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Progesterone Regulation of Endometrial Estrogen Receptor and Cell Proliferation during the Late Proliferative and Secretory Phase in Artificial Menstrual Cycles in the Rhesus Monkey[1]

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ABSTRACT

Progesterone (P) down-regulation of uterine estradiol (E) receptor (ER) appears to be a general mechanism by which P modulates E action in the uterus. Our present studies focus on the regulation of ER by P during the changeover from E to P dominance during artificial menstrual cycles in the rhesus monkey. Because of differential cell-type response and the cellular zonation of the primate uterus, we used immunohistochemical analysis in addition to biochemical assays to study the regulation of ER by P. Ki-67 immunoreactivity was used as an index of endometrial proliferation. We performed our analyses on Days 13 (peak of E), 14 (declining E and rising P), 17 (basal E and rising P), and 21 (basal E and peak P). ER immunoreactivity was present throughout the endometrium in luminal and glandular epithelia and stromal fibroblasts on Day 13. As E was withdrawn and P rose on Day 14 there were few distinct changes in ER staining in stromal and epithelial cells. On Day 17, immunoreactive staining showed a distinct reduction for stromal cells in all zones. Although luminal epithelial cells showed a decrease in immunoreactivity on Day 17, zones II, III, and IV retained positive staining for ER in glandular epithelia. ER staining in stromal cells on Day 21 was similar to the pattern observed on Day 17, whereas epithelial cells in zones I, II, and III showed a reduction in staining. Glandular epithelia in zone IV maintained strong positive staining for ER on Day 21. According to biochemical assays, total and occupied nuclear ER and cytosolic ER were significantly decreased on Day 14 and remained suppressed through Day 21. Endometrial proliferation (Ki-67 immunoreactivity) occurred throughout the endometrium on Day 13, showed little change on Day 14, and was dramatically suppressed in stromal fibroblasts and epithelial cells of zones I, II, and III on Days 17 and 21. Proliferation continued in glandular epithelium of zone IV during the changeover from E to P dominance. Removal of secretory estradiol (all E implants removed on Day 13) did not affect the proliferation pattern in zone IV on Day 21. Therefore, proliferation of glandular epithelial cells in zone IV is not dependent on secretory E. These results describe the temporal changes in ER immunostaining during the onset of P action in artificial menstrual cycles and demonstrate that not only are there zone-dependent differences in response to P but also that the sensitivity of different cell-types within the endometrium is subject to temporal differences in response. The coincident temporal and zone-dependent decrease in proliferation and ER in zones I, II, and III suggests a close relationship between P-dependent down-regulation of ER and endometrial proliferation.

INTRODUCTION

The availability of a monoclonal antibody to the estrogen (E) receptor (ER) now permits immunohistochemical identification of receptor distribution and regulation in steroid target tissues composed of different cell types. In the primate endometrium, this approach is of particular value because of the classical studies of Bartelmez [1, 2], which demonstrated a horizontal zonation based on epithelial, stromal, and vascular differences. In addition, the uterus is composed of several different cell types that have been shown to respond differently to the same hormonal stimulation [3, 4]. 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.

Our previous studies in the rhesus monkey have shown different zonal patterns of mitotic control in the endometrium during E versus progesterone (P) dominance in the natural menstrual cycle [5, 6]. These studies show that different zonal patterns of proliferation in response to the same hormonal milieu are a property of the nonhuman 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 luteal phase [6]. Concomitant with this increase in proliferation in the basalis, epithelial cells in the other zones of the endometrium were dramatically reduced in proliferation as serum P rose during the secretory phase of the menstrual cycle. Thus, zone IV of the endometrial basalis becomes the dominant proliferating tissue during P dominance.

Because of its important role in growth and differentiation, we studied the regulation of the ER by both biochemical and immunohistochemical analyses in the rhesus uterus during the changeover from E to P dominance. In addition, we monitored zone-dependent changes in endometrial proliferation by immunohistochemistry using an antibody to the Ki-67 proliferation antigen [7]. Because this changeover in hormonal dominance begins at ovulation in the natural menstrual cycle, it is difficult to obtain precisely timed

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endometrial samples for analysis. To circumvent this problem, these studies were conducted with endometrial tissue obtained from ovariectomized rhesus monkeys in which the hormonal pattern of the natural menstrual cycle was simulated by sequential insertion or removal of silastic implants packed with crystalline E and P. Control of the rising and falling serum titers of E and P during these artificial menstrual cycles allows endometrial tissue to be obtained at more precisely timed periods.

