Zonal Changes in Proliferation in the Rhesus Endometrium During the Late Secretory Phase and Menses (44079)

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Abstract. **The objective of the present study was to examine the zonal changes in endometrial proliferation that occur during the late secretory phase, menses, and postmenstrual endometrial regeneration. We used as our model ovariectomized rhesus monkey in which artificial menstrual cycles were simulated. Our marker of proliferation was the immunohistochemical detection of the Ki-67 antigen. On Day 26, as progesterone (P) levels are falling in the late secretory phase, proliferation in zone IV of the basalis decreased compared with Day 23 (peak P level). Proliferation in the upper regions of the endometrium remained suppressed. Three days after a single bolus injection of the potent antiprogestin RU-486 on Day 20, proliferation in zone IV was virtually absent compared with Day 23 of an artificial cycle. No distinct changes in the pattern of proliferation were observed in the upper regions of the endometrium. On Day 1 of menses (P levels undetectable, estradiol [E] levels of 70–100 pg/ml), there was little proliferation throughout the endometrium. On Day 3 or menses, proliferation returned to zones II–III of the basalis and the functionalis. This proliferation was primarily observed in the glandular epithelia whereas little or no proliferation was observed in zone IV of the basalis. By Day 5 proliferation continued in the glandular epithelia of zones I, II, and III, and was now clearly observable in the stromal cells. Only minimal proliferation was observed in glandular epithelia of zone IV. In the absence of basal E stimulation the return of proliferation to the glandular epithelia in zones I, II, and III was dramatically reduced. These data demonstrate a reciprocal pattern of proliferation in glandular epithelia that is dependent on the prevailing hormonal stimulation. Under P dominance, proliferation is inhibited in zones I, II, and III, and maintained in zone IV, whereas under E dominance (Day 3 or 5) proliferation is driven by E stimulation in zones I, II, and III with little or no proliferation present in zone IV. In addition, the inhibition of proliferation in zone IV by the antiprogestin RU-486 and the decline of zone IV proliferation associated with falling P levels provide further evidence that proliferation of glandular epithelia in zone IV is mediated in part by P.**

[P.S.E.B.M. 1997, Vol 214]

The process of cyclic renewal of the endometrium in women and menstruating primates attests to the powerful regenerative capacity of this tissue and its central importance in fertility. The endometrial regression and reconstruction that occurs during the menstrual cycle is accompanied by dramatic changes in the hormonal milieu. The loss of progesterone (P) influence in the late secretory phase leads to regression and sloughing of the superficial endometrium and subsequently estradiol (E) becomes the dominant hormonal influence during reconstruction.

The rhesus endometrium has been characterized by Bartelmez (1), using histological criteria, as composed of four horizontal zones: the transient functionalis is composed of zone I, the luminal epithelia (ciliated and secretory) and densely packed stroma, and zone II, the upper third segment of the glands; the germinal basalis is composed of zone III, the middle third of the glands, and zone IV, the deepest portion of the glands adjacent to the myometrium. In addition, the endometrium's complexity is further defined by the number of different cell types that it harbors. These cell

This publication was supported in part by a grant (HD-31620) from the National Institutes of Health (NIH). Its contents are the responsibility of solely the authors and do not necessarily represent the official views of the NIH.

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Received November 20, 1995. [P.S.E.B.M. 1997, Vol 214]
Accepted September 10, 1996.

0037-9727/97/2142-0132$10.50/0

types include: luminal and glandular epithelia, stromal fibroblasts, vascular smooth muscle cells, and cells of the lymphocytic system (2, 3). Different cell types in the uterus have been shown to respond differently to the same hormonal stimulation (4, 5). Therefore, a differential cell-type response to hormonal stimuli may play an important role in the coordination of hormonal signals that permits the uterus to achieve appropriate endpoints in reproduction.

