Rapid loss of oestrogen and progesterone receptors in human leiomyoma and myometrial explant cultures

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Introduction

Leiomyomata are the most common tumours of the uterus, occurring in 25% of women of reproductive age. Although these tumours are benign, symptomatic uterine leiomyomata (commonly called fibroids) are a major public health problem and the predominant indication for hysterectomy (National Center for Health Statistics, 1987; Vollenhoven et al., 1990; Cramer, 1992). Their growth appears to be dependent on ovarian function since uterine leiomyomata tend to develop during the reproductive years, often increase in size during pregnancy and regress following the menopause. These clinical observations have led to the belief that oestrogens promote leiomyoma growth and influence their pathogenesis (John and Martin, 1971; Frankel and Benjamin, 1973). More recent evidence suggests that progesterins also play an important role in regulating the growth of leiomyomata (Carr et al., 1993; Rein et al., 1995a). Reports of sequential gonadotrophin-releasing hormone agonists with oral contraceptive treatments have demonstrated a consistent stimulating effect of either oestradiol or progesterone on myometrial or leiomyoma tissue in vitro (Kassis et al., 1984a; Cramer et al., 1985; Krosl, 1989; Sadovsky et al., 1992; Rauk et al., 1995). However, more recent studies, including those from our laboratory, have established that the mRNA expression of both ER and PR as well as the receptor content measured by ligand binding and by specific immunoassay, are over-expressed in leiomyoma when compared to autologous myometrium (Brandon et al., 1993, 1995). Another recent study has shown that ER numbers rise gradually in the myometrium during the follicular and early luteal phases and then decline; whereas ER in leiomyoma tissue remains elevated throughout the menstrual cycle (Andersen et al., 1995). Thus, elevated expression of PR in leiomyomata is not surprising because oestrogen induces transcription of the PR gene (Brandon et al., 1993).

Although these findings suggest that leiomyomata are hypersensitive to oestrogen and possibly progesterone in vivo, it has been difficult to demonstrate a consistent stimulating effect of either oestradiol or progesterone on myometrial or leiomyoma tissue in vitro (Kassis et al., 1984a; Cramer et al., 1985; Krosl, 1989; Sadovsky et al., 1992; Rauk et al., 1995). This has led to the hypothesis that sex steroid hormones influence these tissues indirectly in vivo, and may be mediated by extracellular growth factors such as epidermal growth factor (EGF) or insulin-like growth factor (IGF)-1 (Harrison-Woolrych et al., 1994; Rauk et al., 1995; Strawn et al., 1995). Alternatively, a loss in oestrogen (or progesterone) responsiveness in vitro may be due to a decline in receptor numbers after tissue dispersion, as has been demonstrated in cultured rabbit uterine myocytes (Sadovsky et al., 1992). To further investigate this possibility, we measured the status of ER and PR in human leiomyoma...
and myometrial explant cultures in the presence and absence of oestradiol and progesterone.

Materials and methods

This study was performed in accordance with the Oregon Health Sciences University’s Human Subjects Review Committee policy and procedures. Seven pre-menopausal women aged 39-49 years, without hormonal therapy within one year, consented to hysterectomy (n = 6) or myomectomy (n = 1). The surgeries were scheduled at patient convenience and not at a particular phase of the menstrual cycle. Tumours with obvious degenerative changes, i.e., calcification, cystic degeneration, were not included. Tissue samples of leiomyoma and adjacent normal endometrium were obtained from each patient. An effort was made to harvest leiomyomata of similar diameter (2-5 cm).

Tissue preparation

Specimens were obtained and immediately transported to the laboratory in sterile saline on ice. Portions of each myometrium and leiomyoma sample were frozen for subsequent baseline measurements. Remaining specimens were minced into pieces of ~3 mm$^3$ and placed in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% glucose and 0.5% gentamicin under aseptic conditions. Minced tissue explants (~100 mg) were placed in medium (DMEM, Phenol Red-free, supplemented with 10% steroid-stripped, fetal bovine serum (FBS), 10% glucose, 10 mM HEPES and 1% gentamicin) supplemented with either 1 nM oestradiol or vehicle (50% ethanol) and placed in a humidified incubator containing 5% CO$_2$ at 37°C. In addition to the control vehicle and 1 nM oestradiol, explants were incubated with 1 µM progesterone or a combination of 1 nM oestradiol or 1 µM progesterone for 8, 24, 48 and 72 h. At the completion of the incubation interval, the media were decanted and explants were quick-frozen in liquid nitrogen and stored at -70°C. Tissue specimens were analysed using an enzyme-linked immunosorbent assay (ELISA) technique to quantify the amount of progesterone and oestrogen receptors.

