Tissue differences but limited sex steroid responsiveness of c-fos and c-jun in human fibroids and myometrium

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Sex steroids influence the growth of mammalian uterine tissues and the proto-oncogenes c-fos and c-jun have been implicated in the cascade of cellular events induced by the cyclic influence of oestrogen and progesterone. To investigate the role of these proto-oncogenes for fibroid growth their mRNA expression was measured in myometrium and fibroids under different hormonal conditions, using a solution hybridization method. Fibroids and myometrium were collected at surgery from premenopausal, postmenopausal and pregnant women as well as women treated with a gonadotrophin releasing hormone agonist (GnRHa; Goserelin). The phase of the menstrual cycle was determined in all the untreated, premenopausal, non-pregnant women. The mRNA expression of c-fos and c-jun in fibroids was significantly lower than in homologous myometrium. No significant differences in c-fos expression were observed in myometrium, or fibroids, due to menstrual cycle phase, GnRHa treatment, pregnancy or the menopause. The c-jun expression in myometrium from pregnant women without fibroid disease was significantly higher than the corresponding control myometrium from premenopausal, non-pregnant women. These results demonstrate a tissue difference in the expression of c-fos and c-jun between myometrium and fibroids.

Key words: fibroids/hormones/human/proto-oncogenes

Introduction

Uterine fibroids represent a major cause of gynaecological morbidity, including menorrhagia, pelvic discomfort, pain and, less frequently, infertility. The aetiology of these tumours is largely unknown but their development is apparently related to ovarian steroid production. Fibroids do not occur before menarche (Healy, 1991). When the concentrations of oestrogen and progesterone decline, e.g. after menopause or following treatment with agonists to gonadotrophin releasing hormone (GnRHa), fibroids usually decrease markedly in size (Healy, 1991). During pregnancy individual fibroids may grow rapidly but the majority decrease in size during the third trimester (Lev-Toaff et al., 1987). Although fibroids are benign, the regulation of their growth involves mechanisms implicated in the development of malignant uterine tumours. Thus, the growth of both fibroids and endometrial cancer is influenced by sex steroids and this effect has been postulated to be mediated by a complex series of events, involving activation of proto-oncogenes and peptide growth factors (Koutsilieris, 1991).

The proto-oncogenes c-fos and c-jun are closely involved in cell growth and differentiation. The protein products encoded by these genes, Fos and Jun, play a regulatory role during initiation of cell growth (Pai and Bird, 1994). c-fos and c-jun are transiently expressed early in the cell cycle following stimulation of quiescent cells by growth factors and are therefore called ‘immediate early genes’ (Angel and Karin, 1991). Fos and Jun form stable dimeric complexes which bind to DNA sequences containing AP-1 sites and regulate gene transcription (Chiappetta et al., 1992). Their basal concentration is low in many different types of cells and tissues but can be transiently and rapidly induced by a variety of extracellular signals involved in mitogenesis, differentiation and cell death (Curran et al., 1993).

In general, oestrogens induce a rapid and dramatic growth response of the mammalian uterus, in terms of DNA synthesis and cell division. In the immature rodent uterus both c-fos and c-jun are transiently increased by oestradiol (Loose-Mitchell et al., 1991; Chiappetta et al., 1992; Kirkland et al., 1992). The oestrogen-induced increase in c-fos in these young rats was also shown to be inhibited by progesterone (Kirkland et al., 1992). In adult rats the uterine induction of c-fos by oestrogen has been shown to be restricted to the luminal and epithelial cells of the endometrium whereas no effects on myometrial cells were seen (Papa et al., 1991). In the region upstream of the human c-fos gene an oestrogen responsive element (ERE) has been identified that binds to the complex of oestrogen and the oestrogen receptor (Weisz and Rosales, 1990). So far limited knowledge is available concerning the regulation of steroid responsive genes in human uterine tissues. The aim of the present study was to investigate the expression of c-fos and c-jun in fibroids and myometrium under various endocrine conditions, i.e. in different phases of the menstrual cycle.
cycle, during treatment with GnRHAs, in pregnancy and the menopause.

Materials and methods

Patient material and hormone treatment

 Fibroids and/or myometrium were obtained at hysterectomy, myomectomy or Caesarean section from 17 premenopausal and five postmenopausal patients with fibroids, 10 women preoperatively treated with s.c. implants of GnRHα (Goserelin: 3.6 mg every 4 week) for 3 months and five pregnant women with fibroids. We also collected myometrium from seven premenopausal, five postmenopausal and seven pregnant patients without fibroid disease. Pieces of tissue were cut out either per-operatively or immediately after removal of the uterus/fibroid. Myometrium from term pregnant women was collected at Caesarean section, from the upper flap of the incised lower uterine segment. All specimens were immediately frozen in liquid nitrogen and stored at −70°C.

