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# Expression of Aromatase Cytochrome P450 Protein and Messenger Ribonucleic Acid in Human Endometriotic and Adenomyotic Tissues but Not in Normal Endometrium[1]

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## ABSTRACT

**To determine whether local estrogen production takes place in endometriotic or adenomyotic tissues, in eutopic endometrium from patients with endometriosis or adenomyosis, and in normal endometrium, tissue specimens were examined by immunohistochemistry, catalytic activity, and mRNA expression for aromatase cytochrome P450 ($P450_{arom}$). $P450_{arom}$ was immunohistochemically localized in the cytoplasm of glandular cells of endometriotic and adenomyotic tissues, and of eutopic endometrium from patients with the respective diseases, whereas estrogen receptors and progesterone receptors were localized in the nuclei of the glandular cells and stroma. Aromatase activity in the microsomal fraction of adenomyotic tissues was inhibited by the addition of danazol, aromatase inhibitors, and anti-human placental $P450_{arom}$ monoclonal antibody (mAb3–2C2) in a manner similar to such inhibition in other human tissues. Reverse transcription polymerase chain reaction and Southern blot analysis also revealed $P450_{arom}$ mRNA in these tissues. However, neither $P450_{arom}$ protein activity nor mRNA was detected in endometrial specimens obtained from normal menstruating women with cervical carcinoma in situ but without any other gynecological disease. These results suggest that at a local level, endometriotic and adenomyotic tissues produce estrogens, which may be involved in the tissue growth through interacting with the estrogen receptor.**

## INTRODUCTION

Endometriosis is defined by the presence of endometrial glands and stroma outside of the uterine cavity. Pain such as that of dysmenorrhea and deep dyspareunia, and chronic pelvic pain, as well as infertility are associated with endometriosis. Two main theories of the pathogenesis of endometriosis have been proposed: 1) metastatic implantation such as reflux of endometrial cells, and vascular and lymphatic transplantation, and 2) metaplastic development such as coelomic metaplasia. Adenomyosis, defined by the presence of endometrial tissue within the myometrium, is a separate entity with a different patient population, etiology, and clinical course [1]. However, both endometriosis and adenomyosis develop mostly in women of reproductive age and regress after menopause or ovariectomy, suggesting that the growth is estrogen-dependent. Estrogen receptors (ER) and progesterone receptors (PR) have been detected in endometriotic tissue by hormone-ligand binding assays [2, 3] and immunohistochemistry [4, 5].

The conversion of androgens to estrogens occurs predominantly in the placenta and ovary, and is catalyzed by aromatase, the major component of which is aromatase cytochrome P450 ($P450_{arom}$). Considerable biochemical evidence suggests that estrogen-dependent diseases of the uterus such as endometrial carcinoma [6–8], leiomyomas [9, 10], endometriosis [11], and adenomyosis [12, 13] have aromatase activity and $P450_{arom}$ mRNA expression, suggesting that these tissues may grow not only by reacting with circulating estrogens but also by producing estrogens at a local level.

We studied the immunohistochemical localization of $P450_{arom}$ in endometriotic and adenomyotic tissues using a specific antibody raised against it [14]. In addition, we studied the enzymologic property of aromatase and mRNA expression, and the immunohistochemical localization of ER and PR.

Although histologically the ectopic endometriotic implant more or less resembles eutopic endometrium, ER and PR contents were found to be lower in endometriotic implants than in the corresponding eutopic endometrium, and the cyclic changes of steroid receptors in ectopic endometriosis are not similar to those observed in eutopic endometrium [2–5]. We therefore studied aromatase expression in the eutopic endometrium obtained from patients with endometriosis and adenomyosis.

A number of investigators [15–23] have reported the presence of aromatase activity in normal endometrium of premenopausal women. By contrast, three groups reported the lack of aromatase activity [24, 25] or mRNA expression [26] in normal endometrium. Careful review of those reports, however, reveals that the endometrial specimens defined as "normal" endometria were obtained mostly by hysterectomy conducted for various diseases including leiomyoma, adenomyosis, and endometriosis. The specimens were thus not necessarily from disease-free uteri. To clarify the controversy, we strictly defined as normal endometrium only eutopic endometrium obtained from patients with cervical cancer in situ but with no other gynecological diseases.

