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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, 1992).

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, during treatment with GnRHa, 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 pre-operatively treated with s.c. implants of GnRHa (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 GnRHa 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 GnRHa 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 17β-oestradiol, progesterone, and follicular stimulating hormone (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 GnRHa 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). [^{35}S]UTP-labelled (Amersham) cRNA probes were synthesized using a 3.1 kb long genomic *Xho*1–*Nco*1 fragment of c-*fos* and a 996 bp long *BamH*1–*Hpa*1 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 GnRHa 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

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

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

[a]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.
[b]Represents five patients in proliferative phase and two in secretory phase.
[c]n.d. = not determined.
FSH = follicle stimulating hormone; GnRHa = gonadotrophin releasing hormone agonist.

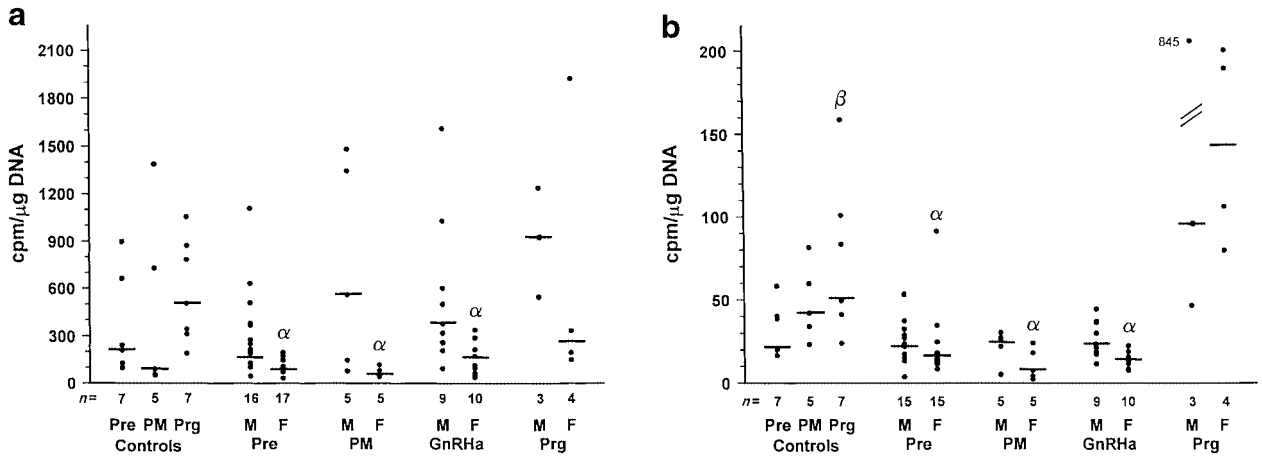

Figure 1. mRNA expression (c.p.m./μg DNA) of c-*fos* (**A**) and c-*jun* (**B**) in fibroids (F) and myometrium (M) from untreated premenopausal (pre), gonadotrophin releasing hormone agonist-treated premenopausal (GnRHa), postmenopausal (PM) and pregnant (Prg) women. Control myometrium (Controls) was collected from premenopausal, postmenopausal and pregnant women without uterine disease. Individual observations are indicated for each group and the solid lines represent medians. $^{\alpha}$Significantly different from the corresponding myometrium (Wilcoxon signed rank test; $P < 0.05$. For the postmenopausal and pregnant groups the standardized *t*-test was applied; $P < 0.05$). $^{\beta}$Significantly different when compared with control myometrium from untreated premenopausal women (Mann–Whitney *U*-test; $P < 0.05$).

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)

	c-*fos* (c.p.m./μg DNA)		c-*jun* (c.p.m./μg DNA)	
	Myometrium	Fibroids	Myometrium	Fibroids
Proliferative (n = 10)	203.5 (46–1111)	87 (33–196)[a]	22.2 (3.9–53.7)	14.5 (8.5–91.5)[a]
Secretory (n = 7)	371.5 (102–509)	100.7 (70–193)[a]	20.9 (13.3–53.1)	14.2 (12.7–24.8)

[a]Significantly different from the corresponding myometrium (Wilcoxon signed rank test, $P < 0.05$).