Previous studies in the rhesus monkey have shown different zonal patterns of proliferation in the endometrium during E versus P dominance in both natural menstrual cycles (6, 7) and artificial menstrual cycles (8, 9). These studies showed that different zonal patterns of proliferation under the same hormonal milieu are a property of the primate endometrium. Of particular importance was the observation that the deepest glandular portion of the basalis (adjacent to the myometrium) increased 10-fold in proliferation during the secretory phase (7). Concomitant with this increase in proliferation in zone IV of the basalis, proliferation was dramatically reduced in epithelial cells in the upper zones of the endometrium as serum P rose during the secreotry phase of the menstrual cycle. Thus, zone IV of the endometrial basalis becomes the dominant proliferating tissue during P dominance.

In our present study, we focus on the proliferative changes that occur during the late secretory phase and during menses and reconstruction. In order to study zonal and differential cell type changes in proliferation, we used the immunohistochemical detection of the Ki-67 antigen (a marker of proliferation) that correlates with the pattern of *in vivo* [^{3}H]thymidine uptake in the rhesus endometrium (7, 8). In addition, we used two approaches to study the hormonal dependence of endometrial proliferation: (i) administration of the antiprogestin, RU-486, during the mid-secretory phase; (ii) withdrawal of E during menses and reconstruction.

The ability to produce artificial menstrual cycles in ovariectomized rhesus monkeys provides the experimental means necessary to analyze in detail the regulation of endometrial response to E and P. The development and use of artificial menstrual cycles in the rhesus monkey was first described by Hodgen (10). These studies showed that simulation of the menstrual cycles by the timed insertion and removal of silastic implants of E or P was sufficient to allow the endometrium to support implantation and eventual delivery (IVF and surrogate transfer). By using this potentially important model we can avoid the irregularity of natural menstrual cycles and obtain precisely dated endometrial samples.

Materials and Methods

Animals. Mature female rhesus monkeys (*Macaca mulatta*) obtained from commercial sources (Hazelton Labs, Alice, TX) were ovariectomized for at least 2 months prior to initiation of artificial menstrual cycles. Animals that had undergone a previous endometriectomy were rested for at least 6 months. A total of three or four animals were used for all experimental paradigms. Previous studies by Barthelmez have shown that following endometriectomy the endometrium is restored to a state that can support potential implantation (1). All protocols were approved by the Institutional Animal Care and Use Committee. Silastic implants were prepared from Dow-Corning (Midland, MI) silastic tubing (medical grade; 0.335-cm i.d. by 0.465-cm o.d. by packing the tubing with crystalline E or P (Steraloids, Wilton, NH) and sealing the ends with silastic adhesive. Silastic implants were placed subcutaneously in the intrascapular area under ketamine anesthesia (10 mg/kg). Removal of implants was also performed under ketamine anesthesia. Endometrial biopsies were collected by endometriectomy and flash frozen in liquid nitrogen until further processed. Discomfort following surgery is minimized by administration of pentazocine (2–5 mg/kg), burprenorphine (0.01 mg) or orphanol (0.025 mg/kg). Choice of analgesic depended on the time following surgery (up to 48–72 hr) and individual animal recovery.

The following protocol previously described (11) for placement or removal of the implants was used: basal E levels (70–100 pg/ml of serum) are maintained with a single 3.0-cm implant throughout the cycle; the E surge is created by sequential insertion of three 2.3-cm E implants on Days 10–12, followed by their removal on Day 13; one P implant 3.0 cm in length is inserted on Day 13, and two P implants are inserted on Day 16. All blood sampling or tissue harvesting was performed prior to insertion or removal of implants. Blood samples were measured for E and P by radioimmunoassay (RIA) as previously described (11, 12). Serum E and P in these artificial menstrual cycles have been studied (11) and shown to mimic those observed during the natural cycle (13). Serum E and P data for the experimental paradigms used in these studies are shown in Table I.

Preliminary studies showed that using a single bolus of RU-486 at a dose of 15 mg/animal administered intramuscularly in corn oil, (animal weight range was held to 10–12 lb) menses was induced 3–4 days later. RU-486 treatment was administered on Day 20, and endometrial tissue was harvested 3 days later to correspond to tissue harvested on Day 23 of a normal cycle. Tissue samples taken on a normal Day 23 are taken prior to the removal of one P implant.

