Apoptosis in endometrium of mouse during estrous cycle

S J Dharma, S D Kholkute & T D Nandedkar*

Institute for Research in Reproduction (ICMR) Jehangir Merwanji Street, Parel, Mumbai 400 012, India

Received 4 November 1999; revised 28 November 2000

The present study was carried out to evaluate apoptosis in endometrium and to correlate these changes with the circulating levels of estradiol and progesterone in the mouse. Apoptosis was observed in various compartments of mouse uterus i.e. stroma, glandular epithelium and luminal epithelium depending on the stage of cycle. Stromal cell apoptosis was observed during various stages of cyclicity except on estrus day. Luminal epithelial cells showed apoptotic changes during all stages of cyclicity except on diestrus day. During metestrus, apoptosis was observed in glandular and luminal epithelia as well as stromal cells. Steroid antagonists such as tamoxifen and onapristone altered the apoptotic changes in the uterus. The results suggest that epithelial cell apoptosis is regulated by estrogen while stromal cell apoptosis is under the control of progesterone.

During normal estrous cycle, changes in the circulating levels of steroid hormones regulate the uterine endometrium. In human, the endometrial lining is sloughed off at each cycle during menstruation. Similarly, in rodents during estrous cycle, the uterine gland degenerates and regenerates again in cyclic manner. The stromal cells also undergo proliferation and regression. However, it is not clear whether these degenerative changes in the uterine endometrium during the estrous cycle are apoptotic or necrotic. Further, it is also not known whether both stromal and endometrial cells undergo these changes and if so, whether these changes are regulated by the steroid hormones.

Therefore the present study has been undertaken to evaluate the apoptotic changes in the uterine endometrium and to correlate these changes with the circulating levels of steroid hormones — estradiol (E₂) and progesterone (P₄).

In addition, tamoxifen, an estrogen antagonist and onapristone, a progesterone antagonist were administered to block E₂ and P₄ levels respectively to study the role of these hormones in endometrial apoptosis.

Materials and Methods

Animals — Regularly cycling female Swiss mice (30) of 3 months of age weighing 20-25 g were used. The animals were bred in Institute’s colony and were housed in a controlled environment. (14:10 hr L:D cycle, 23±1°C, and 50% RH). They were supplied with standard diet and water ad libitum. Estrous cycles were monitored by daily examination of vaginal smears and animals displaying at least two consecutive cycles of 5 days duration were selected for the study. Animals were divided into 6 groups of 5 animals each. Mice of first four groups were autopsied in each phase of the cycle i.e. diestrus, proestrus, estrus and metestrus days. One group of animals (i.e. fifth group) was treated orally with 400 μg/kg tamoxifen on proestrus day. This dose has been reported to antagonize E₂ action in rats. Sixth group was injected with 1 mg/kg onapristone on estrus day. The animals were sacrificed 24 hr later. The animals were anaesthetized with ether and blood samples were collected by cardiac puncture. Serum after separation was stored at −20°C until analyzed.

Evaluation of apoptosis — The uterine horn was removed, freed of surrounding fat and connective tissue, snap frozen and 5 μ sections were cut on Leitz cryostat at −20°C. The sections were picked up on aminopropyl triethoxy silane coated glass slides and fixed in paraformaldehyde. Apoptosis was evaluated by in situ 3' end labeling, TUNEL (TdT mediated dUTP Nick End Labeling) technique in which terminal deoxynucleotidyl transferase (TdT) labeled double stranded DNA breaks were visualized using the kit from Boehringer Mannheim, Germany. Briefly, the DNA fragments in the frozen tissue section, after fixing with paraformaldehyde, were labeled with labeling mixture containing cobalt chloride, Dig dUTP, dATP and terminal transferase.
for 2 hr at 37°C. Nonspecific sites were blocked by Triton X-100 and normal sheep serum to reduce the background. This was followed by addition of primary anti-digoxigenin antibodies coupled to enzyme alkaline phosphatase. The cells containing digoxigenin labeled DNA fragments were visualized after addition of nitroblue tetrazolium (NBT) and bromo-chloro-indolyl phosphate (BCIP) to produce purple blue precipitate at the site where the enzyme activity was located.

Sections were incubated without TdT for negative controls. Staining intensity was graded as absent, weak, moderate and intense.

**Radioimmunoassay** — Estimation of estradiol and progesterone in serum was carried out by RIA as reported earlier. The antisera were obtained from ICN Pharmaceuticals, USA and the label was procured from Amersham Radiochemicals, England, U.K.

**Statistics** — The results are expressed as mean ± SE. Differences between the experimental groups were evaluated using Student’s t test. Differences were judged to be significant if P < 0.05.

**Results**

Apoptotic changes were observed in various compartments of mouse uterus i.e. stroma, epithelia of lumen and glands depending on the stage of cyclicity (Table I). During diestrus, the stromal cells showed apoptosis as evidenced by moderately stained, fragmented cells (Fig. 1a). The epithelial cells however were weakly stained on the diestrus day indicating minimal apoptosis.

On proestrus day, moderately stained apoptotic cells were seen in the stroma. The luminal epithelial lining showed maximum apoptosis as evident by intense staining (Fig. 1b). On the day of estrus, a weak staining in the luminal epithelial and in the stroma was observed (Fig. 1c). On metestrus day of the cycle, a distinct and intense positive staining could be seen in the luminal and glandular epithelial cells of the endometrium and also in the stromal cells (Fig. 1d). In the tamoxifen treated mice, stromal cells revealed enhanced uterine apoptosis on the estrus day (Fig. 1e). The treatment with onapristone caused apoptosis only in the epithelial cells of the glands but not in the stromal cells (Fig. 1f). The controls without TdT showed negative staining (Fig. 1g).

