Expression of proliferating cell nuclear antigen in luminal epithelium during the growth and regression of rat uterus

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Abstract

Proliferating cell nuclear antigen (PCNA), a processivity factor of DNA synthesis, has often been used as a marker that reveals proliferating cells. However, it also plays a role other than in DNA replication. The aim of this study was to examine the relationship between the expression of PCNA and cell proliferation, and also its relation to cell death in the uterine epithelium under various hormonal conditions. Rats with regular estrous cycles were killed at various stages of the cycle, and their uteri were removed for the detection of PCNA and apoptosis by immunohistochemical and terminal deoxynucleotidyl transferase-mediated nick end-label staining respectively. There was an inverse relationship between the expression of PCNA and apoptosis in the uterine epithelium during the estrous cycle. From diestrus to proestrus, the expression of PCNA increased, and few apoptotic cells were detected in the luminal epithelium. However, at estrus, apoptosis occurred markedly, and the expression of PCNA disap-

peared. To study further the effects of estrogen on PCNA expression and cell growth in the uterus, rats were ovariectomized and then implanted s.c. with estrogen capsules 2 weeks later. In ovariectomized rats, only a few PCNA-positive cells were observed in the uterine epithelium. After estrogen treatment, PCNA was expressed strongly in the luminal and glandular epithelia. In these rats, the removal of estrogen capsules resulted in apoptotic death and surprisingly strong PCNA expression in the cells of luminal epithelium. Our results demonstrate that PCNA is expressed not only in the estrogen-stimulated uterine growth, but also in the processes of regression induced by the withdrawal of estrogen. Although the expression of PCNA has been reported to represent cell proliferation, our results implicate functions other than cell replication for PCNA in the uterus.

Journal of Endocrinology (2000) **166**, 87–93

Introduction

Tritiated thymidine or bromodeoxyuridine (BrdU), a thymidine analog that labels cells undergoing DNA synthesis, is often used as a marker of cell proliferation. However, dependence on the use of radioisotope and the administration of exogenous substance to an *in vivo* system have somewhat limited their application. Recently, proliferating cell nuclear antigen (PCNA) has been used to identify replicating cells. PCNA functions as a processivity factor of polymerase δ and ϵ and is essential for DNA replication (Kelman 1997, Tasurimoto 1998). The detection of PCNA in animal studies has provided results that are comparable to those achieved with quantitative analysis using tritiated thymidine (Eldridge *et al.* 1993, Foley *et al.* 1993, Li 1994). These results suggest that PCNA can serve as an endogenous marker of cell proliferation. In addition to its role in DNA replication, PCNA is also involved in DNA repair. The overexpression of PCNA without accompanying cell proliferation

was observed in cells after u.v. irradiation-induced DNA damage (Celis & Madsen 1986, Toschi & Bravo 1988, Nichols & Sancar 1992). PCNA was also expressed during tissue regression, such as in the corpus luteum during structural luteolysis, the prostate gland after castration and the neural apoptosis after dopamine stimulation (Colombel *et al.* 1992, Kiya *et al.* 1998, Shirvan *et al.* 1997). Furthermore, recent studies have suggested that PCNA can act as a docking site to interact with numerous proteins that are involved in cell cycle regulation and DNA repair (Kelman & Hurwitz 1998, Tasurimoto 1998). Thus the interaction of PCNA with other proteins that have various cellular activities suggests that PCNA may have multifunctional roles in addition to DNA replication.

The uterus, which undergoes growth and regression under the regulation of ovarian hormones, is a good model with which to evaluate the role of PCNA in cell proliferation and cell death. Animal studies have shown that the administration of estrogen stimulates the proliferation of uterine epithelial cells and that the withdrawal of estrogen

Figure 1 PCNA expression and apoptosis in the uterine luminal epithelium during the estrous cycle. Rats were killed at proestrus (A, E), estrus (B, F), diestrus I (C, G) and diestrus II (D, H). Their uteri were removed for immunostaining of PCNA (A–D) and detection of apoptotic cells by TUNEL (E–H). Arrowheads indicate apoptotic cells. Scale bar represents 100 μ m.