Immunohistochemical Analyses of the Ki-67 Antigen. Endometrial biopsy tissue was oriented in a small aluminum foil cup and frozen immediately in Tissue Tek OCT embedding compound (liquid-propane-liquid nitrogen or liquid nitrogen). All samples were subsequently stored at −80°C. Cryostat (−25°C) sections (6 μm) were fixed in freshly prepared 4% paraformaldehyde in Sorensen's buffer (pH 7.4) for 15 min at room temperature. Sections were washed twice in phosphate-buffered saline (PBS, pH 7.4). All subsequent incubations with immunochemicals were performed in a humidified chamber.

Monoclonal antibody to the Ki-67 antigen was obtained commercially from Transbio (Paris, France). The avidin-

biotin complex technique was used (Vector Labs, Burlingame, CA). Ki-67 localization was performed as described previously (14). Briefly, sections were incubated with Ki-67 antibody (1/30 in PBS) or control (preimmune mouse IgG, 1/30 in PBS) overnight at 4°C. Sections were washed twice in PBS for 10 min at room temperature. Anti-mouse biotinylated secondary antibody (Vector Labs) was found to produce the best results at the levels suggested by the manufacturer. Incubations with secondary antibody were for 1 hr at foom temperature. After washing the sections twice in PBS, the sections were treated with streptavidin-peroxidase complexes for 30 min at room temperature followed by washing in PBS as described above. The antigen-antibody complex was detected by incubation with freshly prepared 3′,5′-diaminobenzidine (DAB) solution (0.5 mg/ml) in Tris-HCl (0.05 *M*, pH 7.6) containing 0.0025% hydrogen peroxide and 10 m*M* imidazole. Incubation of sections in DAB solution was allowed to proceed for 10 min followed by immersion in distilled water for 15 min. Slides were dehydrated through ascending grades of ethanol and two changes of xylene (2 min for each treatment) and mounted with Permount (Fisher, Fair Lawn, NJ). Photomicrographs were made with a Zeiss photomicroscope (Carl Zeiss, NY) using Ektachrome 400 (Kodak, Rochester, NY) and Zeiss panneofluar lenses at magnifications of ×200 or ×400. Methodological controls included omission of the first antibody or second antibody or streptavidin-peroxidase complex or omission of all of the above with the DAB detection system alone. No staining was observed with the above controls.

Other Methods. Statistical comparisons were done by the Student's *t*-test for comparison between two means with correction for multiple *t*-tests where appropriate (Duncan's New Multiple Range Test [15]).

The percentage of cells that stained positively for the Ki-67 antigen was assessed following counterstaining with toluidine blue (brown-positive nuclei appear black, whereas nonproliferating cells have a clear blue nucleus). A minimum of 250 cells were counted for each tissue section. The labeling index (LI) was expressed as a percentage of proliferating cells of all cells counted.

RESULTS

For purposes of endometrial orientation, Figure 1 shows an H&E low power (×50) photomicrograph of a typical endometrial biopsy obtained on Day 23 with the endometrial zones and myometrium identified. Figure 2 also shows an H&E stained panel of zones I, II–III, and IV (myometrium is at bottom of panel and not seen) from a Day 23 sample, but at higher magnification. On Day 23 (peak serum P level), glandular epitehlial in zones I and II–III show little or no proliferation, whereas the glandular epithelia in zone IV exhibited considerable proliferation (LI 36 ± 2.9, Fig. 2b, myometrium below panel). A few scattered stromal cells showed proliferation throughout the endometrium (LI 2.5 ± 0.4). These results are comparable to those observed previously on Day 21 of artificial menstrual

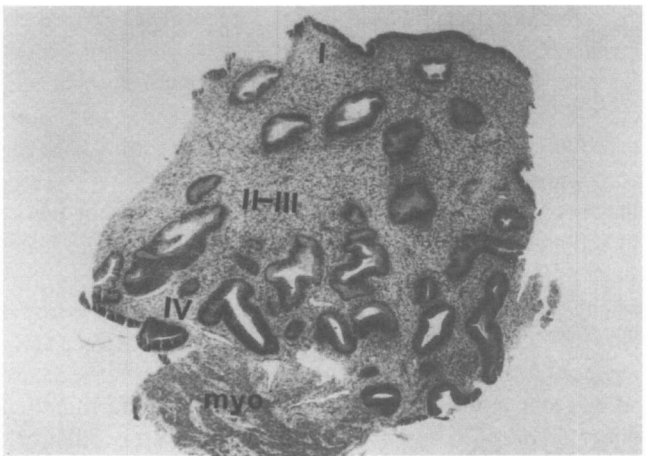

Figure 1. An H&E-stained section of a typical endometrial biopsy at low magnification (×50). Endometrial zones (I, II–III, IV) and the myometrium (myo) are identified in the photomicrograph.