 In a few patients with fibroids, only fibroid or myometrium was collected. In the group of pregnant women with fibroids only two paired samples were available and one woman, 46 years of age, underwent hysterectomy in the 12th week of pregnancy, due to a wish for abortion, which due to multiple large fibroids could not be performed by vacuum aspiration. All other samples from pregnant women, except from this patient, were collected at term pregnancy (38th to 42nd week).

 The design of the study was approved by the Ethics Committee of the Huddinge University Hospital and the Research Ethics Committee of the Uppsala University. Informed consent was obtained from all participating women.

 Determination of hormonal status and efficiency of treatment

 To determine the phase of the menstrual cycle, the effect of GnRHα treatment and to verify the endocrine status of postmenopausal women, several different tools were used. (i) An ultrasound examination was performed before and after treatment with GnRHα to determine the effect on uterine size. (ii) Menstrual data were recorded. (iii) A blood sample from an antecubital vein was collected on the morning of surgery and the serum concentrations of FSH were determined using a commercially available non-isotopic immunoassay based on enhanced luminescence (Amerlite, Amersham, Bucks, U.K.). (iv) Endometrial histology was routinely examined in those cases where a hysterectomy was performed. Nevertheless, in several cases it was not possible to classify the patient into any of the above-mentioned categories due to menstrual irregularity/constant bleeding and/or inconsistencies between menstrual history, hormone concentrations and endometrial histology. These cases were not included and neither were women with an additional histological diagnosis of adenomyosis, endometriosis or metropathia haemorrhagica cystica.

 Determination of menstrual cycle phase was based on endometrial histology, serum hormone concentrations and date for last menstrual period. Data concerning age, parity are presented for all groups (Table I). The serum concentrations of 17β-oestradiol and progesterone are shown for all groups except the pregnant patients with fibroid disease. Follicle stimulating hormone (FSH) data are shown for the postmenopausal women. Ten of the premenopausal women were in the proliferative phase and seven were in the secretory phase of the cycle. All GnRHα treated women included in the present study had serum 17β-oestradiol concentrations <100 pmol/l. All postmenopausal women had FSH concentrations >26 IU/l, verifying the postmenopausal status.

 mRNA analysis

 Preparation of total nucleic acids (TNA) and quantification of mRNA expression by hybridization of TNA to a complementary RNA (cRNA) probe in solution were performed as previously described (Durnam and Palmiter, 1983). [35S]UTP-labelled (Amersham) cRNA probes were synthesized using a 3.1 kb long genomic Xhol−NcoI fragment of c-fos and a 996 bp long BamH1−HpaI cDNA fragment of c-jun, subcloned into a Bluescript vector. All samples were analysed several times. The results presented here represent a typical experiment where all samples in duplicate have been hybridized with the same probe, within a week. The background in terms of c.p.m./vial without TNA, expressed as a percentage of input values, was 2–5%. At least double background levels were reached, within a range where a linear relationship between increasing amount of TNA and c.p.m./μg TNA was achieved. The concentration of nucleic acids in the TNA samples was measured spectrophotometrically and the DNA content was measured fluorometrically to determine the RNA:DNA ratio. The RNA versus DNA content varied with the endocrine situation. As the DNA content per cell is a more stable parameter than RNA or TNA content, in a situation where the rate of proliferation differs between the different groups, we have chosen to present our data concerning mRNA expression as c.p.m./μg DNA.

 Statistical analyses

 Statistical calculations were performed using the Kruskal–Wallis test, the Wilcoxon signed rank test and the Mann–Whitney U-test. The Wilcoxon signed rank test was used for paired samples of myometrium and fibroids in those groups where a sufficient number of paired samples was available. In all calculations the mean values derived from a number (one to six) of fibroids for each patient was used. The average number of fibroids per patient in the whole material was 1.8. For the groups of pregnant and postmenopausal women the standardized t-test was used because the number of observations was too low for the Wilcoxon signed rank test. The Mann–Whitney U-test was used for comparisons between groups.

 Results

 Patient characteristics and hormone concentrations in serum for the different groups of patients are presented in Table I. The fibroid mRNA concentration of c-fos was significantly lower than in myometrial specimens obtained during the menstrual cycle, during GnRHα treatment and after menopause (Figure 1a). No significant differences between the cycle phases were present either for fibroids or for myometrium (Table II) and data from the proliferative and secretory phases are therefore presented together in Figure 1. No significant difference between fibroids and myometrium was observed in the group of pregnant women. There were no significant differences in c-fos concentrations between the various endocrine conditions, neither in fibroids nor in myometrium. Moreover, the c-fos mRNA expression in myometrium from patients with fibroid disease was not different from that in myometrium of control patients under the same endocrine conditions.