Enzyme-linked immunosorbent assay

Frozen tissue specimens were thawed on ice and homogenized (1:10, wt:vol) in 0.6 M KCl in TEDG-Mo buffer (0.05 M Tris-HCl, 1.5 mM sodium EDTA, 0.5 mM dithiothreitol, 10% glycerol, and 10 mM sodium molybdate, pH 7.5). Tissue homogenates and all reagents were maintained at 4°C during processing and assay. Homogenates were incubated for 1 h and centrifuged at 40,000 g for 20 min, then diluted 1:1 with TEDG-Mo buffer to yield a protein concentration of 0.5-1.0 mg/ml as determined by the method of Lowry as previously described (Fritz et al., 1993). ER and PR concentrations from both cytosolic and nuclear fractions were determined by enzyme-linked immunosorbent assay (Abbott Laboratories, North Chicago, IL, USA) according to the manufacturer’s specifications. Leiomyoma and myometrium ER and PR concentrations were expressed in fmol/mg DNA after quantification of tissue DNA content by the method of Burton (1956).

RNA isolation and Northern analysis

Total RNA was extracted from tissue by the guanidine isothiocyanate–caesium chloride method as previously described (Brandon et al., 1993). Tissue pieces (0.2-0.5 g) were removed from liquid nitrogen storage and pulverized into a powder with a steel mill precooled with liquid nitrogen. Tissue powder was disrupted in 4 M guanidine isothiocyanate (8 ml) with a motor-driven tissue homogenizer (Tekmar, Cincinnati, OH, USA) and the lysate layered onto 5.7 M caesium chloride (4 ml). After ultracentrifugation at 174,000 g, the RNA pellet was suspended in nuclease-free sterile water and precipitated with ethanol. The RNA concentration was determined by absorbance at 260 nm. The total RNA (10 µg) from each sample of leiomyoma and adjacent myometrium was electrophoresed through a denaturing formaldehyde 1% agarose gel and transferred to a nylon membrane (GeneScreenPlus; New England Nuclear, Boston, MA, USA). Total RNA samples from leiomyoma and adjacent normal myometrium of a given patient were examined simultaneously in adjacent lanes of the same gel. Transcript sizes were estimated by ethidium bromide staining of RNA size markers (Gibco–BRL, Gaithersburg, MD, USA), and 28S and 18S ribosomal RNAs. Leiomyoma and myometrial RNA samples were then sequentially analysed with four different probes: the membrane was hybridized with a [32P]-deoxycytidine random-primed human PR cDNA fragment (3.8 kb) pair full-length coding region, washed at high stringency (twice in 2X saline/sodium citrate (SSC) for 5 min at 22°C, twice in 2X SSC containing 1% sodium dodecyl sulphate (SDS) for 30 min at 60°C and twice in 0.1X SSC for 30 min at 22°C), and autoradiographed after 1-5 days of exposure. This same technique was repeated for each, using ER cDNA (1.96 kb full-length coding region).

To examine the quality and quantity of mRNA, the hybridization transfer membrane was reprobed with a [32P]-β-actin cDNA fragment. An abundant housekeeping mRNA, CHO-A, which is expressed in lesser amounts than β-actin mRNA, was probed for qualitative comparison (1.1 kb probe). Band density for PR mRNA and β-actin mRNA was compared between leiomyoma and adjacent myometrium by laser densitometry (GelScan XL 2.1; Pharmacia–LKB, Piscataway, NJ, USA) (Brandon et al., 1993).

Hormone analysis

On the day of surgery venous blood samples were obtained and serum was frozen at −70°C for subsequent assay of oestradiol and progesterone. Radioimmunoassays were performed for total oestradiol and progesterone serum content and correlated with the last menstrual period to confirm cycle phase. Four specimens were in the secretory phase and two specimens were in the proliferative phase. No correlation was found between the expression of ER or PR and cycle phase for either myometrial or leiomyoma tissue.

Statistical analysis

Results from explant cultures were subjected to the Mann–Whitney rank sum test for non-parametric data with population size below 30.

Results

ER and PR receptor analyses

In general, the mean starting concentrations for ER and PR were higher in the leiomyoma tissue than in the normal adjacent myometrium. With regards to ER, the mean starting concentrations were 2168 ± 689 fmol/mg DNA and 1874 ± 78% from the initial concentration (Figure 1). Although oestradiol is known to be the major inducer of PR expression in other steroid responsive tissues, incubation in oestradiol had
Loss of ER and PR in human explant cultures

Figure 1. Percentage of total (nuclear and cytosolic) oestrogen receptors (ER) and progesterone receptors (PR) remaining in myometrium (M) and leiomyoma (L) explant cultures. Both M and L explants were incubated with 1 nM oestradiol (n = 6) or in a steroid-free environment (control, n = 6). ER and PR protein was measured with a highly specific enzyme-linked immunosorbent assay before (t = 0 h) and after 8 h and 24 h of incubation. Data points represent mean values.

