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Immunohistochemical analysis of oestrogen receptors, progesterone receptors and Ki-67 in leiomyoma and myometrium during the menstrual cycle and pregnancy

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Summary. Immunohistochemical distribution of oestrogen receptors (ER), progesterone receptors (PR), and the cell proliferation-associated antigen Ki-67 was investigated in leiomyomas and the myometrium during the menstrual cycle and pregnancy. In the myometrium, ER expression was observed in the proliferative phase, but was suppressed in the secretory phase and during pregnancy. In leiomyomas, ER expression was observed throughout the menstrual cycle, but was suppressed during pregnancy. However, PR was expressed both in the myometrium and leiomyomas throughout the menstrual cycle and pregnancy. In both the myometrium and leiomyomas, a higher number of Ki-67-positive cells was observed during pregnancy than in the secretory phase, and Ki-67 was negative during menopause. The Ki-67-positive cell count in leiomyomas was significantly higher than that in the myometrium throughout the menstrual cycle and pregnancy. Thus both myometrium and leiomyomas have high growth activity under the hormonal milieu of high progesterone levels. The growth potential of leiomyomas is apparently higher than that of myometrium throughout the menstrual cycle and during pregnancy.

Key words: Oestrogen receptor – Progesterone receptor – Ki-67 – Leiomyoma – Myometrium

Introduction

Leiomyomas are the most common tumours of the uterus and female pelvis and are active during the years of greatest ovarian activity. Following the menopause, with regression of ovarian steroid secretion, growth of leiomyomas usually ceases. Investigators have thus suggested that the growth of leiomyomas is dependent on oestrogen production (Tamaya et al. 1978; Otubu et al. 1982; Soules and McCarty 1982). However, pregnancy is also associated with a rapid increase in the size of leiomyomas and we have also demonstrated recently that increased mitotic activity in leiomyomas under the hormonal milieu of the secretory phase of the menstrual cycle suggests that the growth of these tumours is affected by progesterone levels (Kawaguchi et al. 1989). It is therefore of interest to study the effects of both oestrogen and progesterone on the development of this tumour.

Studies on the concentration of cytosol oestrogen and progesterone receptors demonstrated that their levels are similar in leiomyomas and in adjacent myometrium (Tamaya et al. 1978; Wilson et al. 1980; Soules and McCarty 1982). However, when total cellular (cytosol plus nuclear) receptor levels are measured (Tamaya et al. 1985), there appear to be more oestrogen receptors (ER) in leiomyomas than in myometrium. The ratio of oestrogen to progesterone receptors (PR) is also significantly higher in leiomyomas than in normal myometrium (Tamaya et al. 1985), suggesting that the relative concentrations of these receptors may influence the growth of leiomyomas. However, it is difficult to predict the growth activity of leiomyomas from these studies, since cell kinetics of the tumour cells and myometrial smooth muscle cells have not been studied simultaneously with receptor levels.

The relatively recent production of monoclonal antibodies against a specific ER protein (Greene and Jensen 1982) and a PR protein (Greene et al. 1988) has provided a new histochemical approach for localization of ER and PR (Iwai et al. 1990). Using this method, the histochemical expression of ER and PR in leiomyoma cells and myometrial smooth muscle cells throughout the menstrual cycle and during pregnancy was anlaysed. In addition, the relationship between the expression of these steroid receptors and the cell kinetics of these cells was investigated using a monoclonal antibody, Ki-67,

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which recognizes a protein that is present only in the nucleus of cycling cells, but not in resting cells (Gerdes et al. 1984). The role of ovarian steroids in the growth of uterine leiomyomas was thus examined.

Materials and methods

Hysterectomy specimens from 40 women with typical leiomyomas were selected for study. Donors were women (age 31–49 years) who had regular menstrual cycles (28–30 days) and who had received no exogenous hormones for at least two cycles prior to surgery. There were also 6 pregnant women (age 27–48 years; 8–38 weeks' gestation). The 6 post-menopausal women had also received no exogenous hormones for several months prior to surgery. The menstrual cycle was estimated histologically according to the method of Noyes et al. (1950). Among the 34 uteri from non-pregnant women, 12 were in the proliferative phase, 16 were in the secretory phase, and 6 were in the menopause. Women with malignancies or smooth muscle tumours such as cellular leiomyoma were excluded from this study.

