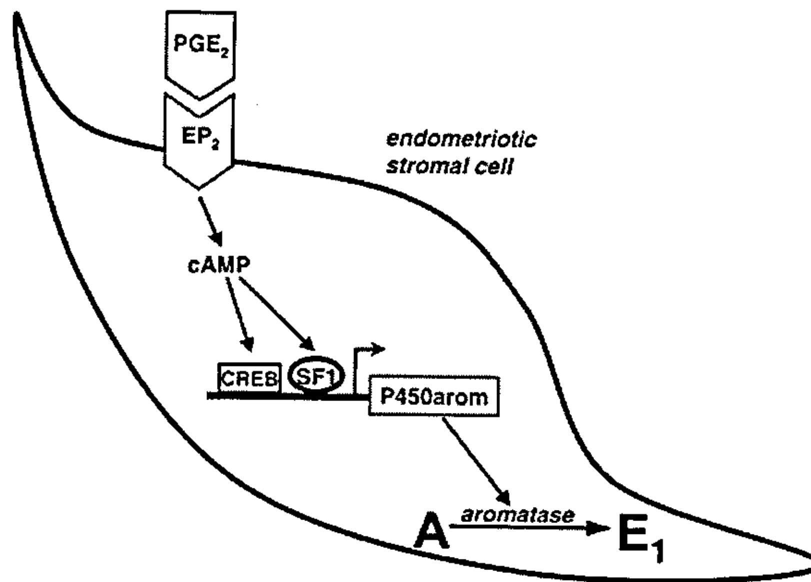

FIGURE 3. Proposed mechanism of regulation of aromatase (P450arom) expression by SF-1 and CREB in endometriosis. Upon binding of PGE_2 to its cell surface EP_2 receptor, intracellular cAMP levels increase. This gives rise to binding of cAMP response element binding protein (CREB) and SF-1 to specific motifs upstream of aromatase promoter II. The stimulatory type transcription factor SF-1 binds as a monomer to a nuclear receptor half-site with a higher affinity compared with that of the inhibitory factor COUP-TF (not shown in the figure), which binds to the same site relatively loosely as a dimer. SF-1 then synergizes with CREB (bound to upstream CRE) and possibly other factors to activate transcription of the P450arom gene in response to cAMP (Zeitoun *et al.* 1999).

et al. 1997). On the other hand, neither cAMP analogs nor PGE_2 was capable of stimulating any detectable aromatase activity in eutopic endometrial stromal cells in culture. The obvious question became: what are the molecular differences that give rise to aromatase expression in endometriosis and its inhibition in eutopic endometrium? To address this, we first determined that the cAMP-inducible promoter II was used for *in vivo* aromatase expression in endometriotic tissue (Zeitoun *et al.* 1999). Then, a stimulatory transcription factor, SF-1, and an inhibitory factor, chicken ovalbumin upstream promoter transcription factor (COUP-TF), were found to compete for the same binding site in aromatase promoter II. COUP-TF was ubiquitously expressed in both eutopic endometrium and endometriosis, whereas SF-1 was expressed, specifically in endometriosis but not in eutopic endometrium, and binds to aromatase promoter more avidly than COUP-TF (Zeitoun *et al.* 1999). Thus, SF-1 and other transcription factors (e.g. CREB) activate transcription in endometriosis, whereas COUP-TF, which occupies the same DNA site in eutopic endometrium, inhibits this process (Zeitoun *et al.* 1999) (Fig. 3). In summary, one of the molecular alterations leading to local aromatase expression in endometriosis but not in normal endometrium is the aberrant production of SF-1 in endometriotic stromal cells, which overcomes the protective inhibition maintained normally by COUP-TF in the eutopic endometrium.

Interconversions of estrone and estradiol in endometriosis

The primary substrate for aromatase activity in endometriosis is androstenedione of adrenal and ovarian origins in premenopausal women. The major product of aromatase activity in endometriosis, namely estrone, is only weakly estrogenic and must be converted to the potent estrogen estradiol to exert a full estrogenic effect. We demonstrated that the enzyme 17β-HSD type 1, which catalyzes the conversion of estrone to estradiol, is expressed in endometriosis (Andersson & Moghrabi 1997, Zeitoun *et al.* 1998). In contrast, the enzyme 17β-HSD type 2 (encoded by a separate gene)

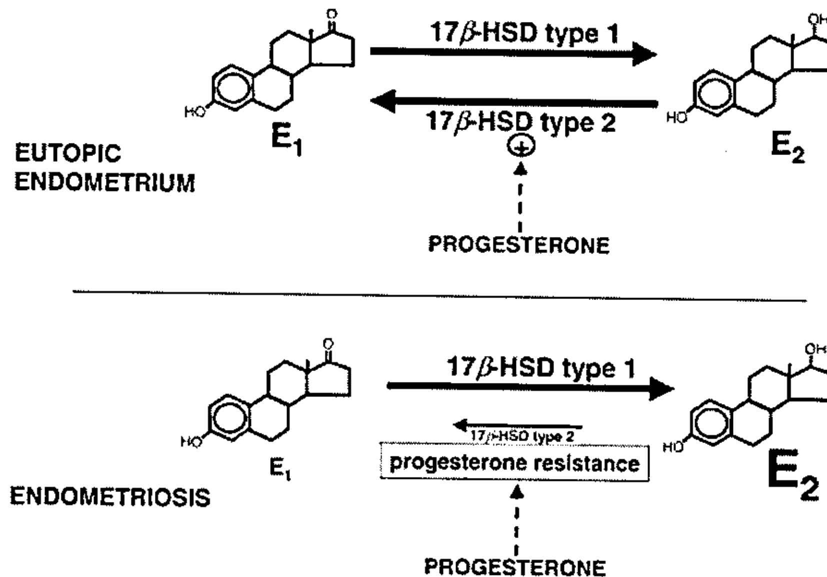

FIGURE 4. Defective inactivation of estradiol (E_2) in endometriosis: E_2 reaches the endometriotic lesions via the blood stream. Additionally, estrone (E_1) is produced in the stromal cell via aberrant aromatase activity. Estrone is further reduced to E_2 by 17β-HSD type 1 in the endometriotic tissue. Estradiol is normally inactivated by conversion to E_1 by 17β-HSD type 2 in epithelial cells of the eutopic endometrium in response to progesterone during the secretory phase. In endometriotic tissue, however, E_2 is not metabolized owing to the lack of 17β-HSD type 2, giving rise to increased local concentration of this potent estrogen. The absence of 17β-HSD type 2 expression in endometriosis despite high levels of progesterone during the secretory phase is indicative of selective progesterone resistance in this tissue.

inactivates estradiol by catalyzing its conversion to estrone in eutopic endometrial glandular cells during the luteal phase (Andersson & Moghrabi 1997). Progesterone actually induces the activity of this enzyme in endometrial glandular cells in culture, making inactivation of estradiol to estrone one of the anti-estrogenic properties of progesterone (Satyaswaroop *et al.* 1982). The expression of 17β-HSD type 2 is absent from endometriotic glandular cells, as demonstrated in paired samples of eutopic endometrium and pelvic endometriosis obtained simultaneously during the luteal phase (Zeitoun *et al.* 1998). Consequently, this protective mechanism that lowers estradiol levels is lost in endometriotic tissue (Zeitoun *et al.* 1998). The aberrant expression of aromatase, the presence of 17β-HSD type 1 and the absence of 17β-HSD type 2 from endometriosis collectively give rise to elevated local levels of estradiol compared with eutopic endometrium. Additionally, 17β-HSD type 2 deficiency may also be viewed as a defective action of progesterone, which fails to induce this enzyme in endometriotic tissue (Fig. 4).

