Immunohistochemical study of the proliferation index, oestrogen receptors and progesterone receptors A and B in leiomyomata and normal myometrium during the menstrual cycle and under gonadotrophin-releasing hormone agonist therapy

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Introduction

Leiomyomata, which occur in 20–30% of women over 30 years of age, are the most common benign tumours of uterine smooth muscle cells. In the United States, they are the most common indication for hysterectomy due to menorrhagia or hypermenorrhoea (Wilcox et al., 1994). Although the mechanism of tumorigenesis is unknown, evidence suggests that leiomyomata are ovarian steroid-dependent (Buttram and Reiter, 1981; Vollenhoven et al., 1990). Indeed, conditions that provoke increased ovarian steroid production (e.g. pregnancy and hormone replacement therapy) result in the rapid growth of leiomyomata.

On the other hand, conditions in which ovarian steroid concentrations are diminished [menopause and gonadotrophin-releasing hormone agonist (GnRHa) therapy] result in a decrease in the size of leiomyomata (Donnez et al., 1989, 1990; Broeckmans, 1996; Gonzalez Barcena et al., 1997). Medical management of fibroids with GnRHa was first described in 1983 (Filicori et al., 1983) and is now approved. But because most leiomyomata return to near-pretreatment size within 4 months following cessation of GnRHa therapy and because of the adverse effects (bone loss with long-term treatment) (Devogelaer et al., 1993), it should be considered as preoperative therapy to reduce tumour size rather than as a definitive alternative to surgery (Maheux et al., 1985; Donnez et al., 1989, 1990, 1993).

The traditional view holds that leiomyomata are only oestrogen dependent, but some experimental and clinical data suggest that progestins may be important for growth. This has led us to reconsider the role of progesterone in the understanding of leiomyoma growth and to reassess the traditional view.

Therefore, the aim of this study was to compare both myomata and adjacent myometrium in terms of proliferation and steroid receptor content throughout the menstrual cycle and during GnRHa therapy. The proliferation index (Ki 67) as well as the oestrogen receptor (ER), progesterone receptor AB (PRAB) and progesterone receptor B (PRB) content of leiomyomata and adjacent myometrium, were evaluated using immunohistochemical techniques.

Materials and methods

Patients

The study included 40 patients ranging in age from 29 to 50 years, suffering from symptomatic submucosal leiomyomata, after myomectomy because of symptomatic leiomyomata (subgroup I). Leiomyoma biopsies were taken from 10 patients suffering from symptomatic submucosal leiomyomata, after 2 month GnRH therapy (subgroup II). During the secretory phase, the proliferation index (Ki 67) was found to be higher in leiomyomata than in myometrium, but the difference was not significant. Oestrogen receptor content was significantly higher in leiomyomata than in myometrium only during the proliferative phase of the cycle. PRAB and PRB content were found to be higher in leiomyomata than in adjacent myometrium with a statistically significant dominance of PRAB over PRB. Under GnRHa therapy, a dramatic decrease was observed in PRAB and PRB content as well as Ki 67 but ER content remained comparable with the results obtained during the menstrual cycle. The results suggest that leiomyomata may be under the influence of progesterone which may play a major role in their growth.

Key words: GnRHa/leiomyomata/proliferation index (Ki 67)/steroid receptors
The proliferative status of muscular cells was measured by evaluating the percentage of nuclei staining positive for Ki 67 antibody at titre 1/100, giving the proliferation index (Gerdes et al., 1984). The monoclonal antibody Ki 67 (Immunotech, Marseille, France) recognizes a nuclear antigen that is expressed in all stages of the cell cycle except G0 (Gerdes et al., 1983, 1984).

Tissue samples were fixed in 10% formaldehyde and embedded in paraffin. Ki 67 labelling was determined by immunoperoxidase techniques using the peroxidase–antiperoxidase (PAP) complex, which increases the reliability and sensitivity of detection to the level obtained with radioimmunoassay. Tissue sections 6 µm thick were mounted on Superfrost Plus slides and stained according to the immunocytochemical assay described by Immunotech (Clone MIB-1, Immunotech, Marseille, France). Labelling was developed with diaminobenzidine and hydrogen peroxide until nuclear brown staining was easily detectable. Positive and negative controls were carried out as previously described (Nisolle et al., 1997). Resultant staining was evaluated by determination of the distribution of the stained nuclei within muscular cells. The tissue was examined on a blind basis by two observers.

The number of stained nuclei per 900–14 000 cells was counted using the ×40 objective of a light microscope (Zeiss, Oberkochen, Germany).

The χ² test and analysis of variance (ANOVA) were used for statistical analysis.

