Virchows Archiv A Pathol Anat (1993) 423:257–263

Virchows Archiv A
Pathological Anatomy and Histopathology

The number of proliferating cell nuclear antigen positive cells in endometriotic lesions differs from that in the endometrium

Analysis of PCNA positive cells during the menstrual cycle and in post-menopause

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Received April 13, 1993 / Received after revision July 24, 1993 / Accepted July 26, 1993

Abstract. Immunohistochemical expression of proliferating cell nuclear antigen (PCNA) was studied in the endometrium and in endometriotic lesions during the menstrual cycle and in post-menopausal patients. During the menstrual cycle, in the basal layer of the endometrium, an increase in the number of positive indices (PI) of PCNA was observed in epithelial cells from the menstrual phase. It reached a maximum in the proliferative phase and decreased in the secretory phase. However, no change was observed in the stromal cells of the basal layer. In the functional layer of the endometrium, the PI of the epithelial cells showed a high peak in the late proliferative phase, decreased sharply in the secretory phase and remained unchanged thereafter. The PI of the stromal cells in the functional layer showed two peaks, one in the late proliferative and the other in the mid and late secretory phase. In the endometriotic lesions, except for the proliferative phase, the number of PI was significantly higher than that of the corresponding endometrium and no significant changes were observed during the menstrual cycle. In post-menopausal endometriotic lesions, the number of PI was also higher than that of the corresponding endometrium. Thus the numbers of PI differed between the endometrium and endometriotic lesions in the same patients. These results imply that the endometriotic lesions are constantly more proliferative than the endometrium irrespective of the hormonal milieu during both the menstrual cycle and in a post-menopausal environment.

Key words: Proliferating cell nuclear antigen – Endometrium – Endometriosis – Menstrual cycle – Menopause

Introduction

Endometriosis is one of the most commonly encountered diseases in gynaecology, and it is usually connected with infertility. The disease is defined as the presence of endometriotic epithelium and stroma in ectopic sites. This is believed to proliferate and differentiate under the influence of ovarian steroids like the eutopic endometrium (Vierikko et al. 1985; Gerbie and Merrill 1988). However, the characteristics of endometriosis seem to be different from those of the eutopic endometrium, as suggested by ultrastructural and sex steroid receptor expression studies (Lyndrup et al. 1987; Lessey et al. 1989; Schweppe 1989). The endometriotic tissues usually grow more or less expansively into the surrounding peritoneum as well as into other organs. Although the disease is not malignant, it often behaves in a tumour-like manner (Bergqvist et al. 1991). However, we have not found any reports describing the proliferative activity of endometriotic lesions.

Proliferating cell nuclear antigen (PCNA), a highly conserved auxiliary protein of DNA polymerase δ, is synthesized during the late G_1 and S-phases of the cell cycle correlating with proliferation (Celis et al. 1984; Fairman 1990). A good correlation of labelling indices has been found between PCNA and Ki-67, a monoclonal antibody that recognizes the nuclei of proliferative cells (Hall et al. 1990; Kamel et al. 1991). A linear correlation has also been demonstrated between PCNA indices and S-phase fractions as determined using bromodeoxyuridine (Wolf and Michalopoulos 1992). PCNA immunohistochemistry has become a valuable tool with which to define proliferative activity in diagnostic pathology because it can be performed on formalin-fixed tissues.

Using this procedure, we compared the proliferative indices between endometriotic tissue and its corresponding eutopic endometrium during the menstrual cycle and in post-menopausal patients, to clarify the proliferative activity of endometriotic lesions.

Materials and methods

Histological materials (endometrium and endometriotic lesions) were obtained from 48 women who had undergone hysterectomy and salpingo-oophorectomy because of leiomyoma of the uterus

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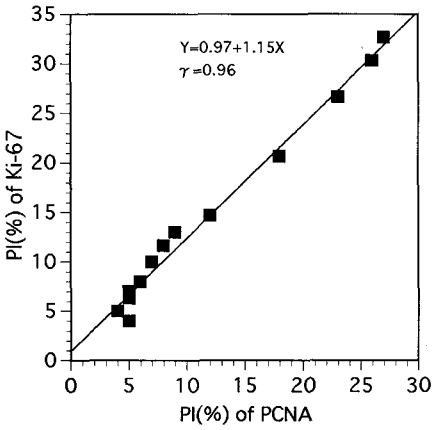