cycles (8). As serum P levels decline between Day 23 and 26, there is a significant reduction in glandular epithelia staining for the Ki-67 antigen in zone IV (LI 18 ± 2.3, $P < 0.05$) with no other apparent changes in zones I and II–III (Fig. 2c, myometrium right of panel). The pattern of stromal cell proliferation remained similar to that observed on day 23 (LI 2.0 ± 0.6).

To assess the role of P on epithelial proliferation in zone IV we used administration of the potent antiprogestin, RU-486. A single bolus injection of RU-486 was administered on Day 20 and endometrial tissue was harvested for analysis 3 days later. A Day 23 endometrial sample from a normal artificial cycle served as control (similar to that shown above but repeated here for comparison; Fig. 3, myometrium below panel). There is a modest decrease in stromal proliferation in all zones. Epithelial proliferation in zone IV is, however, virtually absent following RU-486 treatment (LI 0.8 ± 0.4, *P* 0.05; Fig. 3b, myometrium below far right panel) compared with a normal Day 23 endomerial sample (Fig.3a, far right panel). No distinct changes in the pattern of proliferation were observed in the upper regions of the endometrium.

On Day 1 of an artificial menstrual cycle serum P levels are below the level of detection and E becomes the dominant hormonal stimulation (Table I). Proliferation is virtually absent in zone IV glandular epithelia of the basalis (LI 0.5 ± 0.3; Fig. 4a). Glandular epithelia in zones II–III are also devoid of the Ki-67 antigen (LI 0.7 ± 0.4). Despite the onset of menses (determined by swabbing), one of the endometrial samples contained a section of luminal epithelia that was also negative for Ki-67 staining (Fig. 4a). Similar to observations made on Day 23 and 26, only a few scattered stromal cells showed evidence of proliferation (LI 2.3 ± 0.5).

As endometrial reconstruction proceeds on Day 3 there is a dramatic return of proliferation in the glandular epithelia that is zonally dependent (Fig. 4b). Glandular epithelial proliferation remains absent in zone IV of the basalis but is