Leiomyoma, myometrial and endometrial tissues were cut immediately into small sections, with three to four pieces being quickly frozen in OCT compound (Ames, Elkhart, Ind.) and stored at −70° C for 1–7 days until use. The remaining tissues were routinely processed for light microscopy.

The immunohistochemical staining procedure for ER and PR was performed using ER-ICA and PR-ICA monoclonal kits (Abbott, Chicago, Ill.) according to the manufacturer's instructions. Briefly, thin cryostat sections mounted on glass slides were placed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. After being rinsed in PBS for 5 min, they were immersed in 100% methanol (4° C) for 5 min, and finally in acetone (4° C) for 3 min. After fixation, these slides were incubatd with normal goat serum to reduce the non-specific binding of the primary antibody. Slides were incubated with anti-ER monoclonal antibody or anti-PR monoclonal antibody or control rat IgG for 30 min at room temperature, followed by treatment with goat anti-rat IgG antiserum and peroxidase-antiperoxidase complex (PAP). Finally, the slides were treated with diaminobenzidine (1.2 mg/ml) in TRIS buffer solution containing 0.02% hydrogen peroxide for 6 min. Immunohistochemical staining for Ki-67 was performed with the Histoscan monoclonal detector ABC kit (Biomeda, Foster City, Calif.). Air-dried thin cryostat sections mounted on slide glasses were immersed in acetone (−20° C) for 10 min. After rinsing in PBS, they were treated with normal goat serum followed by incubation with anti-proliferation associated antigen Ki-67 antibody (Dakopatts, Copenhagen, Denmark) or control rat IgG for 30 min at room temperature. Specimens were then treated with biotinylated goat anti-rat IgG antiserum, avidin-biotin peroxidase complexes and stained with diaminobendizine (0.4 mg/ml in 0.5% hydrogen peroxide) for 10 min. Counterstaining was performed with 3% methyl green.

Specific staining was observed as brown-coloured granules, and the control slides treated with control antibody yielded negative results. For positive ER and PR controls, cryostat sections of endometrial tissues in the proliferative and secretory phases, and commercially prepared slides with ER-positive and PR-positive cells were used. The intensity of staining for ER and PR was evaluated by staining the same specimens several times and by the observations of more than two observers. Intensity was graded as (−) for no immunostaining, (+) for weak and (++) for strong immunostaining. The number of Ki-67-positive cells in ten consecutive high-power fields (×40 objective, ×15 eyepiece) was counted. Areas of degeneration were excluded. When multiple leiomyomas were available within one uterus, all were examined and the section yielding the highest number of Ki-67-positive cells in ten consecutive high-powerfields was used in this study. Sections with fewer than ten available high-power fields were excluded.

Histological diagnosis of the specimens was made using both cryostat sections and routinely processed haematoxylin- and eosin stained sections. Statistical analysis of data in this study was performed by Student's *t*-test.

Results

Specific staining with anti-ER antibody, anti-PR antibody, and antibody Ki-67 was confined exclusively to the nuclei. The endometrial tissues and commercially supplied ER-positive and/or PR-positive cells for positive controls were also characterized by positive nuclear staining for both ER and PR.

In the proliferative phase of the menstrual cycle both myometrial smooth muscle cells and leiomyoma cells showed positive staining for ER (Fig. 1A) and PR (Fig. 2A). However, the intensity of reactivity for anti-ER in leiomyoma cells was stronger than that of myometrial smooth muscle cells (Fig. 1A, Table 1). The intensity of reactivity for anti-PR in leiomyoma cells seemed to be stronger than that of myometrial smooth muscle cells, but the difference was not distinct.

Myometrial smooth muscle cells rarely showed Ki-67 positivity, but several leiomyoma cells were positive for Ki-67. The mean number of Ki-67-positive cells in leiomyoma (5.68 ± 2.00 mean $\pm$ SEM) was significantly higher than the count in myometrium (0.31 ± 0.21 mean $\pm$ SEM) ($P < 0.05$) (Table 1, Fig. 3).