Fig. 1. The relationship between the positive indices (PI) of proliferating cell nuclear antigen (PCNA) and that of Ki-67 in the serial sections of epithelial component of 13 endometriotic lesions. There is a linear relationship between the PI of PCNA and that of Ki-67

and endometriosis. Samples were excluded if accurate menstrual cycle dates could not be assigned or if unexpected pathology was found (such as endometrial hyperplasia). All specimens were fixed in 10% buffered formalin and embedded in paraffin. Serial sections were stained with haematoxylin and eosin for light microscopic examination. The endometrial tissues were dated according to the method of Noyes et al. (1950). The menstrual cycle was divided into six categories: menstrual (days 1–5), early proliferative (days 6–9), late proliferative (days 10–14), early secretory (days 15–21), mid-secretory (days 22–24) and late secretory (days 22–28) phases.

Immunohistochemical PCNA staining was performed using labelled streptavidin-biotin, and the Dako LSAB monoclonal detector kit (Dakopatts, Copenhagen, Denmark) according to the manufacturer's instructions. In brief, the sections were dewaxed in xylene, rehydrated through graded alcohol and treated with 0.3% hydrogen peroxide to block endogenous peroxide activity, and incubated with normal goat serum to reduce non-specific binding. The sections were incubated with PCNA antibody (PC10, Dako) at a dilution of 1:20, or with control normal mouse serum at 4° C overnight. Biotinylated goat anti-mouse IgG was used as the linker. After washing, the streptavidin complex was applied, stained with diaminobenzidine and then counterstained with methyl green.

The PCNA positive cells were counted in the basal and functional layer of the endometrium separately. For each section, at least three fields were selected randomly, 100 epithelial and stromal cells in each field were selected and the positive indices (PI) were expressed as the number of positive-cells per 100 cells. In endometriosis, the pathological features were complex; 52% (25/48) of the lesions had both glandular and endometrial-type stromal structures, whereas the other 48% (24/48) had glandular structures but they were surrounded by a few endometrial-type stromal cells intermingled with red blood cells, haemosiderin-laden macrophages, and/or histiocytes. The latter lesions were apparently differentiated from the lesions of mesothelial inclusion. The PCNA positive cells were counted in the epithelial component of all lesions, but they were counted in the stromal component of 25 lesions which had typical endometrial-type stromal cells, and PI was obtained.

In order to confirm the results of PCNA, using the monoclonal antibody Ki-67 (MIB–1 antibody; Immunotech S.A., France) that also recognizes the nuclei of proliferative cells, immunohistochemical Ki-67 staining was also performed in the serial sections of endometriotic lesions (13 cases) processed in the microwave oven. And Ki-67 positive cells were counted in the epithelial component of the endometriotic lesions. The PI of Ki-67 was a little higher (mean: 14.62) than that of PCNA (mean: 11.69), however, a linear relationship ($r=0.96$) between the PI of Ki-67 and PCNA was observed (Fig. 1). Therefore, only the results of PCNA were analysed in this study.

The data were statistically analysed using Student's t-test. A difference between groups was considered significant if P was <0.05.

Results

Specific staining with the PCNA antibody was exclusively confined to the nuclei. The age and endometrial conditions of the 48 patients are shown in Table 1, and the positive indices (PI) of PCNA in the endometrium and endometriotic lesions are shown in Table 2.

Figure 2A shows cells positively stained for PCNA in the basal layer. The epithelial cells showed an increase of positive staining for PCNA from the menstrual phase (3.67 ± 2.57%: mean ± S.D.), then reached a maximum during the early proliferative phase (7.10 ± 2.26%: mean ± S.D.) and maintained the high level in the late proliferative phase (6.88 ± 2.37%: mean ± S.D.). The PI decreased sharply, reaching a very low level in the secretory phase (PI < 2%). The stromal cells showed only a slight increase of PI in the late proliferative phase (3.33 ± 2.45%: mean ± S.D.), then a very low level was revealed in the late secretory phase (less than 2%). Thus, a significant change was not observed in the stromal cells during the menstrual cycle.