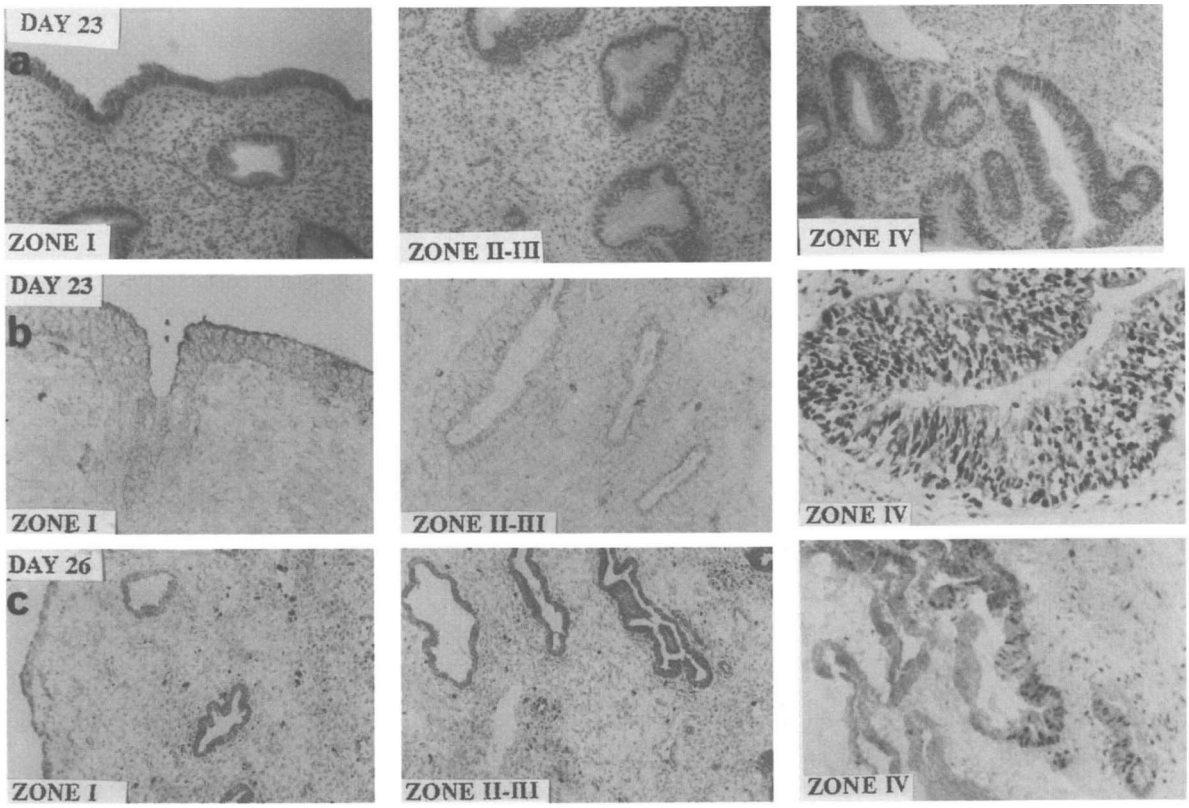

Figure 2. Distribution of endometrial proliferation (Ki-67) in rhesus endometrium on Days 23 and 26 of artificial menstrual cycles. (a) An H&E-stained endometrium for histological orientation. (b and c) Ki-67 staining for Day 23 and 26, respectively. Zones I, II–III, and IV are identified in each panel. Magnification: ×200 (except for zone IV of Panel b [×400]); n = 3–4 for each day of the cycle.

present in zones II–III (LI 31 ± 3.0). This gradient of proliferation is striking because it can be observed within individual epithelial glands emanating from zone IV (no proliferation) into zone III (proliferating) of the basalis (Fig. 4b, far right, myomtrium right of panel). In contrast to the return of proliferation to glandular epithelia in the upper zones of the endometrium, only a few stromal cells throughout the entire endometrium show evidence of proliferation (LI 1.4 ± 0.4). By Day 5, the endometrium has completed reconstruction and endometrial proliferation is present in both glandular epithelia, luminal epithelial and stroma. Although some proliferation is present in glandular epithelia in zone IV of the basalis (LI 8.6 ± 2.0), there is considerably less staining for the Ki-67 antigen than in zones II–III (LI 28 ± 3.1, $P < 0.05$). This pattern of Ki-67 staining in zone IV on Day 5 is very similar to that observed previously on Day 13 (peak serum estradiol) (8).

It has previously been shown that the endometrium regrows following endometriectomy in the rhesus monkey (1). Following endometriectomy in our artificial cycles E and P hormonal support is removed and yet the endometrial wound created by this procedure ceases bleeding and resurfaces (data not shown). In order to determine the importance of E during reconstruction as opposed to normal wound healing mechanisms, we removed all E stimulation (removal of 1 remaining E implant) on Day 26 and harvested tissue on what would correspond to day 3 of a normal cycle. While the endometrium continues to heal, proliferation throughout the entire endometrium is lower in the absence of E stimulation (LI 15 ± 2.3, $P < 0.05$, Fig. 5 versus Fig. 4b). These data show that E is the primary hormonal stimulus for the changes in proliferative response during menses and suggest that normal wound healing mechanisms play only a minor role in the reconstruction and zonation of the endometrium during menses.