In the secretory phase myometrial smooth muscle cells showed weak to negative staining for ER (Fig. 4A). In contrast, leiomyoma cells showed positive staining for ER (Fig. 4B). Both myometrial smooth muscle cells and leiomyoma cells showed positive staining for PR (Fig. 5A, B). The intensity of reactivity for anti-PR in leiomyoma cells seemed to be stronger than that of myometrial smooth muscle cells, but the difference was not distinct.

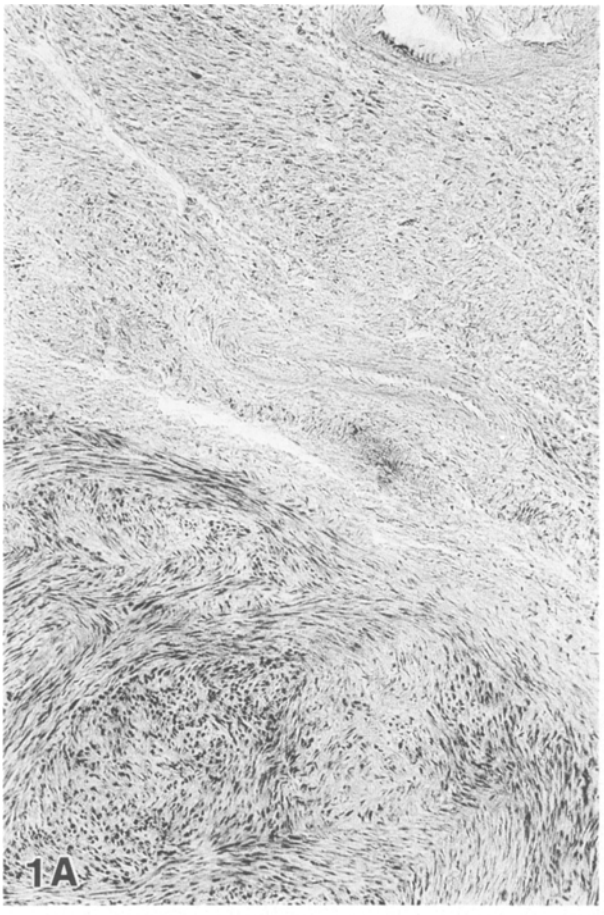
A few myometrial smooth muscle cells showed Ki-67 positivity (1.85 ± 0.93, mean $\pm$ SEM). In contrast, the number of Ki-67-positive cells increased in leiomyoma (18.29 ± 4.79, mean $\pm$ SEM) (Fig. 6A). The Ki-67-positive count in leiomyoma ($P < 0.01$) was significantly higher than the count in myometrium. In addition, the Ki-67-positive count for leiomyoma in the secretory phase was significantly higher than that of the proliferative phase ($P < 0.05$) (Table 1, Fig. 3).

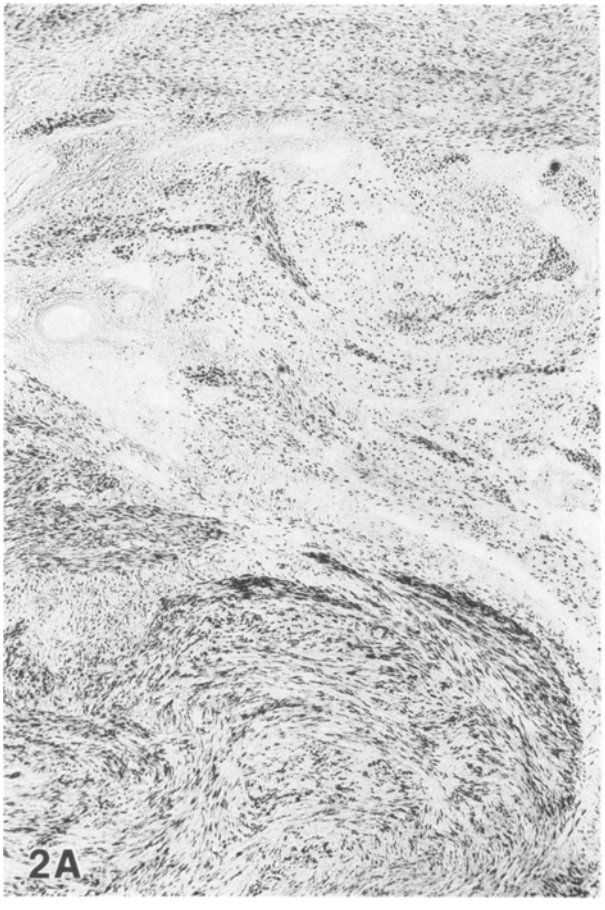
In menopausal patients both myometrial smooth muscle cells and leiomyoma cells showed positive staining for ER and PR (Table 1). A difference in staining intensity was not observed between these tissues.