Figure 3A–C show cells positively stained for PCNA in the functional layer. The PI of epithelial cells in the functional layer increased from the early proliferative phase (7.80 ± 2.26%: mean ± S.D.), reaching a maximum in the late proliferative phase (12.09 ± 2.45%: mean ± S.D.) (Fig. 3A), after which it decreased sharply in the late secretory phase (PI < 2%) (Fig. 3B). The PI of the stromal cells increased from the early proliferative phase (6.02 ± 2.60%: mean ± S.D.), was high in the late proliferative phase (13.25 ± 2.46%: mean ± S.D.) (Fig. 3A), decreased in the early secretory phase (4.02 ± 2.30%: mean ± S.D.), then sharply increased again in the mid (12.80 ± 4.36%: mean ± S.D.) and late secretory phases (13.68 ± 4.58%: mean ± S.D.) (Fig. 3B, 3C). In the late secretory phase, stromal cells that transformed into predecidual cells showed a highly positive rate of PCNA staining. In the secretory phase, the stromal cells in the

Table 1. Age distribution of the patients and stage of the menstrual cycle

Days	Menstrual cycle						
	1–5	6–9	10–14	15–21	22–24	25–28	Post-menopause
Subjects No.	6	10	8	5	7	5	7
Mean Age (years)	41.5	40.0	44.3	42.4	45.0	45.2	59.8

Table 2. PCNA positive cell index (%: mean ± SD) in the endometrium and endometriotic lesions. Classification of endometriotic lesions is not related to their histological features, but the menstrual dating of the corresponding endometrium

Day	Menstrual cycle						
	1–5	6–9	10–14	15–21	22–24	25–28	Post-menopause
Endometrium							
Epithelium (Basal layer)	3.67 ± 2.57	7.10 ± 2.26*	6.88 ± 2.37*	1.92 ± 1.57	1.00 ± 1.57	1.10 ± 2.02	0.36 ± 2.36
Stroma (Basal layer)	1.25 ± 1.35	2.75 ± 2.01	3.33 ± 2.45	1.35 ± 2.06	1.80 ± 2.54	0.80 ± 1.05	0.80 ± 1.84
Epithelium (Functional layer)		7.80 ± 2.26*	12.09 ± 2.45*	2.00 ± 1.81	1.07 ± 0.67	0.50 ± 2.67	
Stroma (Functional layer)		6.02 ± 2.60	13.25 ± 2.46*	4.02 ± 2.30	12.80 ± 4.36*	13.68 ± 4.58*	
Endometriotic Lesion							
Epithelium	9.80 ± 2.07**	11.60 ± 2.47	14.40 ± 4.75	11.56 ± 2.50**	10.68 ± 2.36**	10.88 ± 2.34**	5.62 ± 2.37**
Stroma[a]	7.75 ± 3.18 (*n* = 4)	8.40 ± 2.76 (*n* = 5)	9.50 ± 3.18 (*n* = 4)	5.00 ± 4.03 (*n* = 3)	7.29 ± 4.03 (*n* = 4)	5.00 (*n* = 1)	6.85 ± 3.18 (*n* = 4)

[a] PI was counted in the 25 cases which had typical endometrial-type stromal cells

* Significantly high between the respective PI during the menstrual cycle and in post-menopause

** Significantly high between the PI in the epithelium of the endometrium and the PI in the epithelium of endometriotic lesions

upper two-thirds of the functional layer (the compact layer) were stained more intensely for PCNA than the lower one-third of the functional layer (spongiosa layer) (Fig. 3B, C). The endothelial cells of the artery were also positively stained for PCNA in the mid to late secretory phase (Fig. 3C).

In post-menopausal tissue the endometrium contained considerably less cells positively stained for PCNA. The PI was 0.36 ± 2.36% (mean ± S.D.) in the epithelium and 0.80 ± 1.84 (mean ± S.D.) in the stroma (Fig. 4).