Discussion

The remarkable regenerative capacity of the primate endometrium was demonstrated years ago by Hartman (16) when he showed that the endometrium could completely regenerate following an endometriotomy that would leave only a few endometrial cells on the surface of the myometrium. This regenerative capacity of the endometrium is perhaps not surprising, becuase of the central role it plays in reproduction. Our analysis of endometrial proliferation in this study focused on the changes that occur during the late secretory phase, menses and reconstruction. In addition, we studied the hormonal dependence of proliferation during these periods.

Proliferation in the endometrium during the changeover from E to P dominance decreases in the functionalis and zone III of the basalis and increases in zone IV of the basalis by Day 21 (8). We show in the present study that proliferation in the endometrium is similar on Day 23. This result is

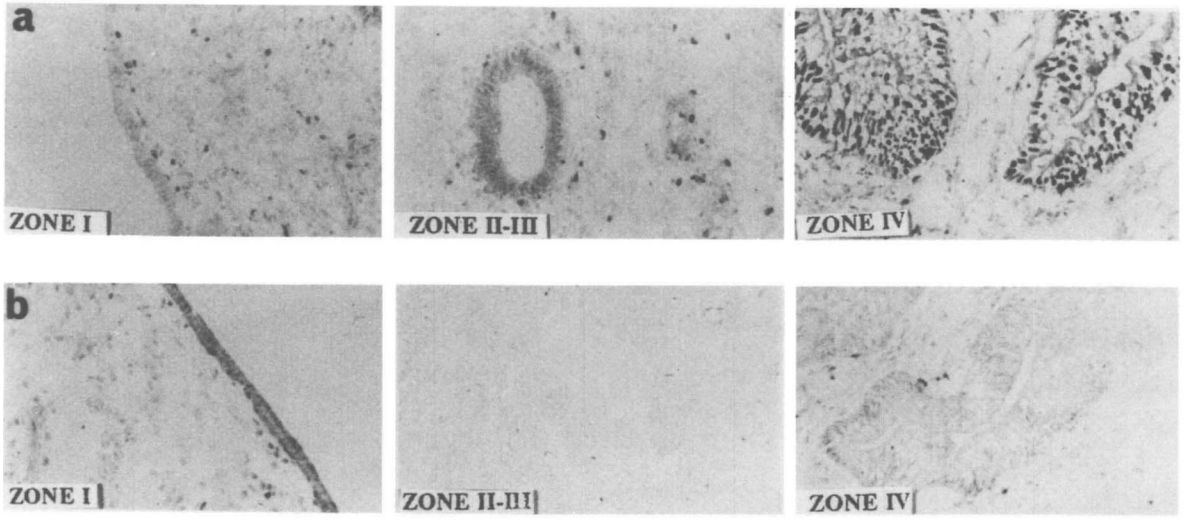

Figure 3. Endometrial proliferation (Ki-67) following RU-486 treatment during the mid-secretory phase of artificial menstrual cycles. Administration of RU-486 (15 mg/animal) was given on Day 20, and tissue biopsies were harvested on day 23. Control animals received no treatment (Fig. 2a, Day 23). Magnification: ×400; n = 3–4 for control (a) or treatment group (b).

Table I. Serum E and P Levels

Cycle day	E (pg/ml)	P (ng/ml)
23	63 ± 3	7.9 ± 2
26	67 ± 4	3.7 ± 1
1	56 ± 3	<0.2
3	42 ± 4	<0.2
5	56 ± 6	<0.2

Note. All values are the mean ± SEM, n = 4.

not surprising because serum P levels are not significantly different on these 2 days of the secretory phase. The relatively small but consistent decrease in proliferation observed on Day 26 and the absence of proliferation on Day 1 suggest that the decline in serum P is associated with decreases epithelial cell proliferation in zone IV of the basalis.

Previous studies in the human (17–19) and nonhuman primate (20–23) have shown that RU-486 can induce menses. In addition, RU-486 has also been previously shown to inhibit proliferation in the nonhuman primate endometrium (23–25). These previous studies together with the availability of the potent anti-progestin, RU-486, provided us with an important tool to study the action of P on zone IV proliferation during the mid-secretory phase. We were able to show that the administration of RU-486 resulted in the inhibition of proliferation in glandular epithelia in zone IV of the basalis during the mid-secretory phase. These results provide further evidence that P may be a regulator of proliferation on zone IV glandular epithelia. Although P is not commonly regarded as a mitogenic stimulus in the endometrium, the above results together with previous data showing that proliferation in zone IV during the mid-secretory phase is independent of secretory E (8) provide additional evidence to support a role for P as a mitogen in zone IV of the primate endometrium.