Ki-67-positive cells were not observed in either myometrium or in leiomyoma (Table 1, Fig. 3).

During pregnancy both myometrial smooth muscle cells and leiomyoma cells showed negative staining for ER, but positive staining for PR.

The number of Ki-67-positive cells increased both in myometrium (5.61 ± 1.39, mean $\pm$ SEM) and leiomyoma ($44.75 \pm$ SEM) (Fig. 3). The Ki-67-positive count in leiomyoma was significantly higher than the count in myometrium during pregnancy ($P < 0.05$). In addition, the Ki-67-positive count in leiomyoma during pregnancy





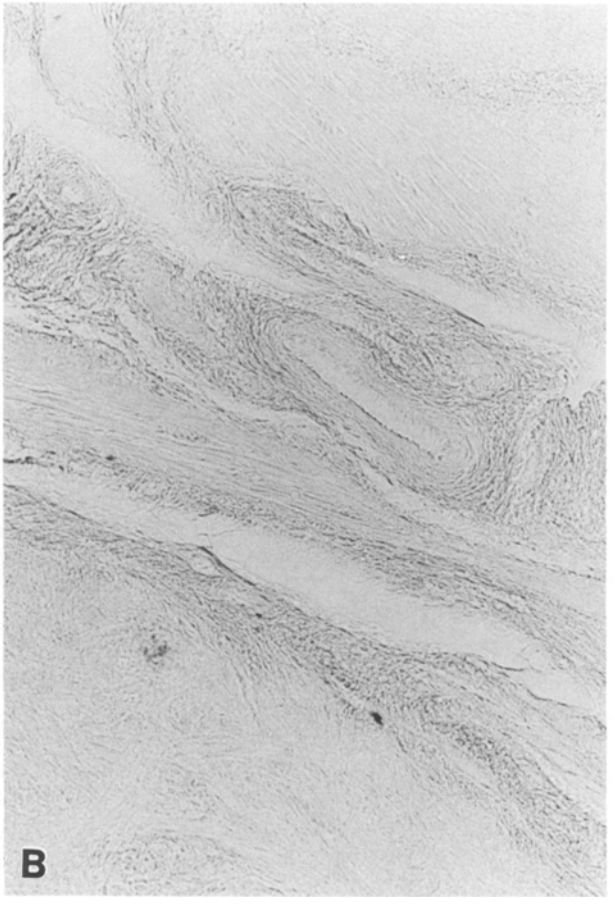


Fig. 1. A Immunohistochemical staining for oestrogen receptors (ER) in the proliferative phase, demonstrating nuclear staining for ER in both leiomyoma cells and myometrial smooth muscle cells. The intensity of reactivity for anti-ER in leiomyoma cells is stronger than that of myometrial smooth muscle cells. **B** Control. ×100

Fig. 2. A Immunohistochemical staining for progesterone receptions (PR) in the proliferative phase, demonstrating nuclear staining for PR in both leiomyoma cells and myometrial smooth muscle cells. **B** Control. ×100

Table 1. Oestrogen (ER) and progesterone receptor (PR) expression, and the mean count of Ki-67-positive cells in myometrium and leiomyomas during the menstrual cycle and pregnancy