Table I. The percentage of oestrogen receptors (ER) and progesterone receptors (PR) remaining in leiomyomata and myometrial explant cultures after incubation with and without 1 nM oestradiol.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>8 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>p</td>
</tr>
<tr>
<td>Percentage PR remaining (n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myometrium without oestradiol</td>
<td>28.5 ± 6.3</td>
<td>0.002</td>
</tr>
<tr>
<td>Leiomyoma without oestradiol</td>
<td>22.3 ± 4.3</td>
<td>0.002</td>
</tr>
<tr>
<td>Myometrium with oestradiol</td>
<td>37.3 ± 10.5</td>
<td>0.002</td>
</tr>
<tr>
<td>Leiomyoma with oestradiol</td>
<td>21.7 ± 5.4</td>
<td>0.002</td>
</tr>
<tr>
<td>Percentage of ER remaining (n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myometrium without oestradiol</td>
<td>22.0 ± 4.7</td>
<td>0.002</td>
</tr>
<tr>
<td>Leiomyoma without oestradiol</td>
<td>31.5 ± 41.1</td>
<td>0.002</td>
</tr>
<tr>
<td>Myometrium with oestradiol</td>
<td>51.8 ± 12.1</td>
<td>0.002</td>
</tr>
<tr>
<td>Leiomyoma with oestradiol</td>
<td>48.2 ± 22.6</td>
<td>0.06</td>
</tr>
</tbody>
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*Mann–Whitney rank sum test indicating the difference from baseline at time zero.

Figure 2. Percentage of total (nuclear and cytosolic) progesterone receptors (PR) protein content remaining in leiomyoma explants measured before and after incubation in different hormonal environments: steroid-free medium (control), 1 nM oestradiol, 1 µM progesterone or a combination of 1 nM oestradiol and 1 µM progesterone.

no effect on the content of PR in these specimens. Similarly, the addition of progesterone did not affect levels of its cognate receptor. From 8 to 24 h of incubation there was no further significant change in the amount of PR measured. However, when the incubation period was extended to 72 h, PR became essentially undetectable in all explants. Furthermore, there was no demonstrable effect on reduction of receptor content in leiomyoma explants when 1 µM progesterone was added alone, or in combination with 1 nM oestradiol, to the incubation medium (Figure 2). Similar results were obtained with myometrial explants (data not shown). The decline in PR was statistically significant in all explants after 8 h and 24 h of incubation when compared with specimens analysed prior to incubation (t = 0), P < 0.002 (Table I). The rapid decrease of PR protein was found in all specimens (n = 6).

A loss of ER protein was found in all tissues (Figure 1). Within 8 h of incubation the mean decline for ER was 62% (range 48–78%) from the initial concentration. An unexplained increase in one of the six specimens occurred in the leiomyoma control group, and this accounted for a 20% rise in measurable ER. Likewise, there was an unexplained increase in ER content in two of six leiomyoma samples in the presence of oestradiol resulting in a 12.1% rise in the mean ER measured in that group. Despite these minor fluctuations in some samples, after
et al., 1984; Rein et al., 826
deiomyoma explants has been demonstrated previously (Daly et al., 1984a; Sadovsky et al., 1992). To our knowledge, this is the first report describing rapid loss of ER and PR in human leiomyoma and myometrial explants.

Previous studies reporting the lack of responsiveness to oestradiol of myocytes in vitro have implicated the loss of ER during tissue culture (Cramer et al., 1985; Sadovsky et al., 1993; Rauk et al., 1995). A recent report shows basic fibroblast growth factor (FGF) to be mitogenic for human uterine myometrial cells grown in culture (Rauk et al., 1995). However, the addition of oestradiol to the incubation media had no effect on mitogenesis of myometrial or leiomyoma cells with or without basic FGF. In a similar study we were able to demonstrate proliferation of myometrial and leiomyoma monolayer cultures with IGF-I in the absence of oestradiol (Strawn et al., 1995). Both studies imply a role for hormones and growth factors other than sex steroids when studying these tissues in vitro.