## MATERIALS AND METHODS

### *Tissue Samples*

The following tissue samples were obtained at the time of hysterectomy or laparoscopy: endometriotic implants on

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the peritoneum (n = 10), adenomyotic tissue in the myometrium (n = 10), eutopic endometrial tissue from patients with endometriosis (n = 10), endometrial tissue from patients with leiomyoma (n = 10), and endometrial tissue from patients with cervical carcinoma in situ but without other gynecological disease (n = 10). All patients were of reproductive age ranging from 28–48 yr with normal menstrual cycles. None of the patients had received endocrine therapy for the treatment of endometriosis or adenomyosis. Endometrial tissue was gently scraped from the uterus. Fresh tissue samples were divided into three portions: one portion was frozen immediately at −80°C until assayed for aromatase and total RNA extraction; two portions were fixed with 4% paraformaldehyde in 0.05 M Tris-HCl buffer (pH 7.6) at 4°C for 24 h; one was subjected to histological diagnosis, and the other was subjected to immunohistochemistry.

### *Immunohistochemistry*

Immunostaining was performed as previously described [27] with modifications using the Dako Labeled Streptavidin Biotin Kit (Dako, Santa Barbara, CA). Briefly, paraffin-embedded tissue samples were cut into 4-μm sections. For immunostaining of ER and PR, the sections were deparaffinized, immersed in 0.01 M citrate buffer (pH 6.0), and autoclaved at 121°C for 20 min. Monoclonal antibodies against ER and PR were purchased from Immunotech (Marseille, France). For immunostaining of $P450_{arom}$, the sections were incubated with rabbit anti-human placental $P450_{arom}$ antiserum (PAb R-8–2, 1:1000) as the primary antibody. The characteristics and specificity of the antiserum were previously reported [14, 28, 29]. Human term placental sections were used as positive controls for $P450_{arom}$. Negative controls for $P450_{arom}$ were incubated with the same dilution of nonimmunized rabbit serum or PAb R-8–2 that had been pretreated with immunopurified human placental $P450_{arom}$ (500 μg $P450_{arom}$ per 1 ml diluted PAb R-8–2) to block the active site. Negative controls for ER and PR were incubated with the same dilution of nonimmunized rat IgG.

### *Aromatase Assay*

Tissues were processed as previously described [30]. Approximately 10 g of adenomyotic tissue and 1–3 g each of normal endometrium and normal myometrium tissue were thawed and minced in 0.067 M potassium phosphate buffer (pH 7.4, PB) containing 1% KCl to remove the blood content. The tissue was homogenized in PB containing 0.24 M sucrose and 1 mM dithiothreitol. The homogenate was centrifuged at 900 × *g* for 10 min, and the supernatant was centrifuged at 105 000 × g for 60 min. The resulting pellet was resuspended in 1 ml PB containing 0.1 mM EDTA and 1 mM dithiothreitol and was subjected to aromatase assay.

Aromatase activity was determined by the tritiated water method as previously described [31]. Briefly, the standard incubation mixture contained 0.5 ml of enzyme preparation (approximately 1.0 mg protein), [1β-$^3$H]androstenedione (Dupont-New England Nuclear, Boston, MA; $6.0 \times 10^6$ dpm, 100 pmol), NADPH (0.5 mg), and varying doses of aromatase inhibitor (aminoglutethimide, kindly provided by Ciba-Geigy, Summit, NJ; or danazol, kindly provided by Tokyo Tanabe Co., Tokyo, Japan) in a total volume of 1.0 ml PB. The reaction was started by addition of the prewarmed mixture of inhibitor and NADPH in 0.1 ml PB. For the suppression assay by anti-human placental $P450_{arom}$ monoclonal antibody (MAb3–2C2) [32], the enzyme preparation was mixed with varying doses of MAb3–2C2 in a total volume of 0.85 ml PB and preincubated for 10 min at 37°C. The assay was started by the addition of substrate-cofactor mixture in 0.15 ml PB.

The incubation was continued at 37°C for 60 min and stopped by addition of 0.5 ml of 20% trichloroacetic acid; 1.0 ml of 5% charcoal was added, and the mixture was incubated for an additional 30 min. The mixture was centrifuged, and the supernatant was filtered through a cotton-plugged disposable pipette. The amount of [$^3$H]water in the eluate derived from the substrate was assessed using the 1 β-elimination mechanism (75% release into water) [30]. [$^3$H]Water release increased linearly with incubation time up to at least 75 min. The tritiated water method was validated by the product isolation method as previously described [33], and the data showed good agreement. Blank incubations contained all reagents except NADPH, and the radioactivity was subtracted from the counts obtained from incubations with NADPH. Activity of less than 10 fmol/h per mg protein was defined as negative. Protein concentrations were determined with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with BSA as standard.