MATERIALS AND METHODS

Animals

Mature female rhesus monkeys (*Macaca mulatta*) obtained from commercial sources (Hazelton Labs, AUS: CITY, PLEASE, TX) were ovariectomized for at least 2 mo prior to initiation of artificial menstrual cycles. Animals that had undergone a previous endometrectomy were rested for at least 6 mo. All protocols were approved by the Institutional Animal Care and Use Committee (AUS: LOCATION, PLEASE). 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. Animals were anesthetized with ketamine (10 mg/kg), and silastic implants were placed s.c. in the intrascapular area. Removal of implants was also performed under ketamine anesthesia.

The following protocol previously described [8] 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 of 3.0-cm length is inserted on Day 13, and two P implants are inserted on Day 16. All blood sampling or tissue collection was performed before insertion or removal of implants. Blood samples were measured for E and P by RIA as previously described [8, 9]. Serum E and P in these artificial menstrual cycles have been shown [8] to mimic those observed during the natural cycle [10]. Serum E and P data for the experimental paradigms used in these studies are shown in Table 1.

Chemicals and Buffers

[2,4,6,7-$^3H_4$]Estradiol-17β ([^{3}H]E; 93–100 Ci/mmol) was obtained from New England Nuclear (Boston, MA) and stored in ethanol (100 Ci/ml) at −20°C. Radioinert steroids were obtained from Steraloids. All other chemicals were obtained from standard commercial sources and were of reagent grade.

The following buffers were used: saline (0.154 M NaCl) buffered with 10 mM Tris-HCl, pH 7.4; buffer A_{30} (50 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol, and 30% glycerol [v/v], pH 7.5); and buffer A_{30} plus 0.5 M KCl. Dextran-coated charcoal suspension contained 0.5 g Norit-A (Sigma Chemical Co., St. Louis, MO) and 50 mg Dextran T-70 (Pharmacia Fine Chemicals, Piscataway, NJ) in 100 ml of 10 mM Tris-HCl, 1 mM EDTA (pH 7.5). Scintillation counting solution was toluene-Triton X-100 (2:1, v/v) with 5 g diphenyloxazolyl and 50 mg 1,4-*bis*-(2[5-phenyloxazolyl]-benzene) per liter.

ER Assays

Tissue was collected by endometrectomy at hysterotomy as previously described [5], and tissue biopsies for immunohistochemistry were obtained from the fundal region of the uterus. Nuclear and cytosolic ER were determined by procedures described and validated previously [11–13].

Briefly, endometrium was blotted, weighed, and placed in ice-cold buffered saline. All subsequent steps were carried out at 0–4°C unless otherwise indicated. Endometrium was homogenized in buffer A_{30} (1:20 or 1:30, w/v) in a Polytron PT-10 homogenizer (Brinkman Instruments, Westbury, NY) for three 5-sec bursts at a setting of 6. Cytoplasmic and nuclear myofibrillar fractions were separated by centrifugation (800 × *g*, 10 min), and the supernatant was centrifuged at 110 000 × *g* for 1 h to yield the particle-free cytosol fraction. The nuclear myofibrillar pellet was washed and centrifuged at 800 × *g* for 10 min. The washed nuclear myofibrillar pellet was resuspended to the original volume of homogenization with buffer A_{30} plus 0.5 M KCl and incubated for 1 h with frequent mixing, followed by centrifugation at 110 000 × *g* for 1 h. The supernatant was used for determination of nuclear ER.

For determination of total ER binding, aliquots (300 μl) of nuclear extract were incubated (1 h at 30°C) in a total volume of 500 μl with increasing concentrations of [^{3}H]E (0.32 to 5 nM). A parallel set of samples was incubated with a 250-fold excess of radioinert diethylstilbestrol for deter-

TABLE 1. Serum E and P values* during artificial and manipulated menstrual cycles in the rhesus monkey.

Cycle day	E (pg/ml)	P (ng/ml(	(−E)† E (pg/ml)
4	85.8 ± 5.2(16)	<0.05	—
6	86.4 ± 8.1(16)	<0.05	—
8	91.8 ± 8.1(15)	<0.05	—
10	98.9 ± 12 (16)	<0.05	—
11	189 ± 18 (16)	<0.05	—
12	215 ± 18 (16)	<0.05	—
13	258 ± 15 (16)	<0.05	279 ± 16(4)
14	82.6 ± 9.1(5)	2.9 ± 0.7(6)	—
15	84.2 ± 8.0(7)	3.1 ± 0.5(7)	<20(4)
16	99.9 ± 11 (7)	3.5 ± 0.4(7)	<20(4)
17	85.9 ± 12 (7)	8.5 ± 1.0(7)	<20(4)
20	97.9 ± 12 (4)	10.3 ± 1.3(4)	<20(4)
21	85.5 ± 10 (4)	11.3 ± 1.0(4)	<20(4)

*All values are the mean ± SEM. Numbers in parentheses after each value are n.