Figure 2 Immunostaining of PCNA in uterine luminal (A–C) and glandular (D–F) epithelium of estrogen-treated rats. Animals were ovariectomized for 2 weeks and then implanted with E₂ capsules for 1 or 3 days. A, D: ovariectomy; B, E: 1 day after E₂ treatment; C, F: 3 days after E₂ treatment. Scale bar represents 100 μ m.

results in apoptotic death (Martin *et al.* 1976, Finn & Publicover 1984, Quarum & Korach 1984). In the present study, we have investigated the expression of PCNA during the estrous cycle and in ovariectomized rats after estrogen treatment and withdrawal. Our results indicate that PCNA is expressed not only during growth but also during the regression of the uterine epithelium.

Materials and Methods

Animals

Wistar female rats, 8–10 weeks old were obtained from the Animal Center at the College of Medicine, National Cheng Kung University. Animals were maintained on standard chow and water, which were available *ad libitum*, in animal facilities illuminated between 0600 and 1800 h. All procedures were performed in accordance with the Guidelines of the NCKU University Animal Center for the handling and training of laboratory animals in an animal center. The stages of the estrous cycle were confirmed by vaginal smears. Rats with two to three regular cycles were used in these experiments and killed at various stages of the estrous cycle. For the study on hormonal control of the uterus, rats were ovariectomized for at least 2 weeks and then implanted s.c. with silastic capsules containing estradiol 17- β (E₂) powder, prepared according to the procedures described by Legan *et al.*

(1979). After 3 days, E₂ capsules were removed from some animals, and animals were killed at different time intervals.

Immunohistochemical staining

The uterus was fixed in 4% paraformaldehyde solution and embedded in paraffin. Tissue sections 5 μ m thick were mounted on polylysine-coated slides, deparaffinized, rehydrated, and then heated with 10 mM citrate buffer (pH 6) in a 95 °C water bath for 15 min. After two washings with PBS, a primary antibody of PCNA (Zymed Lab. Inc., San Francisco, CA, USA, 1:100 dilution) was added to the slides and incubated at 4 °C overnight. After washing three times, tissue sections were incubated for 1 h with 3 μ g/ml biotinylated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA). The slides were then incubated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After three washings with PBS, slides were incubated with an avidin–biotin complex reagent containing horseradish peroxidase (Vector Laboratories) in PBS and color development was achieved by incubation using the aminoethyl carbazole substrate kit (Zymed Lab. Inc.). The tissue sections were counterstained with hematoxylin. To determine the percentage of PCNA-positive cells, PCNA-staining cells and non-staining cells in high-powered fields were counted in different areas of luminal epithelium. A

minimum of 500 cells in different tissue sections per animal specimen were analyzed. The percentage of PCNA-positive cells was calculated (number of PCNA-positive cells divided by total number of cells counted multiplied by 100).

Terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL)

The procedures as described for the TdT fragELTM DNA fragmentation detection kit (Oncogene Research Product, Cambridge, MA, USA) were followed, with some modification. The tissue sections were deparaffinized, rehydrated and rinsed with buffer. They were incubated with proteinase K (10 µg/ml) at room temperature for 15 min. The endogenous peroxidase was inactivated with 3% H₂O₂ in methanol at room temperature for 10 min. After the tissue sections were rinsed with buffer, they were preincubated with terminal deoxynucleotidyl transferase (TdT) equilibration buffer at room temperature for 30 min, followed with TdT labeling reaction buffer at 4 °C overnight. After being rinsed with buffer, slides were incubated with blocking buffer for 10 min and then with the peroxidase streptavidin conjugate. After 30 min, the slides were washed and incubated with 3,3' diaminol benzidine solution for the detection of apoptotic cells. The slides were counterstained with hematoxylin.

Measurement of the thickness and internal circumference of the luminal epithelium

The thickness of the luminal epithelium was measured in four randomly selected areas in a tissue section, using an ocular micrometer. To determine the internal circumference of the lumen, the tissue section was first screened onto the computer at a magnification of $\times 4$ or $\times 10$ under the optical microscope, depending on the size of the lumen. The preprocessed image was then converted to a binary image using the threshold-based image segmentation method. The optimal threshold for dividing the image into foreground and background was computed from the optimization of the between-class variance of the gray-level histogram. The region-growing method was used to fill the vacancy inside the luminal epithelial cells. Finally, the 3'3 Laplacian operator was used to detect the circumference of the luminal epithelium and the internal circumference of the lumen was computed on a pixel-by-pixel basis.

Statistical analysis

Values are expressed as the means $\pm$ S.E.M. of at least four animals. The statistical significance of differences between respective groups was evaluated by ANOVA followed by Fisher's PLSD test.