Rationale for using aromatase inhibitors to treat endometriosis

Endometriosis is successfully suppressed by estrogen deprivation with GnRH analogs or the induction of surgical menopause. Control of pelvic pain with GnRH agonists is usually successful during and immediately after the treatment, whereas pain associated with endometriosis returns in up to 75% of these women (Henzl *et al.* 1988, Waller & Shaw 1993). There may be multiple reasons for the failure of GnRH agonist treatment of endometriosis. One likely explanation is the presence of significant estradiol production that continues in the adipose tissue, skin and endometriotic implant *per se* during the GnRH agonist treatment. Therefore, blockage of aromatase activity in these extra-ovarian sites with an aromatase inhibitor may keep larger number of patients in remission for longer periods of time (Fig. 5). The most striking evidence for the significance of extra-ovarian estrogen production is the recurrence of endometriosis after successfully completed hysterectomy and bilateral salpingo-oophorectomy in a

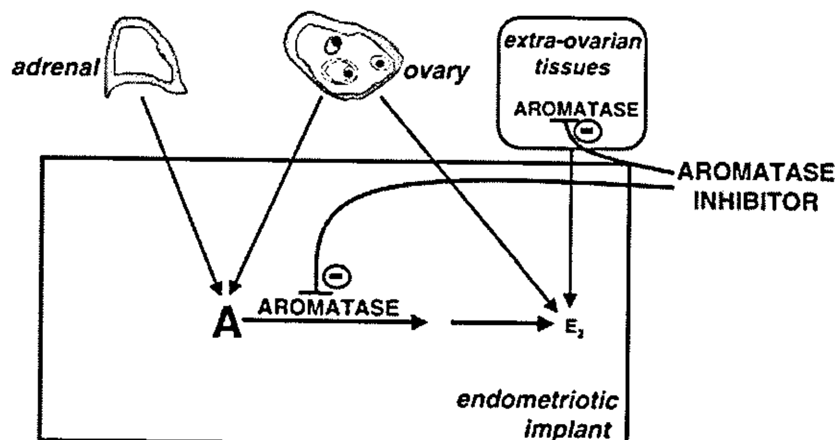

FIGURE 5. Sites of action of aromatase inhibitors to treat endometriosis. In cases resistant to treatment with GnRH agonists or in postmenopausal endometriosis, the use of aromatase inhibitors to block estrogen formation in the skin and adipose tissue as well as in endometriotic tissue may be critical in inhibiting the growth of endometriotic tissue. Recurrent endometriosis, especially after surgical removal of the ovaries, may represent lesions that are sensitive to extremely low levels of estradiol (E_2). Thus, suppression of E_2 production in the extra-ovarian sites (adipose tissue/skin) and in endometriotic tissue may be mandatory for successful treatment of endometriosis.

number of women (Metzger *et al.* 1991, Takayama *et al.* 1998). Endometriotic tissue in one such aggressive case was found to express much higher levels of aromatase mRNA compared with premenopausal endometriosis (Takayama *et al.* 1998). We recently reported the treatment of a 57-year-old overweight woman who had recurrence of severe endometriosis after hysterectomy and bilateral salpingo-oophorectomy. Two additional laparotomies were performed owing to persistent severe pelvic pain and bilateral ureteral obstruction leading to left renal atrophy and right hydronephrosis. Treatment with megestrol acetate was ineffective. A large (3 cm) vaginal endometriotic lesion contained unusually high levels of aromatase mRNA. The patient was given anastrozole (an aromatase inhibitor) for 9 months. Despite the addition of calcium and alendronate (a nonsteroidal inhibitor of bone resorption), bone density in the lumbar spine decreased by 6·2%. The occurrence of significant bone loss in this particular case should be studied further. Dramatic relief of the pain and regression of the vaginal endometriotic lesion were observed within the first month of treatment. At the same time, circulating estradiol levels were reduced to 50% of the baseline value. Markedly high pretreatment levels of aromatase mRNA in the endometriotic tissue became undetectable in a repeat biopsy 6 months later, and the lesion nearly disappeared after 9 months of therapy. Two potential mechanisms may have accounted for this strikingly successful result. Firstly, there was evidence of suppression of extra-ovarian (i.e. skin and adipose tissue) aromatase activity, giving rise to a significant decrease in serum estradiol level (Fig. 5). Secondly, unusually high levels of aromatase expression in the endometriotic lesion disappeared after treatment with the aromatase inhibitor, anastrozole (Fig. 5). Besides the expected direct inhibition of aromatase activity in endometriosis by anastrozole, the disappearance of aromatase mRNA expression in the lesion may be explicable by denial of estrogen that is known to stimulate local biosynthesis of PGE_2, which, in turn, stimulates aromatase expression (Fig. 2).

In summary, the recently developed potent aromatase inhibitors are candidate drugs in the treatment of endometriosis that is resistant to standard regimens. In fact, the use of aromatase inhibitors may be the only available treatment for aggressive postmenopausal endometriosis. It remains to be seen whether aromatase inhibitors alone or together with present lines of therapy in premenopausal women will increase the pain-free interval and time to recurrence after discontinuation (Fig. 5). Studies are under way to address these questions.

SUMMARY

The development and growth of endometriotic lesions are estrogen-dependent. The mechanisms and effectiveness of hormonal treatments for endometriosis should be re-evaluated in view of the new advances that increased our understanding of the body sites of estrogen production in a woman with endometriosis. In addition to ovarian secretion, estradiol is also produced in peripheral sites such as skin, adipose tissue and endometriotic lesions *per se*. We suggest that the intracrine and paracrine effects of estradiol produced in the target tissue amplify the estrogenic action of steroid hormones delivered via the circulation. Additionally, defective inactivation of estradiol in endometriosis in contrast to eutopic endometrium may further enhance this local effect. Aberrant aromatase activity and defective estradiol metabolism in endometriosis are consequences of specific molecular aberrations such as inappropriate expression of a stimulatory transcription factor or progesterone resistance in this tissue. The clinical relevance of these findings was recently exemplified by the successful treatment of a severe case of recurrent postmenopausal endometriosis with an aromatase inhibitor. Future treatment strategies may be designed to target the signal transduction for aromatase expression in endometriosis or to enhance progesterone action in this tissue.

ACKNOWLEDGEMENTS

We thank Margarita Guerrero and Dee Alexander for providing expert editorial assistance. This research work was supported, in part, by the NIH grants HD38691 and CA67167 (Serdar Bulun) and an AAOGF Fellowship Award (Khaled Zeitoun).

REFERENCES

Ackerman GE, Smith ME, Mendelson CR, MacDonald PC & Simpson ER 1981 Aromatization of androstenedione by human adipose tissue stromal cells in monolayer culture. *Journal of Clinical Endocrinology and Metabolism* **53** 412–417.

Andersson S & Moghrabi N 1997 Physiology and molecular genetics of 17β-hydroxysteroid dehydrogenases. *Steroids* **62** 143–147.

Bruner KL, Matrisian LM, Rodgers WH, Gorstein F & Osteen KG 1997 Suppression of matrix metalloproteinases inhibits establishment of ectopic lesions by human endometrium in nude mice. *Journal of Clinical Investigation* **99** 2851–2857.

Bulun SE, Mahendroo MS & Simpson ER 1993*a* Polymerase chain reaction amplification fails to detect aromatase cytochrome P450 transcripts in normal human endometrium or decidua. *Journal of Clinical Endocrinology and Metabolism* **76** 1458–1463.