†Values in the −E column are serum E levels during the secretory phase when all E implants are removed on Day 13.

FIG. 1. Immunohistochemical analysis of ER on Days 13, 14, 17, and 21 during artificial menstrual cycles in the rhesus monkey. Panels a, b, c, d: Endometrial ER immunoreactivity on cycle Days 13, 14, 17, and 21, respectively, in zones I, II-III, and IV as labeled. ×200.

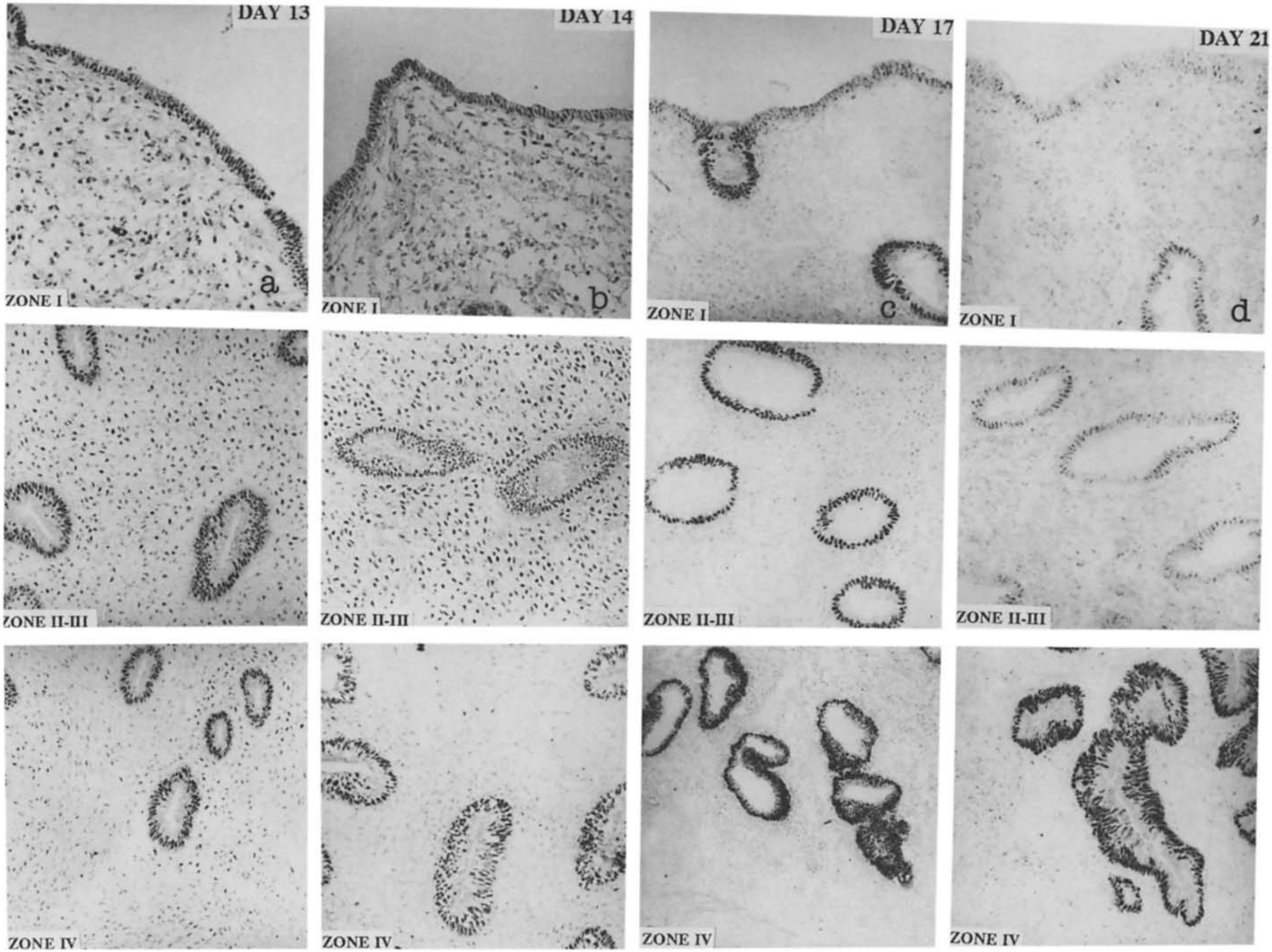

TABLE 2. Endometrial cytosolic and total and occupied nuclear ER on Days 13, 14, 17, and 21 of artificial menstrual cycles in the rhesus monkey.