### *RNA Isolation*

Total RNA was extracted using Trizol reagent (Gibco BRL, Gaithersburg, MD) basically according to the manufacturer's protocol. Approximately 0.1 g of tissue was thawed and homogenized in 1 ml Trizol reagent. The homogenate was incubated for 5 min at 22°C to permit the complete dissociation of nucleoprotein complexes. After the addition of 0.2 ml chloroform, the mixture was vigorously shaken for 3 min at 22°C and centrifuged at 12 000 × *g* for 10 min at 4°C. The aqueous phase was transferred to a fresh tube, and extraction with Trizol was repeated. The aqueous phase was washed with an equal volume of chloroform. An equal volume of isopropanol was added, and the tube was stored at 22°C for 10 min. RNA was precipitated by centrifugation at 12 000 × *g* for 10 min at 4°C. The pellet was washed twice with 75% ethanol, briefly dried under air, and dissolved in 100 μl of diethylprocarbonate-treated water.

### *Reverse Transcriptase (RT) Polymerase Chain Reaction (PCR)-Southern Blotting*

The first-strand cDNA synthesis from total RNA was catalyzed by Superscript II RT (Gibco BRL) using oligo$(dT)_{12-18}$ according to the manufacturer's protocol. The reaction mixture consisted of 4 μg total RNA, 0.5 μg oligo$(dT)_{12-18}$, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM dNTP, 10 mM dithiothreitol, and 200 U Superscript II RT in a total volume of 20 μl of 20 mM Tris-HCl (pH 8.4). The first-strand cDNA was used for PCR amplification with the following primers as described by Price et al. [34]: 5′-TTG TTG TTA AAT ATG ATG CC-3′ and 5′-ATA CCA GGT CCT GGC TAC TG-3′. The PCR mixture consisted of 1 μl first-strand cDNA, 0.5 μM each of primers, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 U *Taq* polymerase (Gibco BRL) in a total volume of 100 μl of 20 mM Tris-HCl (pH 8.4).The PCR condition was 94°C for 3 min to denature the RNA/cDNA hybrid, then 40 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min. The PCR product was electrophoresed in 2% agarose gel and transferred to a nylon membrane. An antisense probe 5′-TAA TGA TTG TGC TTC ATT ATG TG-3′ [34] was 5′-end-