Bulun SE, Price TM, Mahendroo MS, Aitken J & Simpson ER 1993*b* A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcription. *Journal of Clinical Endocrinology and Metabolism* **77** 1622–1628.

Bulun SE, Simpson ER & Word RA 1994 Expression of the CYP19 gene and its product aromatase cytochrome P450 in human leiomyoma tissues and cells in culture. *Journal of Clinical Endocrinology and Metabolism* **78** 736–743.

Halme J, White C, Kauma S, Estes J & Haskill S 1988 Peritoneal macrophages from patients with endometriosis release growth factor activity *in vitro*. *Journal of Clinical Endocrinology and Metabolism* **66** 1044–1049.

Henzl MR, Corson SL, Moghissi K, Buttram VC, Berqvist C & Jacobson J 1988 Administration of nasal nafarelin as compared with oral danazol for endometriosis. *New England Journal of Medicine* **318** 485–489.

Hill JA 1992 Immunology and endometriosis. *Fertility and Sterility* **58** 262–264.

Huang JC, Dawood MY & Wu KK 1996 Regulation of cyclooxygenase-2 gene in cultured endometrial stromal cells by sex steroids. *Proceedings of the American Society of Reproductive Medicine Meeting* **S5** (abstract).

Khorram O, Taylor RN, Ryan IP, Schall TJ & Landers DV 1993 Peritoneal fluid concentrations of the cytokine RANTES correlate with the severity of endometriosis. *American Journal of Obstetrics and Gynecology* **169** 1545–1549.

Kjerulff KH, Erickson BA & Langenberg PW 1996 Chronic gynecological conditions reported by US women: findings from the National Health Information Survey 1984 to 1992. *American Journal of Public Health* **86** 195–199.

MacDonald PC, Rombaut RP & Siiteri PK 1967 Plasma precursors of estrogen I. Extent of conversion of plasma Δ^4-androstenedione to estrone in normal males and non-pregnant normal, castrate and adrenalectomized females. *Journal of Clinical Endocrinology and Metabolism* **27** 1103–1111.

MacDonald PC, Edman CD, Hemsell DL, Porter J & Siiteri PK 1978 Effect of obesity on conversion of plasma androstenedione to estrone in postmenopausal women with and without endometrial cancer. *American Journal of Obstetrics and Gynecology* **130** 448–455.

Metzger DA, Lessey BA, Soper JT, McCarty KS, Jr & Haney AF 1991 Hormone-resistant endometriosis following total abdominal hysterectomy and bilateral salpingo-oophorectomy: correlation with histology and steroid receptor content. *Obstetrics and Gynecology* **78** 946–950.

Michael MD, Kilgore MW, Morohashi KI & Simpson ER 1995 Ad4 BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (CYP19) gene in the ovary. *Journal of Biological Chemistry* **270** 13561–13566.

Michael MD, Michael LF & Simpson ER 1997 A CRE-like sequence that binds CREB and contributes to cAMP-dependent regulation of the proximal promoter of the human aromatase P450 (CYP19) gene. *Molecular and Cellular Endocrinology* **134** 147–156.

Noble LS, Simpson ER, Johns A & Bulun SE 1996 Aromatase expression in endometriosis. *Journal of Clinical Endocrinology and Metabolism* **81** 174–179.

Noble LS, Takayama K, Putman JM, Johns DA, Hinshelwood MM, Agarwal VR, Zhao Y, Carr BR & Bulun SE 1997 Prostaglandin E_2 stimulates aromatase expression in endometriosis-derived stromal cells. *Journal of Clinical Endocrinology and Metabolism* **82** 600–606.

Olive DL & Schwartz LB 1993 Endometriosis. *New England Journal of Medicine* **328** 1759–1769.

Osteen KG, Bruner KL & Sharpe-Timms KL 1996 Steroid and growth factor regulation of matrix metalloproteinase expression and endometriosis. *Seminars in Reproductive Endocrinology* **15** 301–308.

Sampson JA 1927 Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *American Journal of Obstetrics and Gynecology* **14** 422–425.

Satyaswaroop PG, Wartell DJ & Mortel R 1982 Distribution of progesterone receptor, estradiol dehydrogenase, and 20α-dihydroprogesterone dehydrogenase activities in human endometrial glands and stroma: progestin induction of steroid dehydrogenase activities *in vitro* is restricted to the glandular epithelium. *Endocrinology* **111** 743–749.

Sharpe-Timms KL, Penney LL, Zimmer RL, Wright JA, Zhang Y & Surewicz K 1995 Partial purification and amino acid sequence analysis of endometriosis protein-II (ENDO-II) reveals homology with tissue inhibitor of metalloproteinases-1 (TIMP-1). *Journal of Clinical Endocrinology and Metabolism* **80** 3784–3787.

Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, Mendelson CR & Bulun SE 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Reviews* **15** 342–355.

Takayama K, Zeitoun K, Gunby RT, Sasano H, Carr BR & Bulun SE 1998 Treatment of severe postmenopausal endometriosis with an aromatase inhibitor. *Fertility and Sterility* **69** 709–713.

Vessey MP, Villard-Mackintosh L & Painter R 1993 Epidemiology of endometriosis in women attending family planning clinics. *British Medical Journal* **306** 182–184.

Waller KG & Shaw RW 1993 Gonadotropin-releasing hormone analogues for the treatment of endometriosis: long-term follow-up. *Fertility and Sterility* **59** 511–515.

Yue W, Wang JP, Hamilton CJ, Demers LM & Santen RJ 1998 *In situ* aromatization enhances breast tumor estradiol levels and cellular proliferation. *Cancer Research* **58** 927–932.

Zeitoun K, Takayama K, Sasano H, Suzuki T, Moghrabi N, Andersson S, Johns A, Meng L, Putman M, Carr B & Bulun SE 1998 Deficient 17β-hydroxysteroid dehydrogenase type 2 expression in endometriosis: failure to metabolize estradiol-17β. *Journal of Clinical Endocrinology and Metabolism* **83** 4474–4480.

Zeitoun K, Takayama K, Michael MD & Bulun SE 1999 Stimulation of aromatase P450 promoter (II) activity in endometriosis and its inhibition in endometrium are regulated by competitive binding of SF-1 and COUP-TF to the same *cis*-acting element. *Molecular Endocrinology* **13** 239–253.

