Heterogeneity in endometrial expression of aromatase in polyp-bearing uteri

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BACKGROUND: Comparative assessment of expression of aromatase (CYP19A1) messenger RNA (mRNA) in pathological and non-pathological sites within the uterine cavity of mid reproductive age women diagnosed with endometrial polyp (EP).

METHODS: We report a case series of seven premenopausal infertile women undergoing hysteroscopic removal of EP. Directed endometrial biopsies were collected from the EP (P), from the endometrium immediately adjoining the EP (A) and from a normal appearing site remote from the EP (R). Expression of CYP19A1 mRNA within the respective samples in each patient was evaluated by quantitative real-time PCR. Fold changes in the mRNA expression of CYP19A1 within the P versus the R and A endometrial sites were calculated to assess the hypothesis of a ‘field effect’ in the expression of aromatase within EP bearing endometria.

RESULTS: Overall, similar mRNA expression of CYP19A1 gene was demonstrated between P and A endometrial samples. In only one of the seven patients, aromatase expression within P was enhanced by almost 4-fold compared with R (P = 0.14 for comparison with the difference in CYP19A1 expression in P versus R in the remainder of the patients). In contrast, in three of the seven patients, P demonstrated a marked (>1000-fold) under-expression in CYP19A1 mRNA levels compared with the R endometrium (P = 0.22).

CONCLUSIONS: We herein provide evidence of heterogeneity in the expression of endometrial aromatase in premenopausal uteri bearing EPs. Our data suggest that an overexpression of endometrial aromatase may underlie pathogenesis of EP at least in a subset of cases.

Keywords: endometrium; polyp; aromatase; CYP19A1; PCR

Introduction

Endometrial polyps (EPs) belong to a spectrum of proliferative endometrial pathologies that includes endometrial hyperplasia (EH) and endometrial adenocarcinoma (EAC) (Nagele et al., 1996). Epidemiological data suggest that EPs may be regarded as a risk factor for EAC (Pettersson et al., 1985; Anastasiadis et al., 2000; Baskin et al., 2002). In a post-menopausal woman ever diagnosed with an EP, the risk of subsequent EAC may be nine times greater than that of a woman without EP (Armenia, 1967). A point prevalence of EP of up to 16% is reported, both in premenopausal (DeWaay et al., 2002) as well as asymptomatic post-menopausal women (Fay et al., 1999).

Prolonged endometrial exposure to mitogenic effects of estrogen (E), unopposed by progestin, is recognized as a contributor to the various proliferative endometrial disorders (Maia et al., 1998, 2001; Thijs et al., 2000). An increased likelihood of endometrial disorders with advancing age is an apparent paradox, since trophic stimulation from circulating E is minimal in the post-menopausal period, and may be partly explained by local sequestration or synthesis of E within the endometrium.

Aromatase (CYP19A1), a ubiquitous P450 enzyme responsible for the conversion of C19 steroids to E, is a major contributor to circulating E in the post-menopausal women (Bulun et al., 2001). A pathogenic role for an exaggerated local aromatase activity within a spectrum of E-dependent proliferative disorders including breast cancer, mammary ductal hyperplasia, endometrial cancer, endometriosis, adenomyosis and uterine leiomyomas is suggested (Edman and MacDonald, 1978; Kitawaki et al., 1997; Tekmal et al., 1999; Tarkowski et al., 2000; Bulun et al., 2001). Aromatase immunoreactivity has been demonstrated within endometrial stromal cells both in the secretory and proliferative phases of the menstrual cycle (Tseng, 1984; Bulun et al., 1994).

As the current literature supports a role for dysregulated aromatase expression in proliferative endometrial pathologies, we hypothesized that there would be a differential messenger RNA (mRNA) CYP19A1 expression between the EP and the adjoining (A) and remote (R) sites within the same endometrial
cavity. The predicted 'field effect' in premenopausal women diagnosed with EP's would present as a progressive increase in CYP19A1 expression from the normal appearing endometrium at remote tissue regions (R) to the site of EP (P), thereby supporting a pathogenic role for localized excess of endometrial aromatase in the genesis of EP. We designed a cross sectional study utilizing quantitative real-time PCR (QR–PCR) to compare the level of CYP19A1 gene expression in the EP, relative to other endometrial sites in the same individual.

**Cases**

Premenopausal women who were diagnosed with EP either during evaluation for abnormal uterine bleeding, or during routine imaging (sonohysterogram or hysterosalpingogram) during an infertility workup and were anticipating undergoing hysteroscopic removal of EP were offered participation. Participants with submucosal uterine myomas, breast cancer, tamoxifen therapy, a prior history of EAC or diagnosis of EH (within the past 3 months) were excluded. In view of our interest in assessing a site-related differential in gene expression within an individual's endometrium, known diagnoses of endometriosis or uterine fibroids were not considered as exclusion criteria. Patients noted intra-operatively to have multiple EP (>2) were excluded. The protocol was approved by the Center for Clinical Investigation at the Albert Einstein College of Medicine, and the Institutional Review Board at the Montefiore Medical Center, and a written consent was obtained.