Oestrogen receptor localization

Oestrogen receptors were identified using a monoclonal antibody (mouse monoclonal antihuman ER 1D5) which recognizes ERα (Immunotech®, Marseille) (1/300 dilution). A negative control was prepared in each case and consisted of one section incubated with mouse immunoglobulin G (IgG) instead of mouse antireceptor antibody.

The immunostaining quantitative analysis was performed with a computerized microscope image processor. All samples were analysed field by field using the ×100 objective of an Axioskop light microscope through a CCD 72E camera (Dage: MTI, Michigan City, IN, USA). The number of fields of view analysed was 15–20, corresponding to an area of 0.1–0.147 mm² (one field ×100: 7330 mm²). The image features were displayed on a red-green-blue (RGB) monitor and stored for processing by the image analysis program we created and set on the Vidas 2.1 (Kontron Bildanalyse GmbH, Eching, Germany). In each case, positive and negative nuclei (mean: 114; range: 63–505) were selected on a blind basis and evaluated. The only selection criterion was the study of nuclei from longitudinal muscular fibres.

Progesterone receptor localization

Progesterone receptors were identified using a polyclonal antibody which does not distinguish between PRA and PRB (Santa Cruz®: Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1/200 dilution). Since an antibody specific to PRA is not available, all immunohistochemical analysis of PRA is by subtractive deduction. Thus, it is proposed that PRA is the subtype responsible for positive immunoreactivity when the PRB subtype is not specifically detected, as published previously (Wang et al., 1998). Progesterone receptor B was specifically identified using a polyclonal antibody with the same dilution ratio (Dako®, Dako, Glostrup, Denmark). In each case, positive and negative nuclei (mean: 100; range: 50–215) were evaluated.

Evaluation of steroid receptor content

The distribution of specific staining was thus evaluated according to an optical density scale using the values of all the positive nuclei of a given receptor in longitudinal myometrial fibres. The quantitative H-score (QH score) was calculated as follows: QH score = Σpi, where i is the optical density degree from 0 (negative nuclei) to 5 (high optical density) and P the percentage of stained cells for each given i (from 0 to 100%). This method is a modification of the semi-quantitative H-score analysis described (McCarty et al., 1985). This modification was earlier published by our group (Nisolle et al., 1994).

Results

Proliferative activity

Figure 1 illustrates Ki 67 immunostaining of muscular fibre nuclei of a leiomyoma and normal myometrium.

Figure 2 shows the results of the proliferation index in normal myometrium and intramural leiomyomata during the menstrual cycle and in submucosal myomata after GnRHa therapy.

In myometrium, the proliferation index was 0.98% (range 0–2.9) during the proliferative phase and 0.43% (0–2.4) during the secretory phase, but this cyclic change was not statistically significant.

In leiomyomata, there was an increase in the proliferation index during the secretory phase but it was not statistically significant. The proliferation index was 1.10% (range 0–4.9) during the proliferative phase and 1.72% (range 0–10.5) during the secretory phase.

When the proliferation index of normal myometrium was compared with that of leiomyoma, no differences were observed during the proliferative phase. During the secretory phase, the proliferation index was found to be higher in leiomyomata than in myometrium, but this was not statistically significant. In subgroup II, after 2 months of GnRHa therapy, the proliferation index of leiomyomata was 0.52% (range 0–3.0). This value was significantly lower (P < 0.05) than the values observed during both phases of the menstrual cycle.

Oestrogen receptor quantitative H-score

Figure 3 shows the ER content of normal myometrium and intramural leiomyomata during the menstrual cycle and of submucosal myomata after GnRHa therapy. During the proliferative phase, the mean ER QH-score was 23 ± 10 in myometrium and 66 ± 12 in leiomyomata. During the secretory phase, the mean ER QH-score was 24 ± 10 in myometrium.
and 40 ± 15 in leiomyomata. The difference observed between the QH-scores of leiomyomata and myometrium was significant (P < 0.01) during the proliferative phase but not during the secretory phase. In subgroup II, the QH-score was 79 ± 16, not different from the QH-score obtained in leiomyomata during both phases of the menstrual cycle.

**PRAB and PRB quantitative H-scores**

Figure 4 shows the PRAB and PRB content of normal myometrium and intramural leiomyomata during the menstrual cycle and of submucosal myomata after GnRH agonist therapy.  

**Progesterone receptors AB**

In myometrium, the mean PRAB QH-score was 255.7 ± 16.8 during the proliferative phase and 233 ± 21 during the secretory phase. In leiomyomata, the mean PRAB QH-score was 245 ± 26 during the proliferative phase and 262 ± 25 during the secretory phase. No significant changes in PRAB content were observed during the menstrual cycle. In subgroup II, the mean PRAB QH-score was 98 ± 24, significantly lower (P < 0.0002) than that observed in intramural leiomyomata during both phases of the normal menstrual cycle.