RECEIVED 17 December 1999

0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(5):2084–2091
Copyright © 2003 by The Endocrine Society
doi: 10.1210/en.2002-221030

17β-Hydroxysteroid Dehydrogenase Type XI Localizes to Human Steroidogenic Cells

ZHONGLIN CHAI, PHILLIP BRERETON, TAKASHI SUZUKI, HIRONOBU SASANO, VARUNI OBEYESEKERE, GENEVIEVE ESCHER, RICHARD SAFFERY, PETER FULLER, CARLA ENRIQUEZ, AND ZYGMUNT KROZOWSKI

Baker Heart Research Institute (Z.C., P.B., V.O., G.E., C.E., Z.K.), Melbourne, Victoria 8008, Australia; First Department of Surgery and Pathology, Tohoku University, School of Medicine, Sendai, Miyagi 980-8575, Japan (T.S., H.S.); and Murdoch Children's Research Institute (R.S.), Melbourne 3052, Australia; and Prince Henry's Institute (P.F.), Melbourne 3168, Australia

We searched expressed sequence tag databases with conserved domains of the short-chain alcohol dehydrogenase superfamily and identified another isoform of 17β-hydroxysteroid dehydrogenase, 17βHSDXI. This enzyme converts 5α-androstane-3α, 17β-diol to androsterone. The substrate has been implicated in supporting gestation and modulating γ-aminobutyric acid receptor activity. 17βHSDXI is colinear with human retinal short-chain dehydrogenase/reductase retSDR2, a protein with no known biological activity (accession no. AAF06939). Of the proteins with known function, 17βHSDXI is most closely related to the retinol-metabolizing enzyme retSDR1, with which it has 30% identity. There is a polymorphic stretch of 15 adenosines in the 5′ untranslated region of the cDNA sequence and a silent polymorphism at C719T. A 17βHSDXI construct with a stretch of 20 adenosines was found to produce significantly more enzyme activity than constructs containing 15 or less adenosines (43% *vs.* 26%, $P < 0.005$). The C719T polymorphism is present in 15% of genomic DNA samples. Northern blot analysis showed high levels of 17βHSDXI expression in the pancreas, kidney, liver, lung, adrenal, ovary, and heart. Immunohistochemical staining for 17βHSDXI is strong in steroidogenic cells such as syncytiotrophoblasts, sebaceous gland, Leydig cells, and granulosa cells of the dominant follicle and corpus luteum. In the adrenal 17βHSDXI, staining colocalized with the distribution of 17α-hydroxylase but was stronger in the mid to outer cortex. 17βHSDXI was also found in the fetus and increased after birth. Liver parenchymal cells and epithelium of the endometrium and small intestine also stained. Regulation studies in mouse Y1 cells showed that cAMP down-regulates 17βHSDXI enzymatic activity (40% *vs.* 32%, $P < 0.05$) and reduces gene expression to undetectable levels. All-*trans*-retinoic acid did not affect 17βHSDXI expression or activity, but addition of the retinoid together with cAMP significantly decreased activity over cAMP alone (32% *vs.* 23%, $P < 0.05$). Cloning and sequencing of the 17βHSDXI promoter identified the potential nuclear receptor steroidogenic factor-1 half-site TCCAAGG-CCGG, and a cluster of three other potential steroidogenic factor-1 half-sites were found in the distal part of intron 1. Collectively, these results suggest a role for 17βHSDXI in androgen metabolism during steroidogenesis and a possible role in nonsteroidogenic tissues including paracrine modulation of 5α-androstane-3α, 17β-diol levels. 17βHSDXI could act by metabolizing compounds that stimulate steroid synthesis and/or by generating metabolites that inhibit it. (*Endocrinology* 144: 2084–2091, 2003)

SEQUENCING OF THE human genome has resulted in the identification of a huge number of new genes of unknown function. Some insights into their biological roles can be inferred from knowledge of the evolution of proteins in superfamilies. The short-chain alcohol dehydrogenase (SCAD) superfamily is involved in the oxidoreduction and oxidation of secondary alcohol groups and ketones such as in the interconversion of cortisol and cortisone or estradiol and estrone. Members of this family are thought to have evolved from enzymes that metabolized sugars, and over time substrates have come to include polyols, organic acids, retinoids, vitamins, prostaglandins, and steroids. A handful of the 200 or so SCAD members are highly conserved, allowing insights into the nature of the substrate for newly identified proteins, but most proteins are less than 30% identical and have sequences that are uninformative with respect to biological function. To confound matters, dehydrogenases acting on the same substrate can be highly divergent.

The important biological role played by the SCAD superfamily is exemplified by the 11β-hydroxysteroid dehydrogenase (11βHSD), 11βHSD1 and 11βHSD2 (1). These enzymes play pivotal roles in modulating tissue levels of glucocorticoids and are thus excellent mediators of paracrine and autocrine actions. 11βHSD1 activates glucocorticoids by converting cortisone into cortisol and effectively amplifies levels of this hormone in liver, adipose tissue, and brain (2). Deletion of this gene shows that it plays a role in lipogenesis and insulin sensitivity and may thus be potentially important in atherogenesis (3). Recently overexpression of 11βHSD1 in mouse adipose tissue confirmed these observations and underlined the importance of local glucocorticoid activation in visceral obesity and the metabolic syndrome (4). In contrast, 11βHSD2 inactivates glucocorticoids, endowing specificity on the mineralocorticoid receptor in sodium-transporting epithelia (5) and in the placenta protecting the fetus from high circulating levels of maternal glucocorticoids (6). The

Abbreviations: 3α-Adiol, Androstane-3α, 17β-diol, 5α-[9, 11-^{3}H(N); 11βHSD, 11β-hydroxysteroid dehydrogenase; 17βHSD, 17β-hydroxysteroid dehydrogenase; DHT, dihydrotestosterone; EST, expressed sequence tag; GABA, γ-aminobutyric acid; pA, poly adenosine; RT, reverse transcription; SCAD, short-chain alcohol dehydrogenase; SF, steroidogenic factor; TLC, thin-layer chromatography; UTR, untranslated region.

Downloaded from endo.endojournals.org on June 23, 2005

syndrome of apparent mineralocorticoid excess, in which patients exhibit excessive sodium retention and severely elevated blood pressure, is a result of mutations in this gene (7, 8). Similarly, various 17β-hydroxysteroid dehydrogenases (17βHSDs) regulate the levels of active androgens and estrogens in a tissue specific manner (9), and mutations in the 17βHSD3 gene lead to male pseudohermaphroditism (10).

In previous studies a novel dehydrogenase was identified by searching expressed sequence-tagged databases translated in six frames for conserved motifs of the SCAD superfamily (11, 12). The protein has been previously denoted Pan1b or retSDR2 (13), but demonstration of 17βHSD activity necessitated renaming it to 17βHSDXI. Although it is present in the eye extensive studies in cell-free systems have been unable to demonstrate retinoid metabolizing activity (12). However, transfected CHOP cells rapidly metabolize androstane-3α, 17β-diol, 5α-androstane, 7β-diol [3α-Adiol] (13). In the present study, we describe polymorphic forms of the messenger RNA and its effects on enzyme activity and further characterize substrate specificity, tissue distribution, and regulation of 17βHSDXI expression in steroidogenic cells.

Materials and Methods

All steroids and carbenoxolone were from Sigma (St. Louis, MO) or Steraloids (Newport, RI) with the exception of 3α-Adiol, which was from NEN Life Science Products (Boston, MA). CHOP-C4 cells were a gift from Dr. James Dennis (Samuel Lunenfield Institute, London, ONT). Silica gel thin-layer chromatography (TLC) plates with aluminum backing (HPTLC-Alufolien Kieselgel 60) were from E. Merck (Darmstadt, Germany).

Preparation of constructs of 17βHSDXI with various lengths of poly adenosine (pA) in 5′ untranslated regions (UTRs)

Human 17βHSDXI cDNA was amplified by PCR from a pcDNA1 clone using SP6 reverse primer 5′-GATTTAGGTGACACTATAG-3′ and a 17βHSDXI-specific forward primer containing 5, 10, 15, or 20 adenosines at the 5′ end (5′-$(A)_n$CACACCAAAC-3′ where n represents 5, 10, 15, or 20). The PCR products were cloned into pGEM-T EASY vector (Promega Corp., Madison, WI). The 17βHSDXI inserts containing 5′pA of various lengths were excised using *Eco*RI and subcloned into the pcDNA1 expression vector. All constructs were confirmed by DNA sequencing. Several preparations of plasmid DNA were used throughout the study and all gave similar results.