Although the mechanism is not known, oestradiol is accepted as a key promoter of leiomyoma formation and growth (Frankel and Benjamin, 1973; Cramer, 1992). Recent evidence, direct and indirect, suggests that progesterone also has an effect on uterine leiomyoma pathogenesis (Brandon et al., 1993; Rein et al., 1995a). Mitotic counts in leiomyoma are higher in the progesterone-dominated secretory phase than in the proliferative phase or menses (Kawaguchi et al., 1989). Both PR protein and mRNA are over-expressed in leiomyoma in comparison with autologous myometrium (Brandon et al., 1993). Furthermore, the numerous clinical trials involving gonadotrophin-releasing hormone (GnRH) agonists, medroxyprogesterone acetate and RU-486 suggest progesterone as a potentiating factor in leiomyoma growth (Friedman et al., 1988; Carr et al., 1993; Murphy et al., 1993).

The true role of progesterone in leiomyoma pathogenesis is unknown. In this study we did not alter the loss of PR in tissue explants with either progesterone, oestradiol or a combination of oestradiol and progesterone. Although the enzyme-linked radioimmunoassay we use is highly specific and not affected by the amount of progesterone in the media (bound or unbound), this assay does not differentiate between the A and B isoforms of PR. We have shown that the mRNA for both isoforms is expressed in leiomyoma tissue (Brandon et al., 1993). However, these two isoforms may have independent roles in stimulating or repressing cellular functions (Vegeto et al., 1993).

We examined the effect of progesterone alone or in combination with oestradiol on ER and PR content in explants. All explant cultures exhibited similar decreases in ER and PR content despite variation of the hormonal milieu (Figure 2). Likewise, oestradiol dose–response trials did not affect the loss of ER or PR.
Our study, along with previous reports suggests the importance of evaluating ER and PR content prior to studying the effect of sex steroids on human myometrium and leiomyoma cells in vitro (Soules and McCarty, 1982; Cramer et al., 1985; Andersen et al., 1995). One report of receptor maintenance uses a transformed hamster uterine myocyte cell line in which human ER cDNA was successfully transfected (Sadovsky et al., 1993). In that model, induction of PR by oestriol was observed in myocytes. A previous study demonstrated oestriol responsiveness in primary cultures from immature rat uteri (Kassis et al., 1984b). Oestriol induction of the PR in leiomyoma tissue remains unproven. A major difficulty in studying these tissues is the heterogeneity observed between species, cycle phase at harvest time and differences in leiomyoma obtained from the same uterus. This heterogeneity of leiomyoma is exemplified by karyotyping these tumours. Several different cytogenetic abnormalities have been identified in uterine leiomyoma (Meloni et al., 1992). Aberrations in chromosome 12 are frequently reported. For example, a fusion transcript between HMGl-c, a gene encoding for a member of the high mobility group proteins, and mitochondrial aldehyde dehydrogenase gene in various chromosomal regions 12q14–15 and 12q24 has recently been described (Kazmierczak et al., 1995). Two biologically different cell types of uterine leiomyoma have been distinguished using cytogenetic analysis (Brosens et al., 1995). Also, leiomyoma size correlates with a more aberrant karyotype (Brosens et al., 1995; Rein, et al., 1995b).

Besides the genetic heterogeneity found in these tumours, culture environment must also play a role in receptor maintenance. More than a decade ago, an ER activator protein was postulated (Kassis et al., 1984a). This protein has yet to be defined. Growth factors have been shown to initiate mitogenesis and cellular proliferation in vitro (Kawaguchi et al., 1989; Rauk et al., 1995; Strawn et al., 1995); however, myometrium and leiomyoma sex steroid responsiveness seen clinically has yet to be reproducibly demonstrated in culture. Lack of consistent sex steroid responsiveness can be, at least in part, attributed to receptor decline in vitro. Furthermore, our data imply that the loss of ER and PR protein is related to a concomitant loss of respective mRNA. However, data indicating that other transcripts and proteins remain stable in culture imply that a diffusible factor may modulate expression of ER and/or PR in vivo. If an ‘ER activator protein’ or substance exists, it remains undefined.

In animal models the half-life of myocyte ER has been estimated, by hormone binding or oestriol responsiveness studies, to be 12 h to several days (Kassis et al., 1984a). In breast cancer cells the half-life of ER was reported to be as short as 6 h (Eckert et al., 1982). Based upon our data, the in-vitro half-life for ER and PR in both leiomyoma and myometrial tissues is several hours. Therefore, the rapid loss of ER and PR may occur by increased receptor turnover in addition to loss of ER and PR mRNA.

Even the minutes necessary to transport specimens from the operating room to a suitable laboratory may affect receptor status in these tissues. Future studies on ER and PR mRNA should reveal whether loss of these transcripts occurs because of their increased turnover or due to a decline in synthesis. In vitro studies designed to test the effects of sex steroids and their respective inhibitors on growth and function of leiomyoma and myometrial cells should consider these findings.

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References


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