Figure 3 Effect of estrogen on the internal circumference (A), thickness (B) and percentage of PCNA-positive cells (C) in uterine luminal epithelium. Ovariectomized rats were implanted with E₂ capsules and killed at 0, 1, 2, 3, 4, 5 and 6 days after hormone treatment. * $P < 0.05$ compared with day 0.

Results

We first examined the changes in PCNA expression and apoptotic death during the estrous cycle. As shown in Fig. 1A, there was a strong expression of PCNA in uterine luminal epithelium at the proestrus stage. However, the expression of PCNA diminished at estrus (Fig. 1B) and then increased again at diestrus I and diestrus II (Fig. 1C, D). The appearance of apoptotic cells exhibited a pattern opposite to that for PCNA expression: at proestrus, no dead cells were observed (Fig. 1E); in contrast, dead cells were most prominent at estrus and decreased steadily at diestrus I and II (Fig. 1F–H).

To study further the hormonal control of uterine growth and the expression of PCNA, the estrogen-treated ovariectomized rats were used as a model. Rats were

Figure 4 Expression of PCNA after E_2 withdrawal in the uterine luminal epithelium. Ovariectomized rats were implanted with E_2 capsules. After 3 days, the capsules were removed; rats were killed at 0 (A), 1 (B), 1.5 (C), 2 (D), 3 (E), 5 (F), 7 (G) and 14 (H) days after E_2 removal. Scale bar represents 100 μ m.

ovariectomized for at least 2 weeks and then implanted s.c. with a silastic capsule containing 17β -estradiol. After ovariectomy, there were few PCNA-positive cells in uterine luminal and glandular epithelial cells (Fig. 2A, D). Estrogen treatment stimulated the expression of PCNA in both the luminal and the glandular epithelial cells for 1 (Fig. 2B, E) or 3 days (Fig. 2C, F). As shown in Fig. 3, the percentage of PCNA positive cells increased significantly 1 day after the hormone treatment and remained increased throughout the treatment (Fig. 3C). Both the thickness of the luminal epithelium and the internal circumference of the lumen also increased significantly 1 day after estrogen treatment, reaching a maximal size and maintaining a plateau value through the treatment period (Fig. 3A, B).

To investigate the changes in PCNA during uterine regression after hormone withdrawal, E_2 capsules were removed from estrogen-treated ovariectomized rats after 3 days of E_2 treatment. Expression of PCNA in the luminal epithelium did not decrease during the first 7 days after the removal of estrogen capsules (Fig. 4 A–G). By day 14, only a few scattered PCNA-positive cells were detected (Fig. 4H). As shown in Fig. 5A and B, the internal circumference and thickness of the uterine luminal epithelium decreased significantly by day 1.5 and were reduced to basal values by days 7–14 after removal of the hormone. Meanwhile, the percentage of apoptotic cells peaked at 1.5 days after E_2 withdrawal and decreased gradually thereafter (Fig. 5C). Quantitatively, the percentage of PCNA-positive cells did not change in the first 7 days after E_2 withdrawal, and during the same period dramatic cell death and tissue regression had occurred in the uterine epithelium. The expression of PCNA diminished significantly by day 14 (Fig. 5D). These

results demonstrate that PCNA was expressed in uterine epithelium after estrogen treatment and removal.

Discussion

The administration and withdrawal of estrogen have been demonstrated to cause uterine growth and regression (Martin *et al.* 1976, Finn & Publicover 1984). Expression of PCNA has been reported during uterine growth (Li 1994, Tong & Pollard 1999), but no report has linked the expression of PCNA and uterine regression. Our results clearly show that PCNA expression occurred during growth as expected, but also during the subsequent regression of the uterus.

During the estrous cycle, cyclic proliferation and death of uterine epithelial cells occur. It has been shown that mitosis increased from diestrus toward proestrus, whereas apoptotic death was low at proestrus and increased at estrus (Sandow *et al.* 1979, Sato *et al.* 1997). This phenomenon is closely associated with the concentrations of ovarian steroids, which increase from diestrus to proestrus and decrease at estrus (Smith *et al.* 1975). In this study, our results showed that there was an inverse relationship between the expression of PCNA and apoptosis during the estrous cycle. From diestrus to proestrus, there was strong expression of PCNA in luminal and glandular epithelial cells, but few apoptotic cells. At estrus, when ovarian steroid concentrations decrease, PCNA was not expressed and the appearance of apoptosis was obvious. These observations suggest that the expression of PCNA and mitosis in uterine epithelial cells during the estrous cycle were influenced by ovarian steroids.