Estimation of enzyme activity of 5′pA variants and competition studies

For estimation of enzyme activity of the 5′pA variants, CHOP cells were transfected with 3 μg construct DNA using DEAE-dextran (14). Transfection efficiency was checked by transfection with β-galactosidase-expressing plasmid and was always around 75%. Tritiated 3α-Adiol (8 nM) in serum-free RPMI 1640 medium (Invitrogen, Carlsbad, CA) was added 48 h after transfection and incubated further for 18 h. Steroids were extracted from the medium and separated by TLC with standard steroids as previously described (13, 15). For competition studies, serum-free RPMI 1640 medium containing 8 nM tritium labeled 3α-Adiol and 100 μM cold steroid competitors was added to CHOP cells that were transfected with either wild-type 17βHSDXI or empty vector control DNA and incubated for 18 h. The labeled 3α-Adiol and its converted products were separated by TLC and analyzed as previously described (13).

Immunoblotting of Myc-tagged human 17βHSDXI

The HUP1 antibody does not recognize 17βHSDXI on Western blots. Therefore, the human 17βHSDXI fragment was subcloned into pCDNA3-2M vector in frame with the N-terminal double-Myc-epitope (16). Recombinant plasmid and empty vector were separately transfected to HeLa cells by electroporation and cells harvested 24 h later (16). Approximately 20 μg total cell lysate per lane were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. C-myc-tagged proteins were detected by anti-c-Myc monoclonal antibody 9E10 followed by incubation with antimouse IgG-horseradish peroxidase conjugate and chemiluminescence reagent (NEN Life Science Products). The same blot was then washed with PBS and reprobed with anti-α-tubulin monoclonal antibody (Sigma) to show equal loading.

Preparation of the HUP1 antibody

The HUP1 antibody was raised in rabbits against a chimeric peptide consisting of the first and last eight amino acids of 17βHSDXI joined by a cysteine residue included for ease of coupling to affinity columns. The sequence was thus MKFLLDILCIGYKMKAQ. The peptide was conjugated to diptheria toxoid for injection into New Zealand White rabbits and coupled to a Biogel column for immunopurification as previously described for preparation of the HUH23 antibody (17).

Immunohistochemistry

Human tissue was obtained at autopsy and from surgical pathology files at Tohoku University Hospital (Sendai, Japan). The study was approved by the Ethics Committee of the Tohoku University School of Medicine. Specimens were fixed in 10% formalin and embedded in paraffin/wax. Immunohistochemical analysis was performed using the immunopurified HUP1 (Pan1b) antibody as previously described (13). Negative controls incorporated normal rabbit IgG instead of primary antibody. Antibody (2 μg) was preabsorbed with 5 μg peptide antigen at room temperature overnight and incubated with sections of human liver as an additional negative control.

Intracellular localization

A DNA fragment encoding human 17βHSDXI amino acids 2–300 was amplified using *Pfu* DNA polymerase from a cloned full-length cDNA clone in pCDNA1. The PCR primers were: forward 5′-ACAA*AGAAT-TC*GAAATTTCTTCTGGACATCCTC-3′ and reverse 5′-TCAG*TCTC-GAG*AAAACTAGGTGCTTATTGCGCT 3′. An *Eco*RI site was introduced in the forward primer and *Xho*I in the reverse primer (italicized). The PCR fragment was cloned in frame to the pEGFP-C1 vector (CLONTECH Laboratories, Inc., Palo Alto, CA) and confirmed by sequencing. After overnight transfection to mouse Y1 cells cultured on round coverslips, the cells were fixed with 4% formaldehyde and permeabilized with 0.5% Triton X-100. Enhanced green fluorescent protein-tagged human 17βHSDXI protein was examined for intracellular localization by immunofluorescent microscopy with nuclei stained with Hoechst 33342 (Sigma).

Effect of cAMP and all-trans-retinoic acid on 17βHSDXI expression and progesterone production in mouse Y1 cells

Mouse adrenal Y1 cells were grown to confluence in 35-mm plates in DMEM/F12 medium (Invitrogen) with 10% fetal bovine serum (CSL, Parkville, Australia). Cells were washed with OPTI-MEM (Invitrogen) and incubated in OPTI-MEM containing 1 mM dibutyryl cAMP and/or 0.5 μM all-*trans*-retinoic acid (Sigma) for 24 h. The medium was harvested for progesterone assay using the Ultra Progesterone ELISA test kit (Neogen, Lexington, KY). The 17βHSDXI activities in the intact cells were determined after 1 h of incubation with tritium-labeled 3-α Adiol. Total RNA was then extracted from the cells using an SV Total RNA isolation kit (Promega Corp.) to determine the 17βHSDXI expression level by RT-PCR. First-strand cDNA primed with oligo-(deoxythymidine) was synthesized from approximately 0.8 μg total RNA using a first-strand reverse transcription (RT) kit (Invitrogen). One tenth of the RT reaction was used as the template in subsequent PCR to amplify 17βHSDXI using primers 5′-CAGCATTGAGTCTCTTGTC-3′ and 5′-

Downloaded from endo.endojournals.org on June 23, 2005

GCACATTGACTTCGAAAGT-3′. A pair of mouse β-actin primers (5′-GGCTACAGCTTCACCACCAC-3′ and 5′-GCAGATGTGGATCAGCAAGC-3′) were used to amplify actin mRNA from the RT samples as controls.

Results

17βHSDXI cDNA is colinear with retSDR2

The full-length 17βHSDXI cDNA was derived from a pregnant human uterus library after identification of a partial clone in expressed sequence tag (EST) databases with homology to the SCAD superfamily (Fig. 1A). The cDNA is 1407 bp in length and colinear with retSDR2, which was isolated from the retina (accession no. AF126780), but has shorter 5′ and 3′ UTRs. A consensus Kozak sequence was absent although the crucial G at −3 was present. The protein is 300 amino acids long and contains a potential N-linked glycosylation site at residue 228. Recently a clone with 98% identity to 17βHSDXI at the protein level was isolated from an adenocarcinoma of the colon (accession no. BC008650). All three cDNAs contain an adenosine repeat in the 5′ UTR (5′pA). A repeat of 14 adenosines is present in retSDR2, and there are 15 in 17βHSDXI and the adenocarcinoma clone. A single nucleotide C719T silent polymorphism in the position of nt719 of retSDR2 is present in 17βHSDXI and can be detected using the *Bsm*A I enzyme that cuts in the position of the polymorphism with C (GTCTC) but not T (GTTTC). Sequence analysis of genomic DNA from 28 normal individuals showed that the T allele was present in 15% of cases. A 5′pA motif of 15 adenosines was present in all 52 samples of a normal population. In addition, retSDR2 contained two polyadenylation signal sequences (AATAAA) in the 3′ UTR, and 17βHSDXI as well as the colon adenocarcinoma clone used the first polyadenylation signal to add a polyA tail 12 nucleotides after the signal sequence (Fig. 1A). Western blot analysis was performed using an anti-c-myc antibody directed at 17βHSDXI containing two N-terminal c-Myc epitopes (Fig. 1B). The results show that the enzyme migrates on SDS-PAGE gel with a molecular weight of approximately 32 kDA, which compares favorably with a calculated weight of 35,534 Da for the tagged protein.