Figure 2 shows levels of serum estradiol and progesterone during various stages of the estrous cycle and after treatment with steroid antagonists. The concentration of plasma estradiol was highest on the estrus day of the cycle (533.14 ± 391.54 pg/ml) and was minimal on the metestrus day (93.62±84.18 pg/ml). On the other hand, the concentration of progesterone was optimum on the metestrus day (1534.30 ± 368.24 pg/ml) compared to the other days of the estrous cycle (919.21 ± 360.84 pg/ml). Interestingly, E2 levels in tamoxifen treated mice decreased comparable with control group on estrus day (P<.05). However, injection of onapristone on estrus day enhanced P4 concentrations significantly (P<0.01).

**Discussion**

The results of the present study reveal apoptosis in the stromal cells as indicated by TUNEL positive staining and fragmented nuclear chromatin on all the days of the cycle except on the day of the estrus. On the other hand, in the luminal epithelial cells apoptosis was observed on all the days except on the diestrus day of the cycle. Thus, apoptosis in the two endometrial compartments is regulated differently depending on the stage of estrus cycle. Finn and Publicover reported that in ovariectomized mice, estrogen treatment enhanced mitosis in luminal epithelial cells as revealed by 3H thymidine uptake. A decline in 3H thymidine uptake after the cessation of estrogen treatment correlated with cell death. This corroborates with the present observation of occurrence of minimal apoptosis in the uterine epithelial and in the stromal cells on estrus day in cycling mice which could be due to the high estrogen levels on this day. Further, following treatment with the estrogen antagonist, stromal cells were TUNEL positive. This is in agreement with the observations of Sandow et al. indicating decrease in uterine stromal...
Fig. 1—Apoptosis in the mouse uterine endometrium during estrous cycle as detected by TUNEL technique. (a) Diestrus day: moderate staining in the stromal cells but weak in the epithelial cells. (b) Proestrus day: Moderate staining in the stromal cells and intensely stained luminal and glandular epithelial cells. (c) Estrus day: Weak staining in stromal and luminal epithelial cells. (d) Metestrus day: Intensely stained stromal and epithelial cells. (e) Tamoxifen treatment on proestrus day: Intense staining of stromal cells. (f) Onapristone treatment on estrus day: Moderately stained glandular epithelium. (g) Negative control [a-g x250].
but not epithelial cell apoptosis after 24 hrs treatment with tamoxifen, although they have treated animals with higher dose (30 mg/kg) and for a longer duration (7 days). Sandow et al.6 observed apoptotic changes in the uterine epithelial cells after withdrawal of estrogen in ovariectomized hamsters. West et al.3 also reported degenerative changes in the epithelium when the serum estradiol levels were low. Highest cell apoptosis in the stromal cells was observed on diestrus. Treatment with progesterone antagonist showed decrease in stromal apoptosis. This possibly is the reflection of high concentration of serum progesterone levels on the previous day i.e. the metestrus day following ovulation. However, Terada et al.8 failed to observe apoptosis in glandular epithelium as evaluated by the uptake of 5-iodo, 2-deoxy uridine in progesterone treated castrated mice.

In the present study, due to TUNEL being more sensitive method, apoptosis could be detected in the uterine endometrium on metestrus day when the serum progesterone levels were high. The results of the present study suggest that stromal cell apoptosis may not be directly regulated by estrogen as stromal cell apoptosis was observed during all the days of the cycles. In castrated mice and neonatal mice, steroid treatment also failed to affect stromal cell apoptosis. Probably, induction of stromal cell death may be regulated by growth factors. Results of present study indicate that progesterone may enhance stromal cell apoptosis.

Tamoxifen treatment increased E2 levels compared to the control uninjected mice. Treatment with onapristone increased plasma P4 levels. This is possibly due to blocking of progesterone receptor by onapristone thereby resulting in more P4 concentration in circulation. On the other hand, due to blocking of the receptor for E2 or P4, the respective hormone levels were higher in circulation which is also reflected in the decrease in apoptosis in the epithelial and stromal cells respectively. This is in accordance with the earlier reports1,2 showing increased mitotic index in these uterine cells after injection of E2 or P4 respectively in ovariectomized mice.

In human endometrium, epithelial and stromal cell apoptosis was highest during early proliferative and late secretory phases and during menstruation compared to other phases of the menstrual cycle13. Low levels of estrogen during the early proliferative phase and withdrawal of steroid hormones in the late secretory and menstrual phase may be responsible for inducing apoptotic changes in the human endometrium. The results of the present study also demonstrate minimal apoptosis in the epithelial and stromal cells on estrus day in mouse, when the concentration of estradiol was found to be maximum.

In conclusion, the epithelial cell apoptosis in the uterine endometrium is regulated by estrogen while the stromal cell apoptosis may be regulated by progesterone. Understanding the hormonal and cellular mechanisms responsible for apoptosis in higher primates will provide better understanding of the pathological conditions like amenorrhoea or dysmenorrhoea, heavy bleeding, endometrial dysfunctions and endometrial proliferative diseases such as tumour and fibroid development.

Acknowledgement
Authors are grateful to Dr. H.S. Juneja, Director, IRR for encouragement, Dr. Deepa Bhartiya, Scientist, Jerbai Wadia Hospital for help and Ms. Doris D'Souza for typographical assistance.

References