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 (Bigsby 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 differerent 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 GnRHa 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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References

Ambrosini, C., Cicatiello, L., Cobellis, G. *et al*. (1993) Functional antagonism between the estrogen receptor and Fos in the regulation of c-*fos* protooncogene transcription. *Mol. Endocrinol*., **7**, 1472–1483.

Angel, P. and Karin, M. (1991) The role of *Jun*, *Fos* and the AP-1 complex in cell proliferation and transformation. *Biochim. Biophys. Acta*, **1072**, 129–157.

Bigsby, R.M. and Li, A. (1994) Differentially regulated immdiate early genes in the rat uterus. *Endocrinology*, **134**, 1820–1826.

Chiappetta, C., Kirkland, J.L., Loose-Mitchell, D.S. *et al*. (1992) Estrogen regulates expression of the jun family of protooncogenes in the uterus. *J. Steroid Biochem. Mol. Biol*., **41**, 113–123.

Curran, T., Abate, C., Baker, S. *et al*. (1993) The regulation of c-*fos*: Too much is never enough. In Brown, B.L. and Dobson, P.R.M. (eds), *Advances in Second Messenger and Phosphoprotein Research*. Raven Press, New York, Vol. 28, pp. 271–277.

Durnam, D.M. and Palmiter, R.D. (1983) A practical approach for quantitating specific mRNAs by solution hybridization. *Analyt. Biochem*., **131**, 385–393.

Healy, D.L. (1991) The use of GnRH analogs in menorraghia, contraception and uterine fibroids. In Lunenfeld, B. and Insler, V. (eds), *Current Status of GnRH Analogs*. Parthenon Publishing, Park Ridge, NJ, pp. 33–40.

Kirkland, J.L., Murthy, L. and Stancel, G.M. (1992) Progesterone inhibits estrogen-induced increases in c-*fos* mRNA levels in the uterus. *Endocrinology*, **103**, 3223–3230.

Koutsilieris, M. (1992) Pathophysiology of human leiomyomas. *Biochem. Cell Biol*., **70**, 273–278.

Lev-Toaff, A.S., Coleman, B.G., Arger, P.H. *et al*. *(*1987) Leiomyomas in pregnancy: sonographic study. *Radiology*, **164**, 375–380.

Lessl, M., Klotzbuecher, M., Schoen, S. *et al*. (1997) Comparative messenger ribonucleic acid analysis of immediate early genes and sex steroid receptors in human leiomyoma and healthy myometrium. *J. Clin. Endocrinol. Metab*., **82**, 2596–2600.

Loose-Mitchell, D.S., Gardner, R.M., Hyder, S.M. *et al*. (1991) Estrogen regulation of c-*fos* messenger ribonucleic acid. *Mol. Endocrinol*., **2**, 946–951.

Pai, S.R. and Bird, R.C. (1994) c-*fos* expression is required during all phases of the cell cycle during exponential cell proliferation. *Anticancer*, **14**, 985–994.

Papa, M., Mezzogiorno, V., Bresciani, F. *et al*. (1991) Estrogen induces c-*fos* expression specifically in the luminal and glandular epithelia of adult rat uterus. *Biochem. Biophys. Res. Commun*., **175**, 480–485.

Saez, E., Rutberg, S.E., Mueller, E. *et al*. (1995) c-*fos* is required for malignant progression of skin tumors. *Cell*, **82**, 721–732.

Salmi, A. and Rutanen, F.M. (1996) c-*fos* and c-*jun* expression in human endometrium and myometrium. *Mol. Cell. Endocrinol*., **117**, 233–240.

Stancel, G.M., Baker, V., Hyder, S.M. *et al*. (1993) Oncogenes and uterine function. *Oxf. Rev. Reprod. Biol*., **15**, 1–42.

Weisz, A. and Rosales, R. (1990) Identification of an estrogen response element upstream of the human c-*fos* gene that binds the estrogen receptor and the AP-1 transcription factor. *Nucleic Acids Res*., **18**, 5097–5105.

Yamashita, S., Takayanagi, A. and Shimizu, N. (1996) Temporal and cell-type specific expression of the c-*fos* and c-*jun* protooncogenes in the mouse uterus after estrogen stimulation. *Endocrinology*, **137**, 5468–5475.

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