	Myometrium				Leiomyoma			
	Prolife-rative phase	Secretory phase	Menopause	Pregnancy	Prolife-rative phase	Secretory phase	Menopause	Pregnancy
ER	+	−~+	+	−	++	+	+	−~+
PR	+	+	+	+	+	+	+	+
Ki-67[a]	0.31±0.21	1.85±0.93	0.00	5.61±1.39	5.68±2.00	18.29±4.79	0.00	44.75±11.93
Number of cases	12	16	6	6	12	16	6	6

Grading by intensity: −, not detectable; +, weak; ++, strong staining

[a] The count in ten consecutive high-power fields, mean±SEM

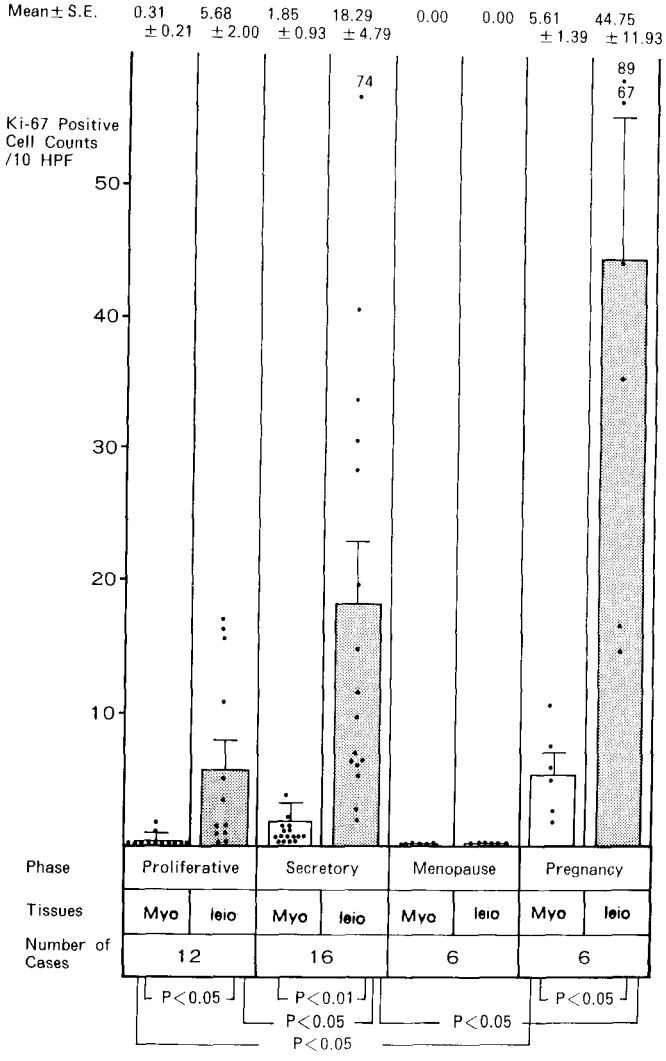


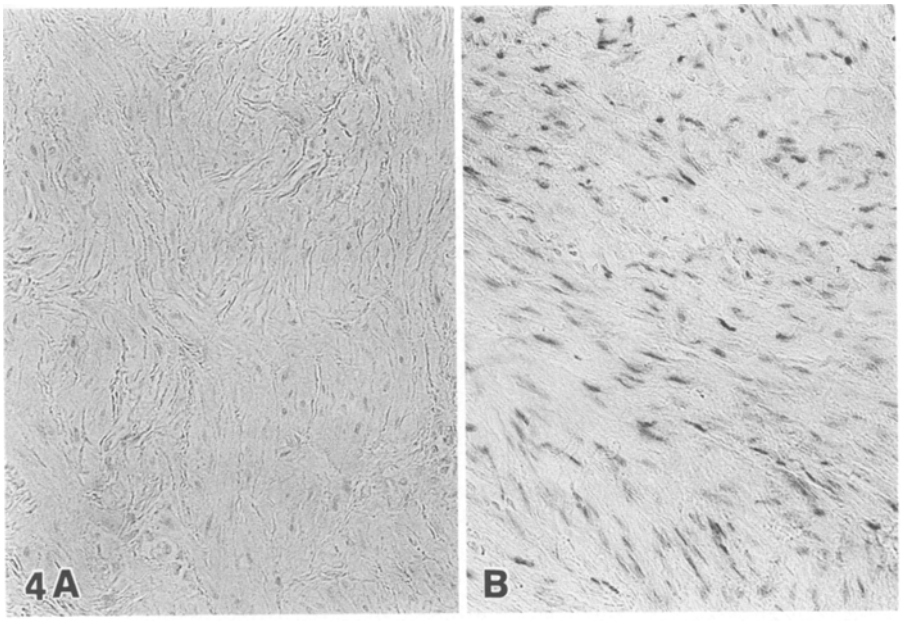
Fig. 3. Ki-67-positive cell counts per ten consecutive high-power fields in myometrium and leiomyomas during the menstrual cycle and pregnancy. Myo = Myometrium, leio = leiomyoma