Despite the presence of basal E levels and the absence of P on Day 1, there is little proliferation throughout the endometrium. These results suggest that both stromal and glandular epithelial cells have become desensitized to the mitogenic effect of E during the early phase of menses. The mechanism that underlies this desensitization is unknown. These cell types (stroma and epithelia) may be arrested in the cell cycle as a consequence of P withdrawal concomitant with the collapse of the vasculature and decreased nutrient supply and may require a certain degree of "healing" to become E responsive (e.g., reepitheliazation of the wound and the return of normal vascular function and nutrient supply). Further studies will be needed to identify the mechanisms involved in this phenomenon.

Our previous experience with this animal model has shown us that despite removal of all hormonal support following endometriectomy the endometrium stops bleeding and heals. This suggested to us that normal wound healing mechanisms may play a role in the reconstruction of the endometrium during menses. As shown above, in the absence of hormonal stimulation the endometrium does heal although proliferation is lower than in the presence of E. These results suggest that in the absence of E other factors may serve to promote proliferation and healing of an endometrial wound. Several autocrine/paracrine factors are known to play a role in wound healing. Because of the known actions of FGF (26), PDGF (27), and TGFβ (28), these factors and others may serve to play a supportive role in normal reconstruction of the endometrium during menses.

A previous study of the rhesus monkey examined endometrial proliferation only in zones II–III following P withdrawal and the ensuing menses (29). These studies reported that E stimulation of DNA synthesis in the upper glands was E dependent but that cells lacking ER (presumably not capable of responding to E) showed evidence of proliferation. Our results also shown that E-dependent pro-