Forty-six endometriotic lesions were observed in specimens from 48 patients. They were distributed in the ovary (45), the oviduct (7), the uterine serosa (3), and the pelvic peritoneum (3). The histological features of endometriotic lesions did not always seem to be correlated with the corresponding endometrium. The dating of endometriotic lesions was difficult if the stromal cells were replaced by red blood cells and/or haemosiderin-laden macrophages. Therefore, we abandoned the attempt to date endometriotic lesions. Figures 5 and 6 show the immunohistochemical expression of PCNA in endometriotic lesions. The histological features of the endometriotic lesions varied, some lesions contained both glandular and stromal cells (Fig. 5A, ovary; B, oviductal peritoneum), which were intensively stained for PCNA. Some lesions had only the glandular structure surrounded by red blood cells and haemosiderin-laden macrophages, and these glandular cells were also stained positively (Fig. 6C, in the pelvic peritoneum). Table 2 demonstrates the comparison of PI in the endometrium and endometriotic lesions during the menstrual cycle and in post-menopausal samples. The PI in the epithelial cells of the endometriotic lesions was higher than that of the endometrium during the menstrual cycle. No significant PI fluctuation was observed in the endometriotic lesions during the menstrual cycle, although the highest level (14.40 ± 4.75%: mean ± S.D.) occurred in the late proliferative phase and the lowest (9.80 ± 2.07%: mean ± S.D.) in the menstrual phase. The comparison of PI between the endometrium and the endometriotic lesions revealed that except for the proliferative phase, the PI of the endometriotic epithelial cells was significantly higher than that of the endometrium. The PI in the stromal cells were counted in the 25 endometriotic lesions. There was no significant change during the menstrual cycle.

In post-menopausal tissues, the endometriotic lesions were also intensely stained for PCNA (Fig. 6A, B). Among 7 post-menopausal endometriotic lesions, 3 had a high positive rate of PCNA staining. In general, the PI of the endometriotic lesions in post-menopause was higher than that of the corresponding endometrium.

Discussion

Using immunohistochemical staining, we compared the PCNA positive rate in the normal endometrium and endometriotic lesions.

In the basal layer of the endometrium, the PI of the epithelial cells increased from the menstrual phase, reached a peak in the early proliferative phase, and decreased in the secretory phase, while the stromal cells showed only a slight increase in the late proliferative phase, with no significant changes during the menstrual cycle. PI in the epithelium of the basal layer increased earlier than in the stroma cells. Previous studies have shown that repair of the endometrium after menses occurs by regeneration of the gland stumps of the residual spongiosa or the persistent surface epithelium adjacent to the periphery-denuded endometrium (Ferenczy et al.