was signifcantly higher than that of the secretory phase ($P<0.05$). The Ki-67-positive count in myometrium during pregnancy was significantly higher than that of the proliferative phase ($P<0.05$) (Table 1, Fig. 3). In addition, Ki-67-positive cells were observed frequently in leiomyoma (Fig. 6B, C) and rarely in myometrium at 38 weeks of gestation.

Discussion

The current immunohistochemical study demonstrates that ER is expressed in the proliferative phase in myometrial smooth muscle cells; suppression of ER expression occurs in the secretory phase and during pregnancy. This is consistent with previous reports that oestrogen stimulates both ER and PR synthesis, and progesterone suppresses both ER and PR synthesis in the endometrium, oviduct, vagina, anterior pituitary, hypothalamus, and other target organs (Katzenellenbogen 1980; Leavitt et al. 1983). However, PR is consistently expressed in myometrial smooth muscle cells throughout the menstrual cycle and during pregnancy. In myometrial smooth muscle cells, it appears that PR expression is not distinctly suppressed by progesterone in the secretory phase and during pregnancy. This pattern of PR expression is comparable to the results of an immunohistochemical study of endometrial stromal cells which were positive for PR during the late secretory phase Bergeron et al. 1988). Consequently, myometrial smooth muscle cells undergo immunohistochemical changes in nuclear ER expression during the menstrual cycle and pregnancy under the hormonal milieu of high progesterone levels, but this is not distinct with regard to nuclear PR.

Both ER and PR are also expressed in leiomyomas. However, ER expression persists throughout the menstrual cycle, and distinctly disappears during pregnancy. It appears that ER expression is not suppressed in the secretory phase. However, the disappearance of ER during pregnancy suggests that the suppressive mechanism for ER by progesterone is not impaired but that high levels of progesterone are necessary to suppress ER expression in leiomyomas. In addition, leiomyoma cells in the proliferative phase show a stronger affinity for ER antibody than myometrium. This suggests that ER is over-expressed in leiomyomas and that the progesterone levels in the secretory phase are insufficient to suppress ER expression. In contrast, leiomyoma cells con-