**Materials and Methods**

All samples were collected in the follicular phase of the menstrual cycle in an attempt to collect normal endometrium in a similar state across the participants. During the hysterectomy, visually directed endometrial biopsies were obtained (minimal size: 2 mm) directly from three sites within the endometrial cavity (Fig. 1): from the EP itself (P); from the adjoining endometrium within 2 cm of the polyp (A) and from a grossly normal appearing endometrial site remote (R) from the polyp (opposite uterine wall). Samples were transferred into RNALater (RNA stabilization solution, Ambion, Inc.), and the specimens stored at −80°C until RNA analysis.

Endometrial samples were obtained from seven premenopausal patients. Adequate samples for the evaluation of CYP19A1 expression were available from the P and R endometrial sites; A samples were available in five of the seven (patient # 1, 3, 9, 11 and 12). Tissue specimens from the respective sites (i.e. P, A and R) were pooled for cases 1 and 11 due to yield constraints.

Extraction of total RNA was performed using Trizol method (Chirgwin et al., 1979) for specimens larger than 5 mm, or RNeasy Mini-kit (Qiagen, Valencia, CA, USA) for samples of smaller sizes, according to manufacturer’s guidelines. RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) to ensure removal of any contamination with genomic DNA. Only samples of high quality were included in the analysis (as evaluated using the nanobiosizing assay (Agilent Bioanalyzer, Agilent Technologies, Palo Alto, CA, USA). The RNA yield ranged from 200 ng to 3 μg. First strand complementary DNA (cDNA) was reverse transcribed employing a Superscript II RNase kit (Invitrogen) from the non-amplicated RNA, as previously described (Niklaus and Pollard, 2006) and labeled with SYBR green fluorescent dye (Applied Biosystems, Foster City, CA, USA) as part of the PCR Mix. For assay efficiency, smaller primer pairs were designed for CYP19A1 human aromatase (LT, 5'-3' AGG AAG TGA CCA ATG AAT CG, RT 5'-3' CAC GAT AGC ACT TTC GTC CA, 116 base pair product) using Primer3 software (http://frodo.wi.mi.ehu.de/primer3/primer3code.html). PCR was performed in real time using the Prism Gene Amp 5700 (Applied Biosystems). The mRNA abundances for CYP19A1 were determined by normalization of data to the expression levels of 18S ribosomal RNA (rRNA) (Universal 18S Primer Pair, Ambion, Inc.). In preliminary experiments, serial dilutions of cDNA were analyzed to confirm a linear relationship between cDNA content and quality of product across the amplification range. All three endometrial samples, irrespective of the site were analyzed for all seven women on the same plate and run in triplicate. Human placental tissue (15 week gestation) was used as a positive control. Omission of cDNA, as well as water only, served as negative controls.

The relative CYP19A1 aromatase mRNA expression within the three specified sites within each individual was assessed using the 2−ΔΔCT method (Livak and Schmittgen, 2001). The cycle threshold (CT) indicates the fractional cycle at which the amplified RNA target reaches its threshold of detection. The CT was determined from the exponential phase of the PCR by the SDS2.2 software. The ΔCT value was calculated by subtracting the CT of the CYP19A1 in each sample of interest from the CT of the ubiquitous housekeeping 18S rRNA. The reaction products were analyzed by dissociation curve profile and by electrophoresis in 2% agarose gel containing 0.5 mg/ml ethidium bromide and visualized over a UV light box. Utilizing the relative differences in QR–PCR CTs for expression of CYP19A1 and 18S rRNA within each respective sample, a fold change in CYP19A1 aromatase mRNA expression in P compared with A and R samples (paired analysis) was calculated.

A greater than 2-fold difference in the expression of CYP19A1 aromatase mRNA expression between the respective endometrial sites within the same individual was considered clinically meaningful. Linear regression analysis was employed to assess if ΔCT levels were influenced by patient’s age or body mass index (BMI). Mann–Whitney U-test was utilized for the comparison of difference in values of ΔCT from the respective sample sites, i.e. P versus R and P versus A in the patients. STATA (Intercooled 8.2, StataCorp, TX, USA) was used for statistical analysis and _P_ < 0.05 was considered statistically significant.

**Results**

The presented data describe endometrial expression of aromatase gene from samples collected from the P, A and R...
endometrial sites in each of the seven participants (1, 3, 4, 9, 11, 12 and 13). The mean values (± SD) for age and BMI of the participants were 35.43 ± 3.7 years and 25.2 ± 7.84 kg/m², respectively.