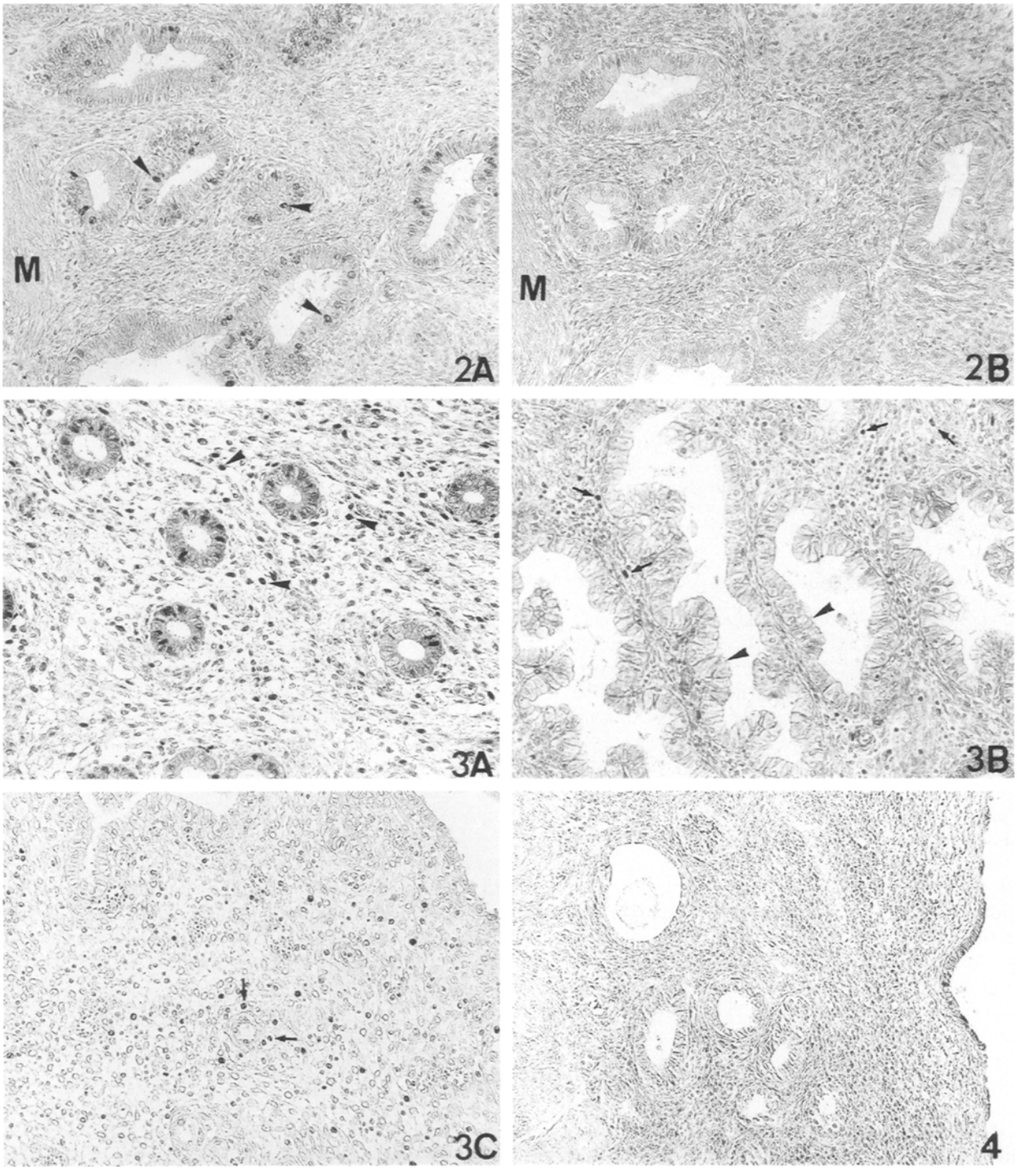
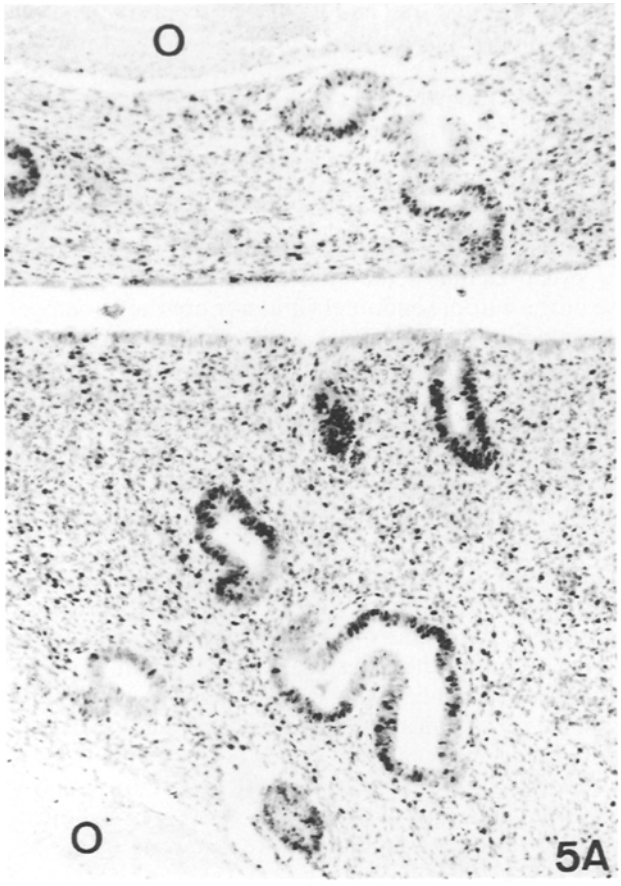
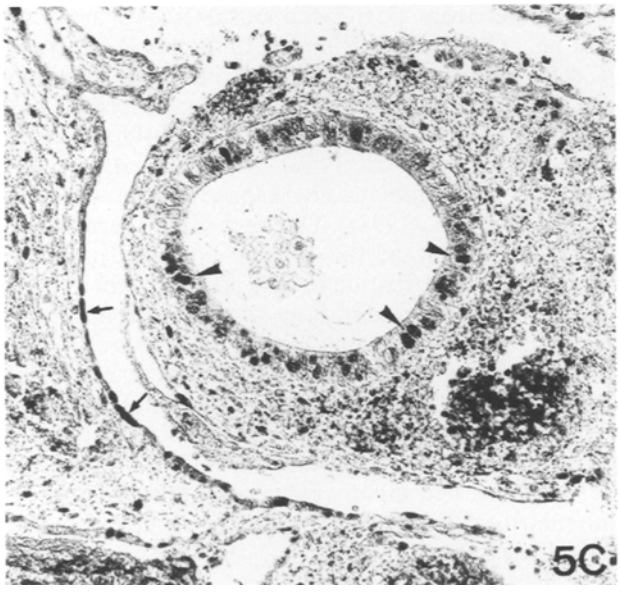