17βHSDXI belongs to the SCAD superfamily. Of proteins with known function, 17βHSDXI is most closely related to the retinol dehydrogenase retSDR1, with which it is 30.3% identical, 22.9% identical with the cortisone reductase 11βHSD1, and only 15.7% identical with 17βHSD6, an enzyme with which it shares some substrate specificity (15). There exists a mouse ortholog 88% identical with the human sequence (accession no. AF304306). We also cloned and sequenced 2.2 kb of the 5′ flanking

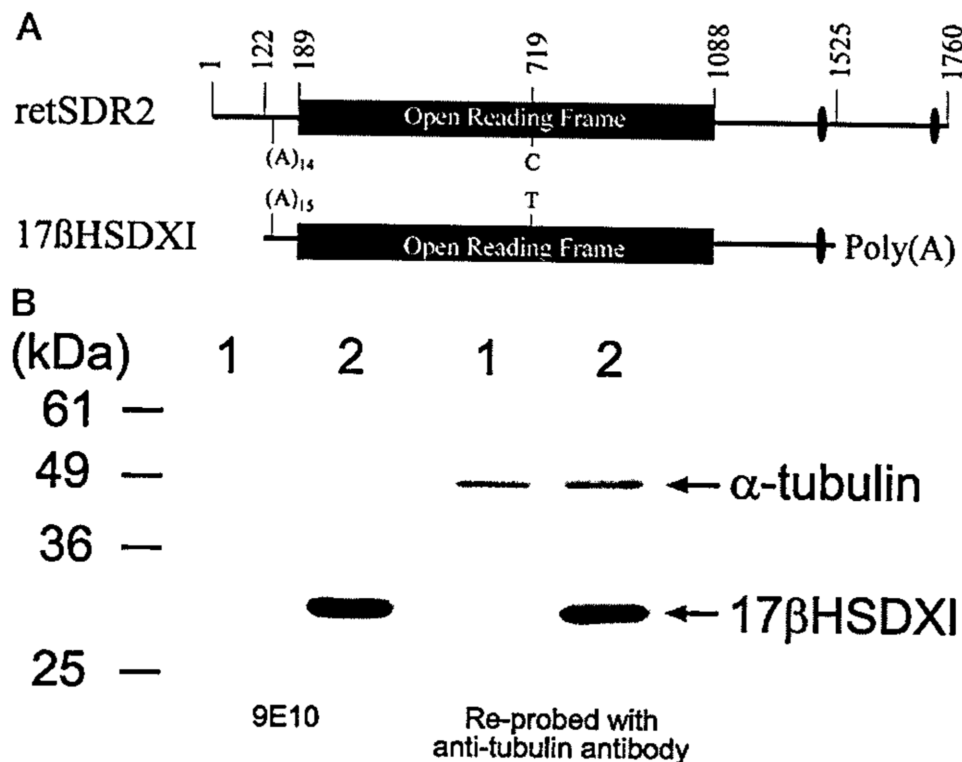

FIG. 1. A, Molecular structure of 17βHSDXI cDNA and comparison with retSDR2. Diagram of 17βHSDXI cDNA is aligned with retSDR2. Nucleotide positions are indicated according to the cDNA sequence of retSDR2 (accession no. AF126780). The open reading frame (*wide bar*), UTRs (*thin bar*), and pA tail are shown. The 5′pA of 14-adenosine sequence [$(A)_{14}$] in retSDR2 and 15 [$(A)_{15}$] in 17βHSDXI are shown. The silent polymorphism at position 719 is shown with C or T, and polyadenylation signals are represented by an *oval-shaped symbol.* B, Western blot analysis of the c-myc-tagged 17βHSDXI protein. Western blotting was performed using anti-Myc monoclonal antibody (9E10) on a blot containing whole-cell lysates of HeLa cells transfected with empty vector DNA (lane 1) and Myc-17βHSDXI construct DNA (lane 2). The same blot was reprobed with anti-α-tubulin monoclonal antibody to show equal loading.

Downloaded from endo.endojournals.org on June 23, 2005

region of the gene and identified the steroidogenic factor (SF)-1-binding site *TCCAAGGCCGG* 347 bp upstream from the translation initiation site (accession no. AY062939). A search of the Celera database identified another three SF-1-binding sites clustered in the distal region of intron 1.

17βHSDXI metabolizes 3α-Adiol to androsterone

Screening of a range of radioactive steroids identified 3α-Adiol as the substrate with the highest rate of metabolism by 17βHSDXI. The following tritiated steroids were not metabolized: androsterone, dihydrotestosterone, DHEA, testosterone, androstenedione, androstene-3β, 17β-diol, and estrone, but there was a small amount of conversion with estradiol (11). The two possible metabolites of 3α-Adiol are androsterone and dihydrotestosterone. Figure 2A shows that 3α-Adiol is converted to androsterone and proves that this enzyme is a 17β-dehydrogenase. Further attempts to define kinetic constants showed nonsaturability with 3α-Adiol up to 100 μM (Fig. 2B). At this concentration DHEA inhibited metabolism of 3α-Adiol by 70%, but androsterone, 3β-Adiol, and carbenoxolone did not affect activity of the enzyme. In other studies 50% inhibition of 3α-Adiol metabolism was achieved with 84 μM DHEA, 50 μM 13 cis-retinol, 31 μM all-*trans*-retinol, and 9 μM all-*trans*-retinal (data not shown), but we have been unable to demonstrate metabolism of these compounds by 17βHSDXI overexpressed in transfected whole cells using HPLC analysis (13).

The length of the 5′pA motif affects enzyme activity

EST database searches showed 10, 11, 13, 14, 15, 16, 17, and 20 adenosines in the 5′pA motif. Next we asked whether there might be a correlation of repeat length with 17βHSDXI activity. We constructed 5′pA variants harboring 5–20 adenosine residues and transiently transfected CHOP cells and determined enzyme activity. Figure 3 shows that there was no significant difference between wild type and constructs with 5, 10, or 15 adenosines. However, the construct with a 5′pA motif of 20 adenosines generated significantly more conversion than the next most active plasmid (43% ± 3.3% *vs.* 26% ± 3.6% for wild type, $P < 0.005$).

Northern blot analysis shows a tissue-specific pattern of distribution

Northern blot hybridization showed a 17βHSDXI mRNA transcript of about 2.1 kb (Fig. 4). High levels of 17βHSDXI mRNA expression were seen in the pancreas, kidney, liver, lung, heart, small intestine, adrenal, and ovary, with significant levels also observed in peripheral blood lymphocytes. Lowest expression was seen in skeletal muscle, brain, stomach, thymus, prostate, and colon, although most tissues displayed some signal.

17βHSDXI is present in steroidogenic cells

A striking cell-specific pattern of distribution was seen for 17βHSDXI in human tissues. Immunostaining specific for

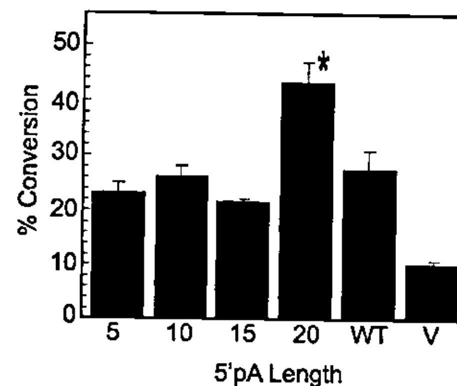

FIG. 3. Effect of length of the 5′pA motif on 17βHSDXI activity. CHOP cells were transfected with p17βHSDXI expression constructs containing 5′pA motifs of various adenosine length (5, 10, 15, and 20) as well as a wild-type p17βHSDXI (15A motif) plasmid (WT) and empty vector DNA (V). The % conversion denotes metabolism of [^{3}H]-3α-Adiol to [^{3}H]-androsterone by the transfected CHOP cells after overnight incubation (n = 3, ± SEM). *, $P < 0.02$, compared with control.