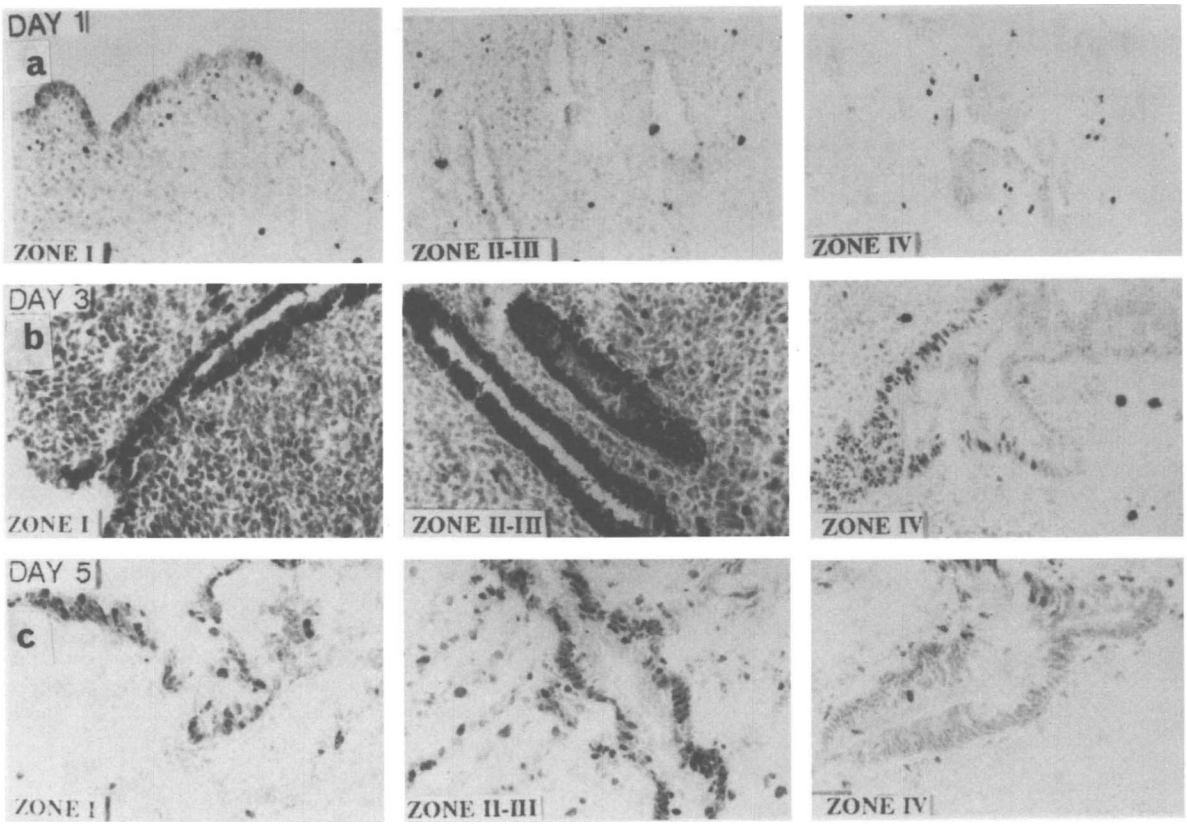

Figure 4. Distribution of endometrial proliferation (Ki-67) in rhesus endometrium on Days 1, 3, and 5 of artificial menstrual cycles. Zones I, II–III, and IV are identified in each panel. Magnification: 400×; n = 3–4 for each day of the cycle.

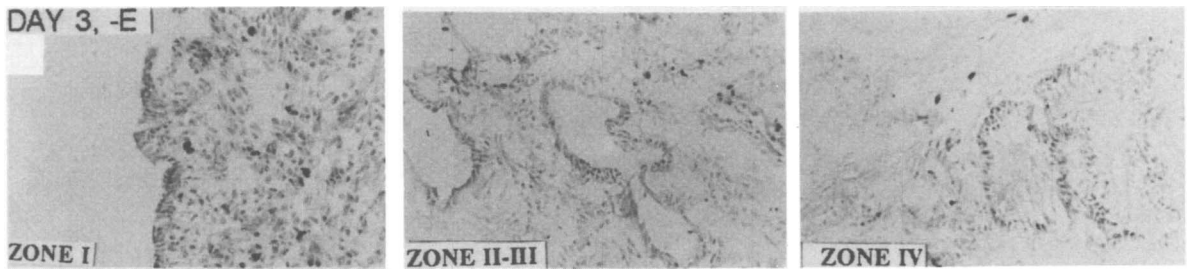

Figure 5. Distribution of proliferation (Ki-67) on Day 3 in the absence of E stimulation. Zones I, II–III, and IV are identified in each panel. Magnification: ×400; n = 3–4.

liferation returns by Day 3 to parts of zones II–III, however, we did not observe proliferation in stromal cells on this day. This difference may be attributable in part to the differences in the hormonal regimen used in the previous study and the present study. As a result, there may be some temporal differences in the appearance of cell proliferation (e.g., proliferation returns to the stromal compartment by Day 5 in our present study).

Zone IV remained distinct in its proliferative response, and glandular epithelia in this zone showed no signs of proliferation through Day 3. Only a minimal proliferative response was observed in zone IV on Day 5 similar to that observed previously on Day 13 (peak E) (8, 9). The gradient of epithelial proliferation observed on day 23 (zone IV, high; upper regions, low) was reversed by Day 3 (zone IV, low; upper regions, high). Whether these gradients of proliferation in the endometrium are a result of zonal differences in the regulation of stimulatory or inhibitory autocrine/paracrine factors or their receptors is also a focus of study in our laboratory (30, 31).

This study has mapped the hormone-dependent changes in proliferation that occur during the late secretory phase through menses and reconstruction in artificial menstrual cycles in the rhesus monkey. Our results suggest that a period of desensitization to E-dependent proliferation occurs during the early days of menses as the endometrium heals. In addition, we provide further data to support a mitogenic role for P either directly or indirectly (e.g., growth factors or their receptors) in the regulation of proliferation of zone IV glandular epithelia. These results also further attest to the unique cell-type- and zonal-dependent hormonal responsiveness of the primate endometrium.

The authors thank Drs. C. Longcope and Janet Tast for their help and support of this work.

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