Fig. 2. **A** Immunohistochemical staining for PCNA in the late proliferative phase of the basal layer of the endometrium, demonstrating the nuclear signals *(arrowheads)* in the glandular cells. **B** control. *M:* uterine myometrium. ×350

Fig. 3. Immunohistochemical staining for PCNA in the functional layer of the endometrium. **A** In the late proliferative phase, demonstrating positively stained cells in both epithelial and stroma cells. **B** In the mid secretory phase, the spongiosa layer, positively stained cells can only be seen in the stroma cells *(arrows)* with no staining in the epithelial cells *(arrowheads)*. **C** Demonstrating positive staining in predecidual cells of the compact layer, and perivascular cells are also positive *(arrows)*. ×350

Fig. 4. Immunohistochemical staining for PCNA in the post-menopausal endometrium. There are very few positively stained cells. ×175





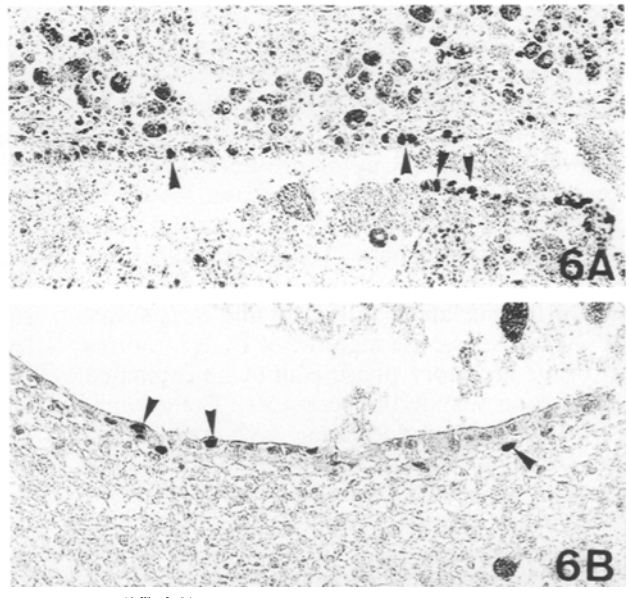


Fig. 5. Immunohistochemical staining for PCNA in endometriotic lesions. **A** In the ovary (*O*: ovary). **B** In the oviductal peritoneum (*OV*: oviduct). Both **A** and **B** are in the proliferative phase. Many PCNA positive cells exist in these glands. **C** The lesion in the ovarian surface in the secretory phase showing PCNA positive cells *(arrowheads)* in the gland embedded in red blood cells and haemosiderin-laden macrophages. Positively stained cells also exist in the peritoneum apposed to the lesion *(arrows)*. **A**, **B** × 250; **C** × 500

Fig. 6. Immunohistochemical staining for PCNA in post-menopausal endometriotic lesions. **A** Endometriotic gland with stroma of pigment-laden *histiocytes* contains PCNA positive cells *(arrowheads)*. **B** Endometriotic gland surrounded by a thin layer of endometrial-type stromal cells also contains PCNA positive cells *(arrowheads)*. × 500

1979a, b). They also showed that the stromal cells are not involved in the surface repair (Ferenczy et al. 1979a, b). The lack of PI variation in the stromal cells of the basal layer suggests that these cells in the basal layer are not involved in the regeneration of the endometrium. With regard to proliferative activity, the stromal cells in the basal layer seem not to be influenced by the sex steroid milieu during the menstrual cycle. This is supported by the ultrastructural findings that the stromal cells in the basal layer do not show morphological changes during the menstrual cycle (Fujimura and Kudo 1984).