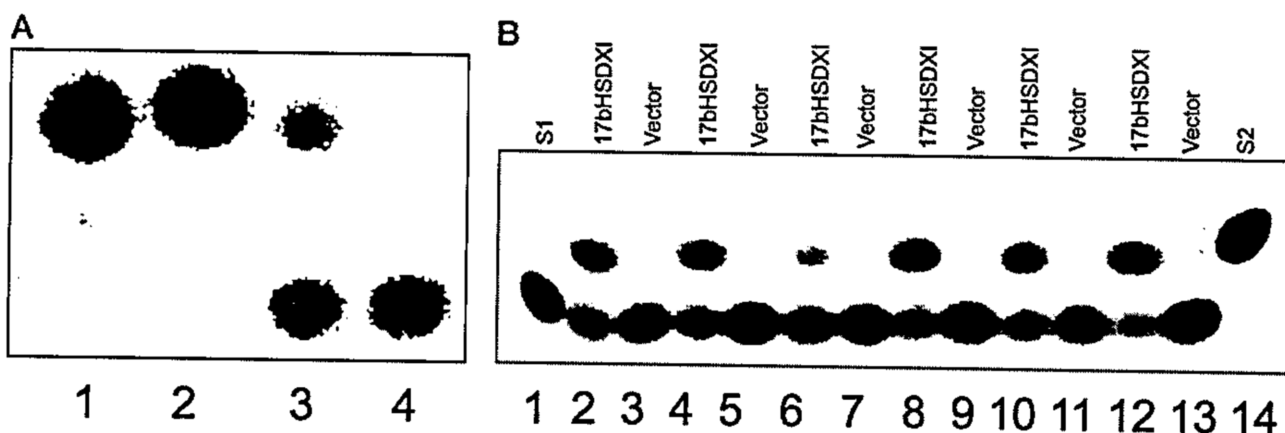

FIG. 2. A, Metabolism of 3α-Adiol to androsterone. Lane 1, [^{3}H]-Androsterone standard; lane 2, [^{3}H]-dihydrotestosterone standard; lane 3, [^{3}H]-3α-Adiol incubated with CHOP cells transfected with p17βHSDXI; lane 4, [^{3}H]-3α-Adiol incubated with CHOP cells transfected with pcDNA1 vector. B, Competition by various compounds for [^{3}H]-3α-Adiol metabolism in transfected CHOP cells. CHOP cells transfected with 17βHSDXI expression plasmid (17βHSDXI) or vector DNA (vector) were incubated with 8 nM [^{3}H]-3α-Adiol and 100 μM competitors. Competitors are: no competitor (2, 3); 3α-Adiol (4, 5); dehydroepiandrosterone (6, 7); androsterone (8, 9); 3β-Adiol (10, 11); and carbenoxolone (12, 13). Standards on the TLC plate are [^{3}H]-3α-Adiol (S1) and [^{3}H]-androsterone (S2).

Downloaded from endo.endojournals.org on June 23, 2005

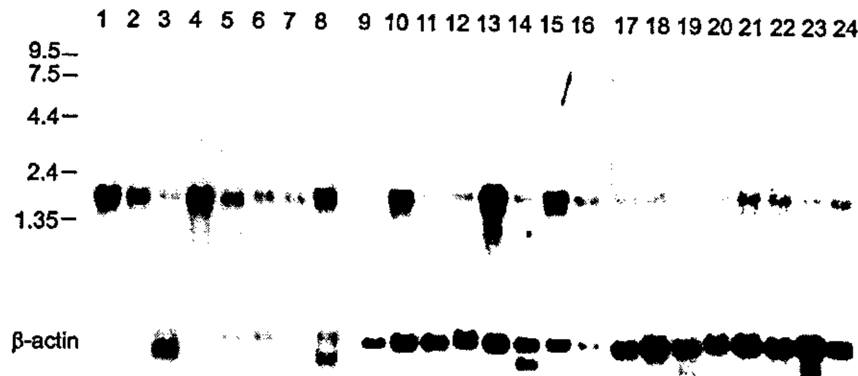

FIG. 4. Northern blot analysis of 17βHSDXI expression in human tissues. Three commercial blots (CLONTECH Laboratories, Inc.) containing 2 μg pA^+ RNA per lane were hybridized with full-length 17βHSDXI cDNA probe. Blots were stripped and reprobed with a full-length β-actin cDNA probe to check for loading of RNA. Lane 1, Pancreas; 2, kidney; 3, skeletal muscle; 4, liver; 5, lung; 6, placenta; 7, brain; 8, heart; 9, stomach; 10, small intestine; 11, thymus; 12; testis; 13, adrenal cortex; 14, thyroid; 15, adrenal medulla; 16, pancreas; 17, spleen; 18, thymus; 19, prostate; 20, testis; 21, ovary; 22, small intestine; 23, colon; and 24, blood leukocytes.

17βHSDXI was evident in parenchymal cells of the liver but was absent from the central vein (Fig. 5a). Preimmune serum did not stain the liver (Fig. 5b) and immunopurified antibody preincubated with synthetic peptide also did not stain sections of human liver (results not shown). Staining was also seen in syncytiotrophoblasts of the placenta (Fig. 5c). In the ovary, only granulosa cells of the dominant follicle stained (Fig. 5d); this distribution differed from that of 17α-hydroxylase, which was restricted to the theca interna cells (Fig. 5e). The endometrium displayed glandular staining (Fig. 5f). In the functioning corpus luteum, luteinized granulosa cells stained for 17βHSDXI (Fig. 5g), but 17α-hydroxylase was present in luteinized theca cells (Fig. 5h). In the testis immunostaining was present in Leydig cells (Fig. 5i). In the adult adrenal gland, 17βHSDXI staining was present in the fasciculata/reticularis with higher levels evident in the outer cortex, but there was no staining in the medulla (Fig. 5j). Staining performed for 17α-hydroxylase in a serial section was coincident with the distribution of 17βHSDXI but was of higher intensity toward the medulla (Fig. 5k). In a 1-d-old neonate, staining for 17βHSDXI was present in the mid to outer cortex and was of lower intensity than in the adult (Fig. 5l). In the small intestine, surface epithelium was positive for 17βHSDXI (Fig. 5m). The skin displayed staining only in sebaceous glands (Fig. 5n). Immunofluorescence studies localized 17βHSDXI to the cytoplasm of the steroidogenic Y1 mouse cell line (Fig. 5o).

Modulation of 17βHSDXI during steroidogenesis

Because 17βHSDXI staining localizes to steroidogenic cells, we sought to obtain evidence for a role of this enzyme in the steroid biosynthetic pathway. Mouse Y1 adrenocortical cells were treated with cAMP and all-*trans*-retinoic acid, two known stimulants of steroid synthesis. 3α-Adiol-metabolizing activity decreased significantly with cAMP treatment (40% *vs.* 32%, $P < 0.05$) but not with all-*trans*-retinoic acid alone (Fig. 6A). However, addition of the retinoic acid together with cAMP significantly decreased activity over cAMP alone (32% *vs.* 23%, $P < 0.05$). Message for 17βHSDXI was reduced to undetectable levels by the addition of cAMP (Fig. 6B). In contrast, parallel measurements of progesterone production showed the expected 2- to 3-fold increases over control cells with all three treatments (Fig. 6C).