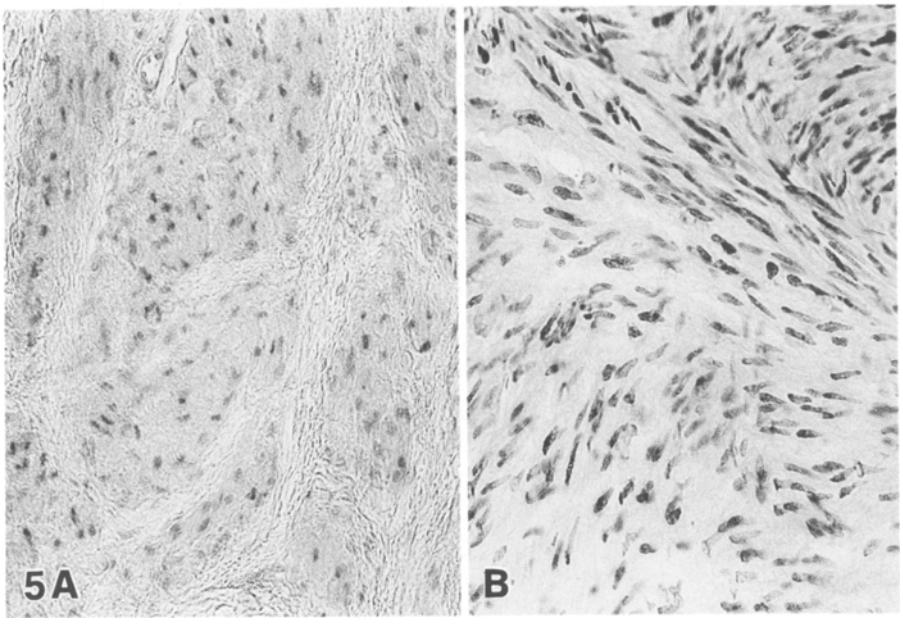


Fig. 4. Immunohistochemical staining for ER in the secretory phase, demonstrating negative nuclear staining for ER in myometrial smooth muscle cells (**A**) and positive nuclear staining in leiomyoma cells (**B**). ×100

Fig. 5. Immunohistochemical staining for PR in the secretory phase, demonstrating nuclear staining for PR in both myometrial smooth muscle cells (**A**) and leiomyoma cells (**B**). ×200

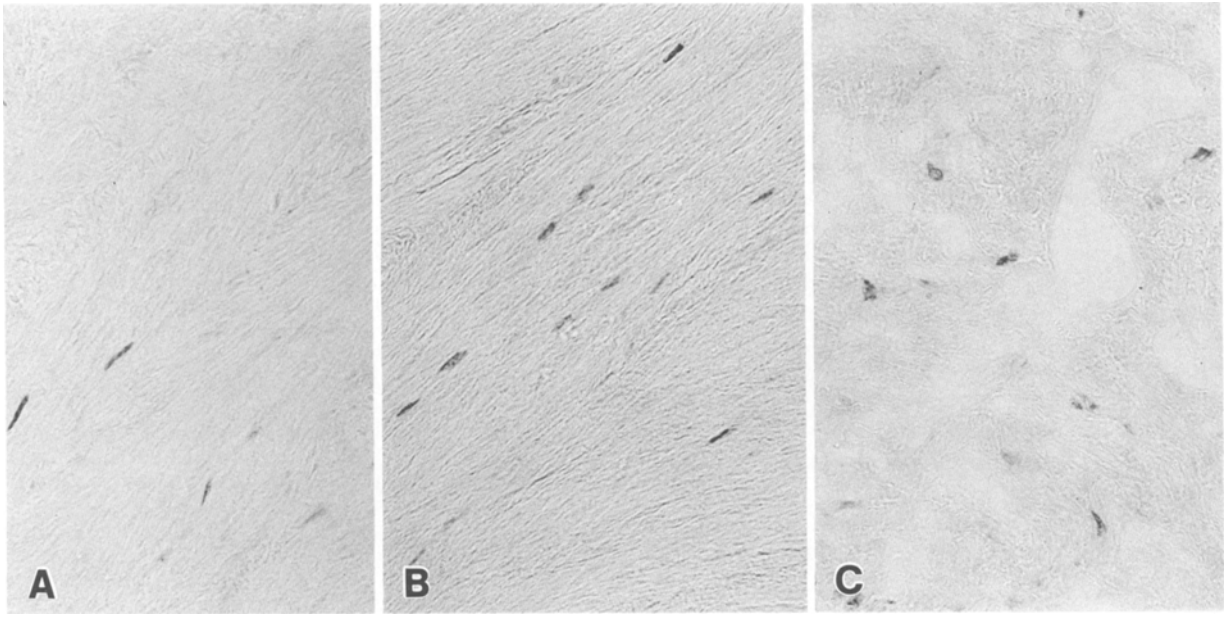


Fig. 6. Immunohistochemical staining for Ki-67 demonstrating nuclear staining in leiomyoma in the secretory phase (**A**), at 20 weeks of gestation (**B**), and at 38 weeks of gestation (**C**). ×200

tain PR in their nuclei throughout the menstrual cycle and during pregnancy as well as in in myometrial smooth muscle cells. The intensity of reactivity for anti-PR in leiomyoma cells seemed to be stronger than that of myometrial smooth muscle cells, but it was not distinct. Therefore, immunohistochemistry for PR did not reveal a difference in PR levels between leiomyomas and myometrium.