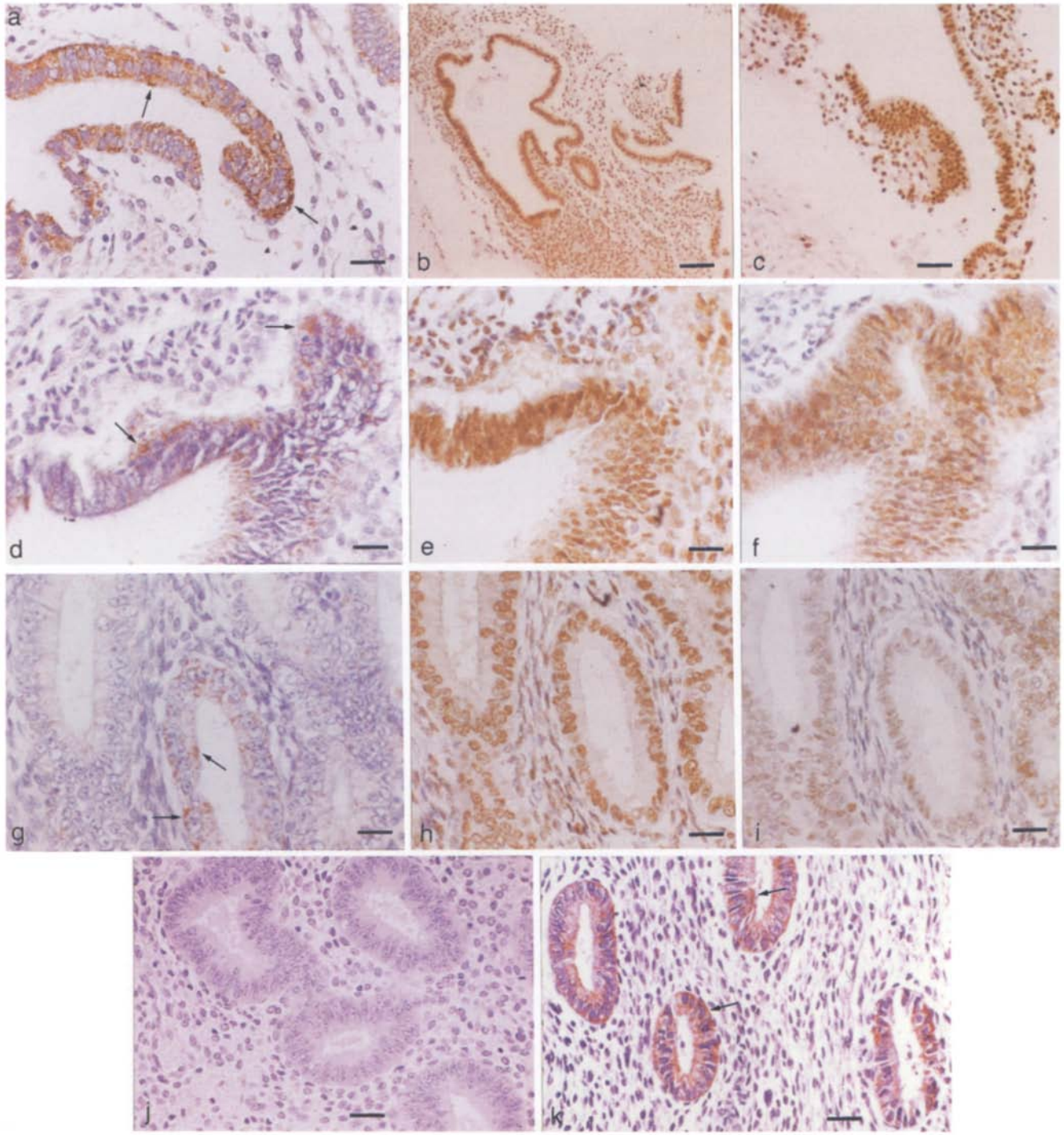


FIG. 1. Immunohistochemical localization of $P450_{arom}$ (**a, d, g, j, k**), ER (**b, e, h**), and PR (**c, f, i**), in endometriotic tissue in a peritoneal vesicle implant (**a–c**), adenomyotic tissue in the myometrium (**d–f**), eutopic endometrium obtained from a patient with endometriosis (**g–i**), eutopic endometrium from a patient with cervical carcinoma in situ but without any other gynecological disease (**j**), and eutopic endometrium from a patient with leiomyomas (**k**). $P450_{arom}$ is immunolocalized exclusively in the cytoplasm of glandular cells (arrows), while ER and PR are localized in the nuclei of the glandular cells and stroma. Original magnification: **a, d–i**) ×132 (bar = 23 μm); **b, c**) ×33 (bar = 91 μm); **j, k**) ×100 (bar = 30 μm).

labeled with [$\gamma$-$^{32}$P]ATP. The membrane was hybridized with the labeled probe overnight at 55°C, and the hybridized signal was analyzed using a bioimaging analyzer BAS 2000 (Fujix, Tokyo, Japan).

## RESULTS

### *Immunohistochemistry*

$P450_{arom}$ was detected in 8 out of 10 endometriotic tissues obtained from peritoneal implants. $P450_{arom}$ was immunolocalized exclusively in the cytoplasm of glandular cells (Fig. 1a). Immunoreactivity was not detected in the stroma. $P450_{arom}$ was also localized in the cytoplasm of glandular cells of adenomyotic tissues (10 of 10) (Fig. 1d) and of the eutopic endometria obtained from patients with endometriosis (8 of 10) (Fig. 1g). However, $P450_{arom}$ was not detected in any of the 10 eutopic endometrial specimens obtained from normal menstruating women with cervical cancer in situ but without any other gynecological disease

TABLE 1. Danazol inhibition of aromatase activity in adenomyotic tissue, and lack of aromatase activity in normal endometrium and myometrium.[a]

| Tissue | Danazol[b] (M) | Aromatase activity (fmol/h per mg protein) |
|---|---|---|
| Adenomyosis | 0 | 168.0 ± 26.0 |
| | $10^{-8}$ | 74.0 ± 5.2[c] |
| | $10^{-7}$ | 50.3 ± 7.7[d] |
| | $10^{-6}$ | 43.9 ± 6.5[d] |
| Normal endometrium | | <10 |
| Normal myometrium | | <10 |

[a] Aromatase activity was measured in the microsomal fraction; n = 4.
[b] Added to the incubate.
[c] $p < 0.02$ and [d] $p < 0.01$ compared to control.