Discussion

We have characterized an enzyme with 17βHSD activity. This is the 11th isoform to be described. The protein belongs to the SCAD superfamily and retains conserved A-to-D domains with other members (18). The cDNA contains a polymorphic adenosine repeat in the 5′UTR, and other examples containing 10–20 adenosine repeats were found in EST databases. However, deviation from a 5′pA of 15 appears to be rare because we did not observe a single occurrence in a normal population of 52 subjects. We found higher levels of 17βHSDXI activity when constructs containing 20 adenosines were expressed in CHOP cells. This effect may be mediated by poly-A-binding proteins that enhance binding of translation initiation factors and result in a higher efficiency of translation (19, 20). However, the structure of the human 5′UTR is not conserved in mouse, and no adenosine repeat exists in that species. The 5′ flanking region of the human gene contains highly methylated CpG islands that bind to the methyl-CpG binding domain of the rat chromosomal protein MeCP2 (21). The gene was found at chromosome 4q21 by radiation hybrid mapping and sequencing by the human genome project.

17βHSDXI is most active with 3α-Adiol as substrate in transfected CHOP cells. Currently we cannot rule out the possibility that 17βHSDXI also has activity with other substrates, given that we observed endogenous 17-keto reductase activity in CHOP cells with a number of substrates during the course of these studies. However, further experiments with Y1, 3T3, and COS cells did not show enhanced 17βHSDXI activity with the above-labeled substrates (results not shown). Nevertheless, the present data suggest that 17βHSDXI participates in the pathway of androgen degradation whereby 3α-Adiol, which is directly produced by the metabolism of dihydrotestosterone, or indirectly from DHEA, is converted to androsterone. We were unable to demonstrate saturation of the enzyme with substrate, a prop-

Downloaded from endo.endojournals.org on June 23, 2005

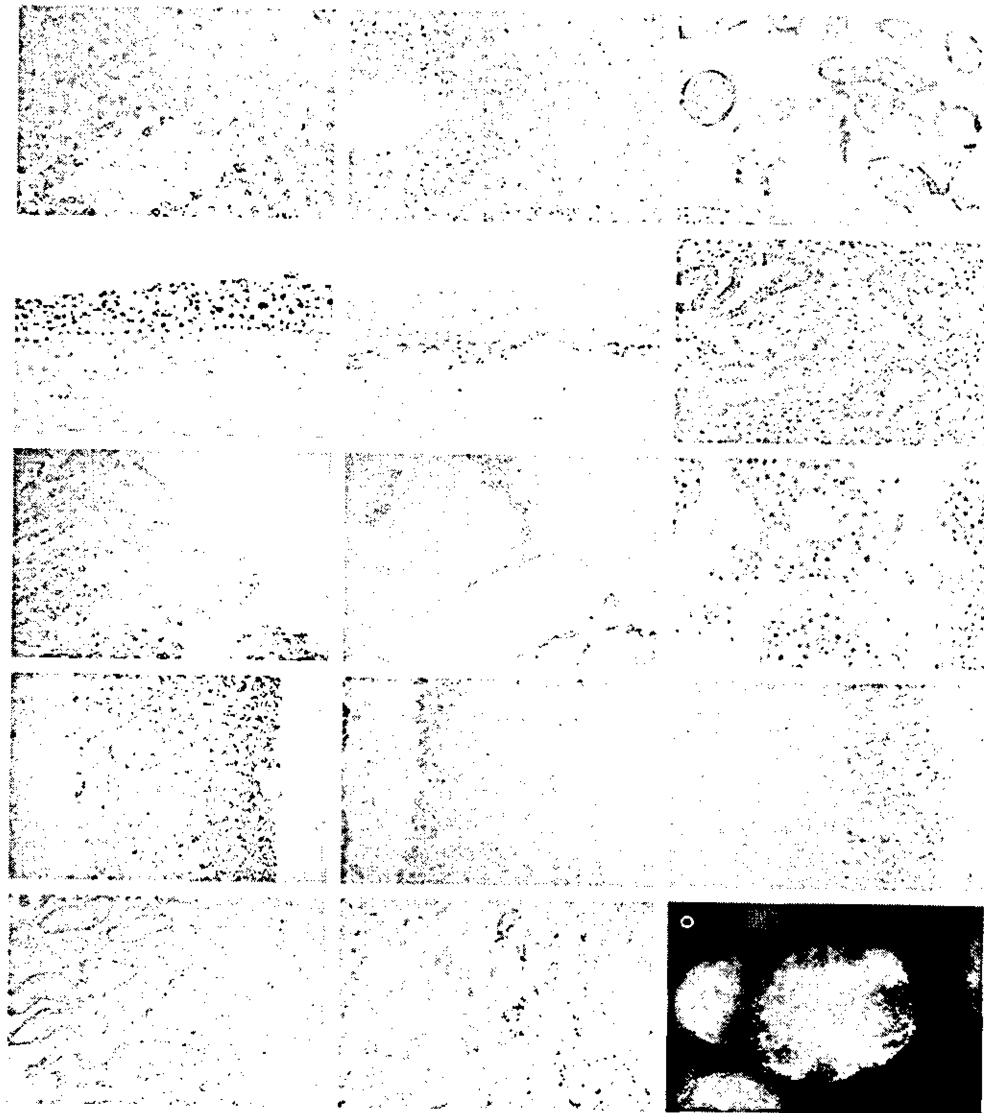

FIG. 5. Immunostaining of human tissues with the immunopurified HUP1 antibody. a, Liver; b, liver negative control; c, placenta; d, ovarian follicle; e, serial section of d stained with an antibody to 17α-hydroxylase (C17); f, uterus; g, corpus luteum; h, serial section of g stained with C17 antibody; i, testis; j, adult adrenal cortex; k, serial section of j stained with an antibody to C17; l, adrenal cortex from 1-d-old neonate; m, jejunum; n, sebaceous gland; o, immunofluorescence labeling of c-myc-tagged 17βHSDXI (*green*) in transiently transfected mouse adrenal Y1 cells with the nucleus shown (*blue*).

erty 17βHSDXI shares with RoDH1 (22); previous studies show there is a considerable overlap in specificity between enzymes that metabolize 3α-Adiol and retinols (15, 23). 3α-Adiol is also metabolized to androsterone by 17βHSD6, an enzyme that is 65% identical with the retinol dehydrogenase RoDH1, but CRAD2 exhibits 3α,17β hydroxysteroid and cis/*trans*-retinol catalytic activities, and RoDH1 converts 3α-Adiol back to dihydrotestosterone (DHT). This suggests that a pocket accommodating 3α-Adiol may also bind retinoids. Indeed, our studies show that high concentrations of retinoids can inhibit the metabolism of 3α-Adiol by 17βHSDXI. However, using HPLC, we were unable to demonstrate metabolism by transfected whole cells of any retinoids. This finding is consistent with previous work employing membranes from insect cells (12). Carbenoxolone, a nonspecific inhibitor that blocks both 11βHSDs and many 17βHSDs, was unable to inhibit 17βHSDXI activity.

Little is known about the biological actions of 3α-Adiol, although emerging evidence suggests that it is a hormone in its own right (24–26), and a few studies indicate an important role in parturition. Administration of 3α-Adiol increased live births in 5α-reductase deficient mice from 27% to over 90%. That this is not due to conversion to DHT is suggested by the observation that administration of DHT only partially restored levels. Furthermore, enzymes that lead to the production of 3α-Adiol are induced in the mouse uterus during

Downloaded from endo.endojournals.org on June 23, 2005