**Progesterone receptors B**

In myometrium, the mean PRB QH-score was 137 ± 16 during the proliferative phase and 161 ± 23 during the secretory phase. In leiomyomata, the PRB QH-score was 158 ± 22 during the proliferative phase and 176 ± 29 during the secretory phase. No significant changes were observed during the menstrual cycle with regard to PRB content in either tissue. In subgroup II, the mean PRB QH score was 36 ± 15, significantly lower [P < 0.0006 (proliferative phase);
Proliferation index and steroid receptors in leiomyomata

Figure 4. Values are mean ± SE. Progesterone receptor (PR) AB and PRB content (QH-score) of intramural leiomyomata and normal myometrium during both phases of the menstrual cycle and submucosal myomata after gonadotrophin-releasing hormone agonist (GnRHa) therapy. ⋆ Significantly higher than PRB content in leiomyomata during both phases of the menstrual cycle (P < 0.03). ★★ Significantly higher than PR content in myometrium during the proliferative phase (P < 0.0001) and the secretory phase (P < 0.02). ★ The PRAB QH-score of submucosal myomata was significantly lower than that observed in intramural leiomyomata during the menstrual cycle (both phases) (P < 0.0002). ★★ The PRB QH-score of submucosal myomata was significantly lower than that observed in intramural leiomyomata during the menstrual cycle (both phases) [P < 0.0006 (proliferative phase) and P < 0.002 (secretory phase)].

Several studies have strongly suggested that myoma growth can be related to progestogens. Back in 1985, it was shown (Tiltman, 1985) that women treated with progestogens before hysterectomy demonstrated significantly higher mitotic activity in their fibroids than women untreated or pretreated with a combined oestrogen–progestogen preparation. When GnRHa and medroxyprogesterone acetate (MPA) are co-administered, the expected regression in leiomyoma size usually observed with GnRHa alone is not achieved, as reported (Friedman et al., 1988). This was the first report to show a direct effect of progesterone on leiomyoma growth, but the mechanism by which MPA inhibits leuprolide-induced shrinkage was not known. The antiprogestogen RU 486 is just as effective as leuprolide acetate in decreasing both uterine volume and blood flow (Reinsch et al., 1994). Others (Harrisson-Woolrych et al., 1995) reported a dramatic increase in leiomyoma size in a menopausal woman taking a high dose of progesterone (megestrol acetate, 160 mg daily) and a good regression in size when the medication was stopped.

Experimental data support the clinical data. Indeed, progestins have been reported to increase uterine weight in rodents. High doses of progesterone combined with low doses of oestriol significantly increased uterine weight in ovariectomized rats when compared with low-dose oestriol treatment alone (Edgren et al., 1961). In castrated rabbits, a 19-nortestosterone progestin caused a significant increase in uterine weight when compared to treatment with vehicle solution (Elton and Edgren, 1958). Oestrogen and progesterone receptors are found within myometrium and fibroids (Wilson et al., 1980) and tissue cultures of both show significant growth when supplemented with progesterone.

All this led us to reconsideration of the role of progesterone...
in leiomyoma growth and reassessment of the traditional view, which holds that leiomyomata are only oestrogen dependent. It was suggested that progesterone might be an important factor in the growth of leiomyomata (Smith, 1993) and the use of an antiprogestin has been proposed as a treatment for leiomyomata (Murphy et al., 1993; Viville et al., 1997).

In this study, leiomyomata were found to contain an increased number of cycling (Ki 67 immunoreactive) cells when compared with myometrium throughout the menstrual cycle. Moreover, the proliferative activity in leiomyomata was greater during the secretory phase, suggesting, as have other reports (Kawagushi et al., 1991; Vu et al., 1998), that progesterone plays a role in the growth of leiomyomata. After GnRHa therapy, a significant decrease in proliferative activity compared to untreated leiomyomata was observed, implicating oestradiol in leiomyoma growth too. Recently, it was suggested (Vi et al., 1998) that the decrease in the number of cycling cells in GnRHa-pretreated leiomyomata plays a key role in myoma shrinkage. In that study, apoptosis was not observed, suggesting that the decrease in growth is mediated through a decrease in cellular proliferation. The current study confirms their hypothesis. According to the findings of in-vitro studies analysing the PR isoforms. Progesterone receptors belong to the superfamily of nuclear receptors. In humans, most authors assume the existence of two progesterone receptor isoforms and recognize both progesterone receptor subtypes A and B (PRAB), which have unique properties of PRA suggest a molecular rationale for their regulation. The two different promoters that are involved may play a role in the modulation of oestrogenic action.