The QRT-PCR data, presented as CT values for individual samples, and the ΔCT (CT values for CYP19A1-18S rRNA) values for the individual patient samples are presented in Table I. The mean ‘fold change in gene expression’ of CYP19A1 in P versus R and A samples was calculated based on the 2^−ΔCT method (2^−(CTP_CYP19A1−CTP_18SRNA)−CTR_CYP19A1−CTR_18SRNA) and 2^−(CTP_CYP19A1−CTP_18SRNA)−(CTA_CYP19A1−CTA_18SRNA), respectively (Livak and Schmittgen, 2001).

As shown in the Table I, a patient and site-dependent heterogeneity in the expression of CYP19A1 is noted. A progressive up-regulation in aromatase expression, i.e. P > A > R, as hypothesized, was noted in only one patient (#9), in whom an almost 4-fold up-regulation in CYP19A1 expression within the P compared with R (P = 0.14 for comparison with the difference in CYP19A1 expression in P versus R in the remainder of the patients), and a 2-fold increased expression of the gene within the P compared with A endometrium (P = 0.18 for comparison with the difference in CYP19A1 expression in P versus R in the remainder of the patients) were observed. Contrary to our hypothesis, in three of the seven patients, a marked reduction in the expression of CYP19A1 was noted within P, compared with R endometrial sites (patient # 3, 1 and 11). In these three patients, the grossly normal appearing endometrium obtained from a site remote from the polyp exhibited a >10 000-fold increased expression of CYP19A1 gene compared with P (P = 0.22). No relationship was noted between CYP19A1 ΔCT values for the respective sites and patient’s age or BMI (P > 0.05).

### Discussion

Pathophysiology of EPs remains elusive. Proposed mechanisms that may contribute to development of EPs include a constitutive lack of progesterone receptor (Maia et al., 1998; Thijs et al., 2000), monoclonal EH (Jovanovic et al., 1996) and genetic causes, which have demonstrated a narrow spectrum of mutations including 6p21–22, 7q22 and 12q13–15 within EPs (Dal Cin et al., 1995). A recent report focusing on aromatase expression within EPs, utilizing histological specimens, demonstrated aromatase protein overexpression within EPs (by immunohistochemistry), compared with normal endometria, albeit obtained from different uterine specimens (Maia et al., 2006). Our findings of EPs yielding positive expression of the aromatase gene are thus consistent with protein data reported by Maia et al. (2006).

We demonstrate herein a pathogenic heterogeneity within the EPs in premenopausal women as relates to expression of aromatase gene. Our hypothesis of a ‘localized overexpression’ of endometrial aromatase within the EP was validated only in a single patient (i.e. aromatase expression in P > A > R, patient #9). Notable is the morbidly high BMI of patient #9 (as well as the relatively older age) who is clearly an outlier in this group of otherwise normal weight individuals. The association

### Table I. Mean CT for expression of CYP19A1 human aromatase and house keeping 18S rRNA gene in endometrial samples from polyp (P), grossly normal appearing, remote endometrium (R) and endometrium adjoining the polyp (A).

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Age (years)</th>
<th>BMI* kg/m²</th>
<th>Gene</th>
<th>CTP</th>
<th>CTR</th>
<th>A Mean fold change in CYP19A1</th>
</tr>
</thead>
<tbody>
<tr>
<td>#3</td>
<td>38</td>
<td>21.3</td>
<td>CYP19A1</td>
<td>33.51</td>
<td>20.10</td>
<td>5.0 fold in P</td>
</tr>
<tr>
<td>#4</td>
<td>37</td>
<td>23.8</td>
<td>CYP19A1</td>
<td>30.49</td>
<td>21.30</td>
<td>5.0 fold in P</td>
</tr>
<tr>
<td>#9</td>
<td>36</td>
<td>42.4</td>
<td>CYP19A1</td>
<td>38.76</td>
<td>10.21</td>
<td>10.0 fold in P</td>
</tr>
<tr>
<td>#11</td>
<td>40</td>
<td>22.4</td>
<td>CYP19A1</td>
<td>36.70</td>
<td>22.23</td>
<td>6.5 fold in P</td>
</tr>
<tr>
<td>#12</td>
<td>29</td>
<td>22.1</td>
<td>CYP19A1</td>
<td>32.79</td>
<td>12.24</td>
<td>2.6 fold in P</td>
</tr>
<tr>
<td>#13</td>
<td>32</td>
<td>20.8</td>
<td>CYP19A1</td>
<td>32.79</td>
<td>12.24</td>
<td>2.6 fold in P</td>
</tr>
<tr>
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<td>CYP19A1</td>
<td>32.79</td>
<td>12.24</td>
<td>2.6 fold in P</td>
</tr>
</tbody>
</table>