 The mRNA expression of c-jun was also significantly
c-fos and c-jun in human fibroids and myometrium

Table I. Patient characteristics and hormone concentrations in serum. Means and ranges are presented.

<table>
<thead>
<tr>
<th>Patient category</th>
<th>No. of patients</th>
<th>Age (years)</th>
<th>Parity (no. deliveries)</th>
<th>17β-oestradiol (pmol/l)</th>
<th>Progesterone (nmol/l)</th>
<th>FSH (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausalb</td>
<td>7</td>
<td>41.6 (31–50)</td>
<td>2.1 (1–4)</td>
<td>398.5 (126–657)</td>
<td>9.1 (0.5–46.4)</td>
<td>8.9 (2.5–19.0)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>5</td>
<td>64.8 (53–84)</td>
<td>2.5 (0–4)</td>
<td>103 (69–134)</td>
<td>0.8 (0.2–1.1)</td>
<td>48.8 (36.9–54.6)</td>
</tr>
<tr>
<td>Pregnant</td>
<td>7</td>
<td>30.1 (24–38)</td>
<td>2.0 (1–3)</td>
<td>&gt;14 000</td>
<td>&gt;150</td>
<td></td>
</tr>
<tr>
<td>Patients with fibroids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative</td>
<td>10</td>
<td>43.2 (38–52)</td>
<td>1.8 (0–3)</td>
<td>590.1 (184–1595)</td>
<td>1.2 (0.5–4.1)</td>
<td>n.d. c</td>
</tr>
<tr>
<td>Secretry</td>
<td>7</td>
<td>42.6 (36–50)</td>
<td>2.1 (0–4)</td>
<td>367.8 (225–765)</td>
<td>22.9 (5.5–45.3)</td>
<td>n.d. c</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>5</td>
<td>64 (53–69)</td>
<td>1.4 (1–3)</td>
<td>106 (25–195)</td>
<td>1.6 (0.4–2.9)</td>
<td>35.4 (26.5–46.9)</td>
</tr>
<tr>
<td>GnRHa</td>
<td>10</td>
<td>41.3 (34–50)</td>
<td>1.2 (0–4)</td>
<td>46.5 (25–95)</td>
<td>0.9 (0.2–1.3)</td>
<td>n.d. c</td>
</tr>
<tr>
<td>Pregnant</td>
<td>5</td>
<td>36.8 (34–46)</td>
<td>1.2 (1–2)</td>
<td>n.d. c</td>
<td>n.d. c</td>
<td>n.d. c</td>
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</tbody>
</table>

The detection limits for 17β-oestradiol and progesterone were 50 and 0.35 pmol/l, respectively. For calculations values below the detection limit were set at 25 pmol/l for 17β-oestradiol and 0.2 nmol/l for progesterone.

bRepresents five patients in proliferative phase and two in secretory phase.

cn.d. = not determined.

FSH = follicle stimulating hormone; GnRHa = gonadotrophin releasing hormone agonist.

Figure 1. mRNA expression (c.p.m./µg DNA) of c-fos (A) and c-jun (B) in fibroids (F) and myometrium (M) from untreated premenopausal (pre), gonadotrophin releasing hormone agonist-treated premenopausal (GnRHa), postmenopausal (PM) and pregnant (Prg) women. Control myometrium (Controls) was collected from premenopausal, postmenopausal and pregnant women without uterine disease. Individual observations are indicated for each group and the solid lines represent medians.

αSignificantly different from the corresponding myometrium (Wilcoxon signed rank test; P < 0.05). For the postmenopausal and pregnant groups the standardized t-test was applied; P < 0.05).

βSignificantly different when compared with control myometrium from untreated premenopausal women (Mann–Whitney U-test; P < 0.05).

c-fos (c.p.m./µg DNA) | c-jun (c.p.m./µg DNA)
<table>
<thead>
<tr>
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<th></th>
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</thead>
<tbody>
<tr>
<td>Myometrium</td>
<td>Fibroids</td>
</tr>
<tr>
<td>Proliferative (n = 10)</td>
<td>203.5 (46–1111)</td>
</tr>
<tr>
<td>Secretry (n = 7)</td>
<td>371.5 (102–509)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Myometrium</th>
<th>Fibroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative (n = 10)</td>
<td>22.2 (3.9–53.7)</td>
</tr>
<tr>
<td>Secretry (n = 7)</td>
<td>20.9 (13.3–53.1)</td>
</tr>
</tbody>
</table>

Table II. mRNA expression of c-fos and c-jun in human myometrium and fibroids obtained during the proliferative and secretory phases of the menstrual cycle. Data are expressed as c.p.m./µg DNA (median and range).

lower in the fibroids than in the corresponding myometrium in all groups except the pregnant women (Figure 1b). For c-jun, no differences between the proliferative and secretory phases were present and data are presented as one group in Figure 1. The c-jun expression was significantly higher in myometrium from pregnant women without fibroids, compared with the corresponding tissue from non-pregnant, premenopausal women without fibroid disease. No other differences in c-jun mRNA expression, related to the endocrine conditions, were observed.