(Fig. 1j), whereas it was detected in eutopic endometrium from patients with leiomyomas (9 of 10) (Fig. 1k). In all cases of endometriotic, adenomyotic, and eutopic endometria, ER (Fig. 1, b, e, and h) and PR (Fig. 1, c, f, and i) were localized in the nuclei of the glandular cells and stroma.

### *Aromatase Activity*

Aromatase activity was detected in the microsomal fraction of adenomyotic tissue, whereas the activity in normal endometrium and normal myometrium was below the detectable level. Aromatase activity in adenomyotic tissue was inhibited by the addition of danazol in a dose-dependent manner (Table 1); it was also inhibited by the addition of two kinds of aromatase inhibitors, aminoglutethimide and pyridoglutethimide, in a dose-dependent manner, similar to the response of human ovarian and placental tissues to these inhibitors (Fig. 2). Moreover, this activity was suppressed by the addition of anti-human placental $P450_{arom}$ monoclonal antibody (mAb3–2C2) in a similar manner to such activity in other human tissue but different from that in rat ovary (Fig. 3).

### *Messenger RNA*

RT-PCR-Southern blot analysis revealed $P450_{arom}$ mRNA in endometriotic tissue (7 out of 10), adenomyotic tissue (5 out of 5), and eutopic endometrial tissue obtained from patients with endometriosis (7 out of 10) (Fig. 4). However, $P450_{arom}$ mRNA was not detected in any of the 10 eutopic endometrial specimens obtained from normal

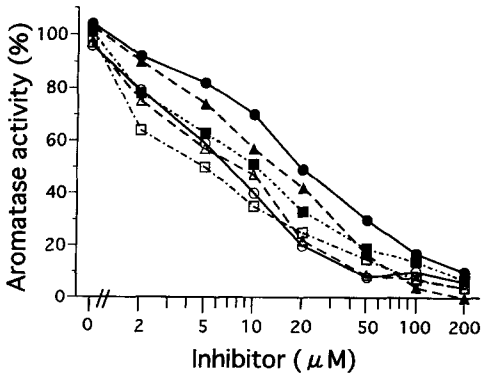


FIG. 2. Inhibition of the microsomal aromatase activity of adenomyotic tissue (circles), human ovary (triangles), and human placenta (squares) by pyridoglutethimide (solid symbols) and aminoglutethimide (open symbols). Data are expressed as the mean of four determinations in two separate experiments.

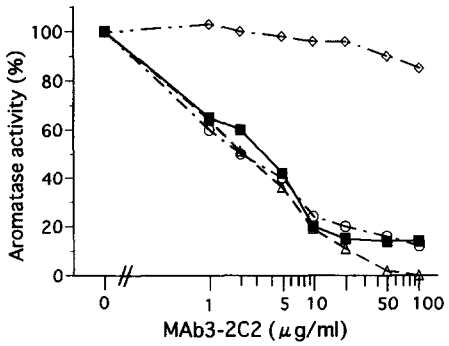


FIG. 3. Suppression of the microsomal aromatase activity of adenomyotic tissue (squares), human placenta (circles), human ovary (triangles), and rat ovary (diamonds) by anti-human placental $P450_{arom}$ monoclonal antibody (MAb3–2C2). Data are expressed as the mean of four determinations in two separate experiments.

menstruating women with cervical cancer in situ but without any other gynecological disease (Fig. 4).

## DISCUSSION

In the present study, we demonstrated that both $P450_{arom}$ protein and mRNA were expressed in endometriotic tissue, adenomyotic tissue, eutopic endometrium of patients with endometriosis, and eutopic endometrium of patients with adenomyosis. The immunoreactivity to $P450_{arom}$ was localized exclusively in the glands but not present in the stroma. By contrast, neither $P450_{arom}$ nor mRNA were expressed in normal eutopic endometrium.