*CT represents the relative expression of aromatase gene CYP19A1 compared with 18S rRNA for each sample, i.e. CT CYP19A1−CT 18S rRNA. A differential in expression of CYP19A1 gene in P versus R and A was calculated by the 2^−ΔCT method: (2^−(CTP_CYP19A1−CTP_18SRNA)−CTR_CYP19A1−CTR_18SRNA) for P versus R and (2^−(CTP_CYP19A1−CTP_18SRNA)−(CTA_CYP19A1−CTA_18SRNA)) for P versus A.
of obesity with peripheral circulating estradiol is well characterized and attributed to enhanced peripheral conversion of androgens by aromatase within the adipose tissue (Edman and MacDonald, 1978) and extreme obesity has been identified as an independent risk factor for endometrial proliferative pathologies including EPs (Hale et al., 2002). A progressive increase in aromatase CP450 transcriptive activity is described, within the adipose tissue at the various body sites with advancing age (Bulun et al., 2001), thus defining a tendency for an overall unopposed estrogenic environment with aging. An association between obesity, aging and endometrial expression of aromatase however is yet to be described.

Much more interestingly, and contrary to our expectations, a converse pattern in the endometrial expression of aromatase gene was noted in three of the seven patients (#3, 1 and 11). In these subjects, a markedly robust expression in the aromatase gene was noted within the grossly normal endometrium remote endometrium compared with the polyp. Our data thus suggest that pathogenesis of at least some EPs may be independent of aromatase-dependent focal excesses of E within the endometrium. These data thus imply that mechanisms other than chronic exposure to focal E excess maybe at play, at least in some patients with EPs. Our findings therefore are supportive of a mechanistic diversity in contributors to the development of EP.

The study design, specifically procurement of hysteroscopically directed tissue biopsies from multiple sites within the same woman’s uterus, allowed each patient to serve as her own control and is a strength of our design. Our data are the first to suggest not only the occurrence of a ‘focal overexpression’ of endometrial aromatase within EP in at least some women with EPs, but also to demonstrate a heterogeneity in the underlying pathogenic mechanisms. We are intrigued by the marked differential in the expression of aromatase gene within the endometrium obtained from P compared with the grossly normal appearing tissue from the R site, as seen in three of the seven patients. Previous reports have described a differential expression of aromatase within the endometrial tissue compartments; the stroma has been shown to preferentially express aromatase, both the gene as well as the enzyme, compared with the glandular tissue (Huang et al., 1991). Different histological characteristics of the respective tissue sites such as an ‘under’ or ‘over’ representation of the stroma from respective biopsy sites may account for the noted heterogeneity in the expression of aromatase gene within the patients and samples. We are however unable to substantiate this conjecture for the presented cases, due to limitations intrinsic to the methodology employed for sample collection for the purpose of gene expression analysis.

Despite the systematic nature of our study, we acknowledge several limitations that hamper our ability to draw firmer conclusions, including the small sample size, and our inability to substantiate our findings by confirmatory assessment of enzymatic expression at the tissue level. We are unable to unambiguously conclude that the alterations in aromatase gene expression within the polyp-bearing uterus is in fact succeeded by concomitant changes in the aromatase protein and enzymatic activity (as reported by others). Nonetheless, we believe that our findings suggest that all endometria-bearing EP may not be considered a uniform entity. Our data suggest that mechanisms other than ‘focal excess of E’ need to be considered as contributors to the genesis of proliferative endometrial pathologies including EP. It remains to be seen whether the prognosis for EP-bearing endometria demonstrating a ‘gradation’ in the expression of endometrial aromatase is more reassuring than those exhibiting a ‘global overexpression’ of the enzyme. Continued overexpression of aromatase has been demonstrated in vitro to be sufficient for maintaining mammary hyperplasia without the need for large amounts of E (Tekmal et al., 1999). Based on existing data linking chronic exposures to E excess with proliferative endometrial disorders, we speculate that polyp-bearing uteri which demonstrate an overexpression of aromatase within the normal appearing endometrium (R) may be at risk for subsequent development of additional proliferative pathologies including recurrent EPs, EH or even EAC (Bakour et al., 2000); this inference merits substantiation by longitudinal follow up studies. If indeed the latter is true, then given the favorable responses to aromatase inhibitor therapy, as seen in women with EAC (Bonte, 2001), these treatments may be of interest as preventive and adjunctive therapies for lesser proliferative lesions of the endometrium.

In conclusion, our data suggest a pathogenic heterogeneity, in terms of endometrial expression of aromatase gene, in premenopausal uteri with EPs. Our findings, if substantiated by future studies, may help identify a disturbed endometrial milieu destined for progression to more sinister pathologies. Future studies need to appreciate this pathogenic diversity within the entity ‘endometrial polyp’.

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