On the contrary, in patients treated with GnRHa, a similar ER content was observed to that seen in untreated leiomyomata during the proliferative phase. A recent report (Englund et al., 1998) observed that during GnRHa treatment, the ER amounts in leiomyomata were similar to those observed in the proliferative phase but significantly higher than those found in the secretory phase. The results of this study could be interpreted as showing a loss of sensitivity to oestrogen suppression (an absence, in leiomyomata, of down-regulation of ER despite the reduced oestrogen concentrations observed after 2 months of GnRHa therapy). It has been shown (Circkel et al., 1994) that the greatest myoma shrinkage was associated with the highest ER content and the poorest reduction did not show any positive staining for ER at all. This association between myoma shrinkage and the ER status of the myoma explains the wide variation seen in myoma shrinkage.

By contrast, the results on the PR content of leiomyomata are more subject to discussion. In this study, PR persisted in both leiomyomata and adjacent myometrium during the secretory phase, as already observed (Lessey et al., 1988). This persistence of PR during the secretory phase in leiomyomata and myometrium could suggest that the regulation of PR is different from that observed in endometrial glandular epithelium. There is also growing evidence that PR are not down-regulated in a variety of situations in which sustained progesterone exposure may be associated with continued responsiveness to progesterone. This demonstrates that PR persist in cells that maintain progesterone responsiveness (Clarke and Sutherland, 1990). Another hypothesis is that PR are synthesized but their activity may be tissue-dependent. For instance, the persistence of PR in endometrial stromal cells and in a perivascular location during the secretory phase supports the role of progestin effects on spiral artery growth at this time (Wang et al., 1998).

Two different types of PR could be distinguished: one oestrogen-induced and one independent of the influence of peripheral oestrogens. This interrelation was investigated by analysing the PR isoforms. Progesterone receptors belong to the superfamily of nuclear receptors. In humans, most authors favour the existence of two original forms of PR: form B (100–120 kDa) and form A (94 kDa). The transcription of the two forms is controlled by two different promoters that are both oestrogen-induced (Gronemeyer et al., 1991). Under certain circumstances, PRA can repress PRB-mediated transcription by a mechanism that requires ligand binding but not DNA binding (Savouret et al., 1994). Hence, PRA can have a dual role as a transcription activator and repressor. The unique properties of PRA suggest a molecular rationale for the existence of two progesterone receptor isoforms and provide a mechanism by which a single hormone promotes different responses, but the exact role of each form remains unclear (Vegeto et al., 1993; Pfeifer and Strauss, 1996). Therefore, it is very important to establish the quantities of both forms (PRA and PRB) in leiomyomata and myometrium for a better understanding of leiomyoma growth and for future medical treatments.

In 1997, it was shown for the first time (Viville et al., 1997) that both forms of progesterone receptors (PRA and PRB) were present in leiomyomata, with higher concentrations of PRA and PRB in leiomyomata than in normal myometrium and with a constant dominance of PRA over PRB. As there was no difference between the concentrations of mRNA encoding PRA and PRB, they suggested post- translational control of progesterone receptors.

An immunohistochemical analysis of antibodies that recognize both progesterone receptor subtypes A and B (PRAB), and antibodies that recognize specifically subtype B (PRB) has been conducted. Indeed, an antibody specific to PRA is not available. The advantage of immunohistochemistry is maintaining the tissue architecture while allowing an assessment of the cellular distribution of receptors (Nisolle et al., 1994). The intensity of stained nuclei in leiomyomata and normal myometrium was determined by an advanced computer-
ized stereographic technology using image analysis (Nisolle et al., 1994).

In all cases in the present study, the PRAB and PRB content were found to be higher in leiomyomata than in normal myometrium but considerable variation was observed between individual cases. This variation shows the importance of comparing tissues (leiomyomata and myometrium) from the same patient. Similar results were observed when Western blotting analysis and quantitative analysis of mRNA were used (Brandon et al., 1993; Viville et al., 1997). Progesterone receptor immunoreactivity was significantly reduced in leiomyomata after GnRH administration compared with untreated tissues.

Three hypotheses have been proposed to explain the effect of progesterone on leiomyomata. Firstly, leiomyomata could escape oestrogen regulation that is present in normal tissue containing progesterone receptors. Secondly, leiomyomata may be more sensitive to oestrogen, leading to permanently high concentrations of oestradiol which could provoke a high PR content. Thirdly, increased expression of PR in leiomyomata could be the consequence of overexpression of functional ER, that results in increased organ sensitivity to oestradiol (Brandon et al., 1995). In conclusion, the high proliferation index and the high PRAB and PRB content of leiomyomata during the secretory phase suggests that progesterone may play a role in myoma growth and that there is a need for prospective studies on the use of an antiprogestin as a treatment for leiomyomata.

References
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