The epithelial cells in the functional layer showed high affinity with PCNA in the late proliferative phase then, in the secretory phase, the PI decreased to less than 2%. This is consistent with the in vitro autoradiographic study on tritiated-thymidine incorporation in the endometrium by Ferenczy et al. (1979b), which showed that the maximum number of endometrial cells engaged in DNA synthesis was seen between cycle days 8–10, and that from day 19, the rate of proliferation of glandular cells decreased to near zero and remained unchanged thereafter. With regard to the stromal cells in the functional layer, this study revealed two high PI peaks, one in the late proliferative phase and the other in the mid to late secretory phase. This is also consistent with the study using Ki-67 by Pickartz et al. (1990). Immunohistochemically, receptors for oestrogen (ER) and progesterone (PR) have been demonstrated in the endometrium during the menstrual cycle (Scharl et al. 1988; Lessey et al. 1988; Pickartz 1990). In the epithelial cells of the functional layer, the most staining for ER and PR appears within 10–14 cycle days and both ER and PR decreased in the mid to late secretory phase. Therefore, the PCNA indices in the glandular cells coordinate well with the increase and decrease of both ER and PR. In contrast, the stromal cells express both ER and PR in the proliferative phase, and down express ER in the secretory phase. However, PR is constantly expressed during the menstrual cycle even in the late secretory phase. Therefore, PCNA indices only coordinate with the changes of ER and PR in stromal cells during the late proliferative and early secretory phases. Nevertheless, the increase of PCNA indices in the mid to late secretory phase cannot be explained solely by changes in sex steroid receptors. The stromal cells transformed into predecidual cells may have proliferative activity under the expression of PR in the mid to late secretory phase. This kind of cell proliferation under the influence of progesterone-induced PR also occurs in the myometrium and in leiomyoma (Kawaguchi et al. 1991). During pregnancy, both decidua and myometrial smooth muscle cells are maintained functionally and in terms of growth under the influence of a large amount of progesterone. Therefore, it is likely that the stromal cells in the endometrium first proliferate under the influence of oestrogen in the proliferative phase and then, under the influence of progesterone, these cells are transformed into predecidual cells in the secretory phase. These cells may then gain proliferative activity again either due to the action of progesterone or due to other growth factors mediated by progesterone.

It is often stated that endometriosis reacts in precisely the same manner to the cyclic changes of ovarian steroids as does the eutopic endometrium. However, ultrastructural studies (Schweppe et al. 1984; Schweppe 1989), which compared the cyclic changes in eutopic endometrium and ectopic endometriotic lesions, demonstrated that ectopic endometriotic glands do not always show parallel changes to those of the eutopic endometrium, and that although some glandular cells may show cyclic changes, they are usually not identical to those of the eutopic endometrium, nor are they homogeneous among the glandular cells of the ectopic endometriotic lesions. Moreover, analysis of the ER and PR concentrations have demonstrated that ectopic endometriotic tissues usually contain many fewer receptors than the eutopic endometrium (Clement 1987). They do not undergo predictable changes in response to endogenous hormones (Lyndrup et al. 1987; Lessey et al. 1989).

This study of PCNA expression showed that, except in the proliferative phase, the PI in the endometriotic lesions was significantly higher than that of the endometrium during the menstrual cycle. Even in the post-menopausal specimens, the PI was high. Furthermore, there were no significant changes in PI in endometriotic lesions during the menstrual cycle. These results suggest that endometriotic lesions have more proliferative activity than the endometrium, and the activity is not effectively suppressed by circulating progesterone in the secretory phase.

The results from post-menopausal tissues are also interesting. The endometrium with atrophic features showed very low levels of PI, but some endometriotic lesions had high levels of PI. In post-menopausal endometriosis, morphologically active lesions have been reported (Kempers et al. 1960), and malignant transformation of the endometriotic lesions has been suggested (DePriest et al. 1992). The fact that there is no proliferative activity in the eutopic endometrium but activity in the endometriotic lesions after the menopause suggests that the proliferation of the endometriotic lesions is controlled by some factors other than sex steroids, or that they have the ability to proliferate autonomously. Recent studies of proto-oncogenes such as EGFR, c-*erb*B–2, c-*fms*, and *ras* expression in the endometriotic lesion (Bergqvist et al. 1991; Prentice et al. 1992) have suggested slightly high expression of c-*erb*B–2 in endometriotic lesions. However, these results were not statistically significant.

The factors controlling proliferative activity in endometriotic lesions remain unknown. As for ovarian tumours, a metaplastic process of the mesothelium has been suggested in the pathogenesis of endometriosis (Fujii 1991), and it is thus necessary to study endometriotic lesions not only from the perspective of eutopic endometrial implants, but from that of benign neoplasia in order to elucidate the pathogenesis this enigmatic disease.

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