Biochemical measurements of ER and PR levels in leiomyomas (Pollow et al. 1978; Tamaya et al. 1985) have shown that both ER and PR levels in leiomyomas are higher than those of myometrium, but others found no difference in receptor contents (Tamaya et al. 1978; Wilson et al. 1980; Soules and McCarty 1982). These conflicting results are probably due to the biochemical methods employed to measure the levels of these receptors in the cytosol, since recent immunohistochemical studies using monoclonal antibodies to ER and PR have revealed that both steroid receptors are present in nuclei but not in the cytosol (Welshons et al. 1984). However, when total cellular (cytosol plus nuclear) receptor levels are measured, there appears to be more ER in leiomyomas than in myometrium, and the ratio of ER to PR is significantly higher in leiomyomas than in normal myometrium (Tamaya et al. 1985). Our immunohistochemical study revealed a difference in staining intensity for ER between leiomyoma and myometrium, but not for PR. Although it is not quantitative, these results suggest that leiomyomas contain more ER than myometrium and the ratio of ER to PR is higher in leiomyomas than in myometrium. This unusual expression of ER may be one of the neoplastic characteristics of leiomyoma cells.

Oestrogen is believed to infuence the growth of leiomyomas as well as myometrial smooth muscle cells. Therefore, unusually high ER expression may influence the growth of leiomyomas. However, to elucidate the influence of ovarian steroids on the growth of leiomyoma, it is necessary to know the cell kinetics of tumour cells during the menstrual cycle and in pregnancy. Recently, a monoclonal antibody Ki-67, which recognizes cellular protein present only in the nucleus of cycling cells, but not resting cells, has been widely used for quick detection of cycling cells without using any DNA precursors (Gerdes et al. 1984). The results using antibody Ki-67 for myometrial smooth muscle cells reveal that the cycling cells are scarce in the proliferative phase, but that they increase slightly in the secretory phase and significantly during pregnancy. In addition, Ki-67 is not expressed in the myometrium of menopause. These results imply that myometrial smooth muscle cells rarely enter the cell cycle under the oestrogen levels in the proliferative phase, but that they can enter the cell cycle under the influence of both oestrogen and progesterone particularly during pregnancy. In addition, this suggests that myometrial smooth muscle cells possibly grow in the secretory phase and during pregnancy and cease to grow during menopause.

In contrast, leiomyomas contain a significantly higher number of cycling cells than myometrium during the menstrual cycle and pregnancy. This implies that leiomyomas grow more rapidly than myometrium. In addition, leiomyomas show significant differences in counts of cycling cells during the menstrual cycle and pregnancy. The highest count is during pregnancy and then in the secretory phase. Leiomyomas in the menopause contain no cycling cells. The significantly higher count of cycling cells in the secretory phase than in the proliferative phase is consistent with our previous report (Kawaguchi et al. 1989) on mitotic activity in leiomyomas during the menstrual cycle. Therefore, the hormonal milieu in the secretory phase and particularly during pregnancy, possibly progesterone, seems to be helpful for the growth of leiomyomas. At present, progesterone becomes a factor which has an important role in the growth of leiomyomas. However, it is unlikely that progesterone alone has a growth effect on leiomyoma cells, because in media containing progesterone alone, both leiomyoma and myometrial smooth muscle cells failed to increase in number in vitro (Kawaguchi et al. 1985). Therefore, the presence of oestrogen or some other, as yet unidentified, growth factor is probably necessary for the growth of leiomyomas.

In conclusion, our current observations suggest strongly that the progenitor cells of leiomyomas which probably reside in the myometrium (Konishi et al. 1983) begin to grow after menarche, and thrive during the years of greatest ovarian activity under the hormonal influence of both oestrogen and progesterone, and following the menopause, with regression of ovarian steroids, growth of leiomyomas usually ceases. However, further study is needed to clarify why progesterone causes increased cycling of cells in the myometrium and in uterine leiomyomas during the secretory phase and pregnancy.

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