Discussion

The present study demonstrates that fibroids express lower mRNA concentrations of c-fos than homologous myometrium, whereas no differences with regard to the endocrine conditions
were identified. The basal concentration of c-jun is generally lower than c-fos, but follows the same pattern, i.e. lower expression in fibroids than in myometrium. These findings are consistent with a recent report where expression of both c-fos and c-jun was shown to be lower in fibroids than in myometrium from premenopausal women, both at the mRNA and protein levels (Lessl et al., 1997). Our data confirm the lack of variation with the phase of the menstrual cycle reported by these authors but also demonstrate that steroid withdrawal, represented by the postmenopausal and GnRHa-treated women, does not influence the mRNA expression of either c-fos or c-jun.

The present data, showing that c-jun expression in myometrium from pregnant women without fibroids was significantly higher than myometrium from the corresponding non-pregnant women, indicate that this gene responds to excessive sex steroid stimulation. This view is consistent with the observations of Yamashita et al. (1996), who demonstrated that administration of oestradiol 17β to adult castrated mice caused differential c-jun expression in all uterine cell types. c-fos was shown to be transiently and rapidly induced only in the epithelium and vascular endothelium with no detectable amounts of c-fos transcript or protein in the stromal or myometrial cells.

In the immature rat uterus a differential regulation of c-fos, c-jun and jun-B has been demonstrated (Biggsby and Li, 1994). In the epithelium oestrogen was shown to stimulate c-fos and jun-B whereas the concentration of c-jun mRNA was reduced. When RNA from the whole uterus was analysed, oestrogen increased the expression of all three genes, indicating that c-jun was increased in the non-epithelial compartment. Pretreatment with progesterone attenuated the increase in c-fos both in whole uterus extracts and in the epithelium, did not alter the oestrogen-induction of jun-B and completely blocked the repressive effect of oestrogen on epithelial c-jun. These differences in regulation of immediate early genes between epithelial and non-epithelial uterine tissues in response to hormone stimulation suggest that tissue-specific transactivating factors are involved.

Studies in human uterine tissues also support the view that different signalling pathways mediate steroid hormone action in endometrium and myometrium. Salmi and Rutanen (1996) demonstrated a weak expression of c-fos and c-jun in myometrium and fibroids. The endometrium exhibited stronger expression, which was significantly higher than the adjacent myometrium only in the proliferative phase. In both endometrium and myometrium from term pregnant women the expression of both proto-oncogenes was almost undetectable (Salmi and Rutanen, 1996). No differences between the different phases of the menstrual cycle were observed in any of the investigated tissues. Whereas the lack of differences between the phases of the menstrual cycle are consistent with our data, their findings, showing low expression of c-fos and c-jun in myometrium from term pregnancy, are contradictory. The observed differences could be due to problems concerning choice of a control gene. When analysing a Northern blot under the present conditions, where pregnant and non-pregnant tissues are compared, it is essential that the control gene used is not influenced by proliferation. We have tried several established house-keeping genes to serve as controls but found that the marked differences that occur in terms of proliferation and inhibition of proliferation during pregnancy, after menopause and during GnRH treatment also influence the expression of all the house-keeping genes studied.

The importance of these considerations is further illustrated by the fact that differences in the relative amounts of RNA and DNA are present in our TNA preparations (unpublished observations). As the rate of proliferation differs between the different endocrine conditions studied here, the DNA content of the cells is a more stable parameter than TNA and RNA and we have chosen to relate all our data to the DNA content of the TNA preparations.

A recent report, utilizing a model for experimental skin carcinogenesis, indicates that c-fos is not necessary for tumour promotion, but could be involved in the progression of a benign to a malignant tumour (Saez et al., 1995). Malignant transformation of a uterine fibroid is an extremely rare event and it could be postulated that the low expression of c-fos and c-jun is part of the benign phenotype which could be of importance to protect the tumour from malignant transformation.

In general, overexpression and misregulation of proto-oncogenes are considered to be associated with tumorigenesis (Stancel et al., 1993). In this case decreased expression of both c-fos and c-jun is part of the fibroid phenotype that is associated with a selective growth advantage compared with normal myometrium in the human uterus. Studies (Ambrosini et al., 1993) suggest that a functional antagonism is present between the oestrogen receptor and the Fos protein in the regulation of c-fos transcription. It cannot be excluded that such an antagonism could influence transcription also of other oestrogen responsive genes. In this case the markedly lower expression of c-fos in fibroids than in myometrium observed here might selectively facilitate stimulation of oestrogen responsive genes in the human fibroid.

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