Figure 5 Changes in the internal circumference (A), thickness (B), apoptotic index (C) and percentage of PCNA-positive cells (D) in uterine luminal epithelium after hormone withdrawal. The day of removing E_2 capsules is denoted as day 0. Treatments of the animals were as described in Fig. 4. * $P < 0.05$ compared with day 0.

Estrogen secreted from the ovary during the estrous cycle is the major hormone to stimulate the proliferation of luminal and glandular epithelial cells in the uterus,

whereas progesterone inhibits estrogen-induced cell proliferation and causes the cells to differentiate. Using ovariectomized rats supplemented with estrogen alone or in combination with progesterone, Tong & Pollard (1999) demonstrated that estrogen stimulated the expression of PCNA and DNA synthesis as measured by the incorporation of BrdU in uterine epithelial cells, and that progesterone treatment inhibited both BrdU incorporation and PCNA expression. To simplify the situation, we used ovariectomized rats supplemented with estrogen only in this study. Our results showing that PCNA was expressed strongly in both luminal and glandular epithelial cells after stimulation by estrogen are in good agreement with previous reports. These observations suggest that PCNA can be a good marker for estrogen-stimulated uterine growth.

Much to our surprise we detected a sustained, high level of PCNA expression for 7 days after hormone withdrawal. During this period, the number of apoptotic cells had peaked and decreased to basal values, and regression of the uterus had occurred as indicated by the decreased thickness and internal circumference of the luminal epithelium. The expression of PCNA during tissue atrophy has also been observed in prostate epithelial cells. After castration, prostate epithelial cells underwent apoptosis and exhibited a transient increase in PCNA expression (Colombel *et al.* 1992). Furthermore, it has been shown that cutaneous burn in mice caused apoptosis in their gut epithelium, atrophy of the tissue being paradoxically associated with an increased expression of PCNA (Wolfe *et al.* 1999). One plausible explanation for these observations may be provided by the interaction of PCNA with p21 – a cyclin-dependent kinase inhibitor (Xiong *et al.* 1993) that is a potent inducer of G1 arrest and can block the onset of DNA replication in response to DNA damage (Gartel *et al.* 1996). *In vitro* studies have shown that p21 mediates the acute inhibition of DNA replication in cells when DNA damage occurs (Li *et al.* 1994, Shivji *et al.* 1994, Waga *et al.* 1994). The interaction between p21 and PCNA may reduce the ability of PCNA to stimulate processive DNA synthesis, hence shifting the balance toward repair-type synthesis of short DNA. If the damage is too severe, apoptosis is likely to occur. It has been reported that exposure of rats to azoxymethanone resulted in apoptosis and inhibition of mitosis in colonic epithelium, with strong expression of PCNA and BrdU detected in damaged cells (Yiroye *et al.* 1996). Thus PCNA expression may not reflect the proliferative state in the early phase after cell damage; instead, a stage of cell cycle arrest or DNA repair is likely to take place. Indeed, in the estrogen-treated mouse model, our current study has shown that the removal of estrogen resulted in a peak of expression of PCNA, accompanied by the incorporation of BrdU but not mitosis (unpublished observations). Although p21 has been detected in human endometrium (Toki *et al.* 1998), further studies are needed to clarify

whether E₂ withdrawal may induce the expression of p21, thereby changing the function of PCNA during uterine regression.

The growth of tissues depends not only on the rate of cell proliferation, but also on the rate of cell death. The imbalance of either one would cause abnormal growth or atrophy of the tissues. Therefore, the PCNA labeling index and apoptotic index are often used to assess the status of cell proliferation and death respectively. Our observation of expression of PCNA together with apoptosis during uterine regression indicates that PCNA expression does not necessarily reflect cell proliferation; functions other than cell proliferation should be taken into consideration.

Acknowledgements

This work was supported by grant NSC86-2314-B006-082 from the National Science Council of Taiwan, Republic of China (to L C W). We thank Dr M J Jiang for comments on this manuscript and Mr W H Ding for the technical support of computing analysis.

Parts of these studies were reported at the 32nd Annual Meeting of the Society for the Study of Reproduction, Pullman, WA, USA, August 1999.

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Received 24 November 1999

Accepted 7 March 2000