Because of very low levels of aromatase activity and small tissue volume, it has been difficult to detect aromatase activity in endometriotic implants. Immunohistochemical technique has the advantage of requiring only a small amount of intact tissue, thus maintaining tissue architecture and allowing the assessment of the cellular distribution of $P450_{arom}$ expression. The antiserum used in this study was raised against immunoaffinity-purified human placental $P450_{arom}$ that showed greater than 97% homogeneity [28], and recognized only $P450_{arom}$ in the Western blot analysis [14]. By use of the antiserum, immunohistochemical localization of $P450_{arom}$ was demonstrated in human placenta [27], normal ovary [29], polycystic ovary syndrome [35], and ovarian tumors [36]. We prepared positive and negative controls for each specimen and detected no false positive or negative data. The RT-PCR analysis for $P450_{arom}$ mRNA expression agreed with the immunohistochemical results and with the data reported by Bulun et al. [26] and Noble

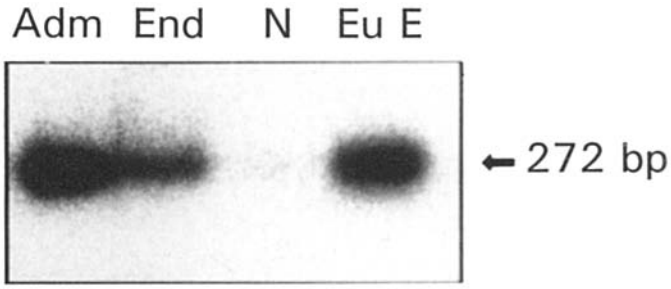


FIG. 4. RT-PCR-Southern blot analysis of $P450_{arom}$ mRNA. Adm, adenomyotic tissue in the myometrium; End, endometriotic tissue in a peritoneal vesicle implant; N, eutopic endometrium from a patient with cervical carcinoma in situ but without any other gynecological disease; Eu E, eutopic endometrium obtained from a patient with endometriosis.

et al. [11]. The results also suggest that the enzymatic property of aromatase in adenomyotic tissue was similar to that in other human tissue. Moreover, the activity was effectively inhibited by danazol, which is widely used for the treatment of endometriosis, in concentrations compatible with the dissociation constants for binding to various steroid receptors [37]. In addition to its antigonadotropin effect, danazol has a direct effect in lowering the local estrogen level.

Since the presence of aromatase activity in normal endometrium was proposed [6], a number of studies [15–23] have shown the regulation of aromatase activity by steroids such as progestogens. However, in most of the previous studies, endometrial specimens obtained from uteri excised for benign gynecological diseases such as endometriosis, adenomyosis, and leiomyomas were used as normal endometrium. The present results support the previous studies, showing the presence of aromatase in the endometrium of patients with such diseases. In contrast, the results do not agree with the presence of such hormonal regulation of aromatase in normal endometrium of disease-free uterus, in view of the failure to detect aromatase throughout the menstrual cycle.

It has been universally accepted through the early work of Cullen [38] that adenomyosis is caused by direct invasion of eutopic endometrium into the myometrium. Given the fact that aromatase is expressed in both the ectopic glands and the eutopic endometrium of patients with adenomyosis, interaction between eutopic endometrium and endometrial glands in adenomyotic tissue may occur. Locally produced growth factors may be involved in the promotion or stimulation of aromatase expression in an autocrine or paracrine manner. This may be supported by the indirect evidence that, in the case of uterine leiomyoma, estrone sulfatase activity was greater in the endometrium located overlying a myoma node than in the endometrium located at the opposite side of the myoma node [13]. In order to understand the mechanism by which aromatase is present both in the ectopic glands and the eutopic endometrium of patients with endometriosis, two hypotheses corresponding to two major theories of pathogenesis are proposed: 1) metastatic theory—aromatase expressed in eutopic endometrium by stimulation of an unknown factor activates transplantation of endometrial implants on the peritoneum; and 2) metaplastic theory—cytokines and growth factors mainly derived from macrophages in the peritoneal cavity [39] stimulate the expression of aromatase in both eutopic and ectopic endometriotic glands.

In conclusion, the present results suggest that at a local level endometriotic and adenomyotic tissues as well as the eutopic endometrium of patients with these diseases produce estrogen, which may be involved in tissue growth through interaction with the ER. Further studies are necessary to elucidate the mechanism by which aromatase is expressed in these tissues, which would contribute to a better understanding of the growth mechanism and pathogenesis of the disease.

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