	ER (pmol/mg DNA)[a]		
		Nuclear	
Cycle day	Cytosolic	Total	Occupied
13	7.56 ± 0.35	3.20 ± 0.21	1.22 ± 0.08
14	2.70 ± 0.54*	0.61 ± 0.13*	0.07 ± 0.06*
17	4.95 ± 0.42*	0.67 ± 0.22*	0.14 ± 0.11*
21	4.28 ± 0.47*	0.60 ± 0.08*	0.13 ± 0.12*

[a]All values are the means ± SEM, n = 3–5 for each value.
*$p < 0.05$ vs. Day 13.

mination of nonspecific binding. Samples were chilled (4°C) after incubation, and free steroid was removed by incubation with 500 μl of dextran-coated charcoal suspension for 10 min, followed by centrifugation at 1200 × *g* for 5 min. Radioactivity was measured in the supernatants, and specific binding (total minus nonspecific binding) was analyzed by the method of Scatchard [14]. Unoccupied receptor was determined in an identical manner except that incubation was for 24 h at 2°C. Occupied nuclear ER was estimated as the difference between total and unoccupied receptor. Total cytosolic ER was determined as described above for total nuclear ER.

Immunohistochemical Analyses of ER and 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 (−30°C) sections (6 μm) were fixed in freshly prepared 4% paraformaldehyde in Sorensen's buffer (pH = 7.4) for 15 min at room temperature for ER and Ki-67 immunohistochemistry. Sections were washed twice in PBS (pH 7.4) and incubated 10 min with blocking serum (Vector Labs., Burlingame, CA). The incubation with blocking serum and all subsequent incubations with immunochemicals were performed in a humidified chamber.

Monoclonal antibody to the human ER (H222) and Ki-67 antibody were obtained commercially from Abbott Labs. (North Chicago, IL) and Transbio (Paris, France), respectively. The avidin-biotin complex technique was used (Vector). ER and Ki-67 localization was performed as described previously [13]. Briefly, sections were treated with ER antibody or control (normal rat IgG) at a dilution of 1:2 in PBS overnight (16–18 h) at 4°C. For Ki-67 antigen localization, 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. Biotinylated secondary antibodies to the ER antibody or Ki-67 antibody (Vector) were found to produce the best results at the levels suggested by the manufacturer. Incubations were conducted with secondary antibodies for 1 h at room temperature. After being washed twice in PBS, the sections were treated with streptavidin-biotin complexes for 30 min at room temperature and then washed in PBS as described above. The antigen-antibody complex was detected by incubation with freshly prepared DAB (3′5′ diaminobenzidine) solution (0.5 mg/ml) in Tris-HCl (0.05 M, pH 7.6) containing 0.0025% hydrogen peroxide and 10 mM imidazole. Incubation of sections in DAB solution proceeded for 10 min and was followed by immersion in tap 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, Inc., NY) using Ektachrome 400 (Kodak, Rochester, NY) and Zeiss panchromatic lenses at magnifications of 200× and 400×. Methodological controls included omission of the first or second antibody or of streptavidin-biotin complex, or omission of all the above with the DAB detection system used alone. No specific staining was observed with the above controls.

Other Methods

DNA was determined by the method of Burton [15], with calf thymus DNA used as a standard.

Statistical comparisons were done by the Student's *t*-test for comparison between two means with the appropriate correction for multiple *t*-tests (Duncan's New Multiple Range test [16]).

The percentage of cells that stained positively for the Ki-67 antigen was assessed after counterstaining with toluidine blue dye. A minimum of 500 cells was counted for each tissue section.

RESULTS

On Day 13 of an artificial menstrual cycle, ER was present in glandular and luminal epithelia and stromal fibroblasts throughout the endometrium (Fig. 1, panel a), as similarly shown previously by immunofluorescence [17]. As E was withdrawn and P rose on Day 14, there was little distinct change in the pattern or intensity of ER immunostaining, although some samples showed a decrease in stromal cell staining in zone IV (Fig. 1, panel b). Glandular epithelia retained strong positive staining in all zones of the endometrium.

As serum P continued to rise on Day 17, ER immunoreactivity in stromal cells in zones I, II, and III showed declines in intensity compared to that on Days 13 and 14 (Fig. 1, panel c). Glandular development increased substantially in the endometrium, and ER staining in epithelial cells remained strong except for a decrease in the luminal epithelium. Cycle Day 21 represented the maximal stimulation of the endometrium by P (as judged by serum P level). Decreases in ER staining intensity were very apparent in zones

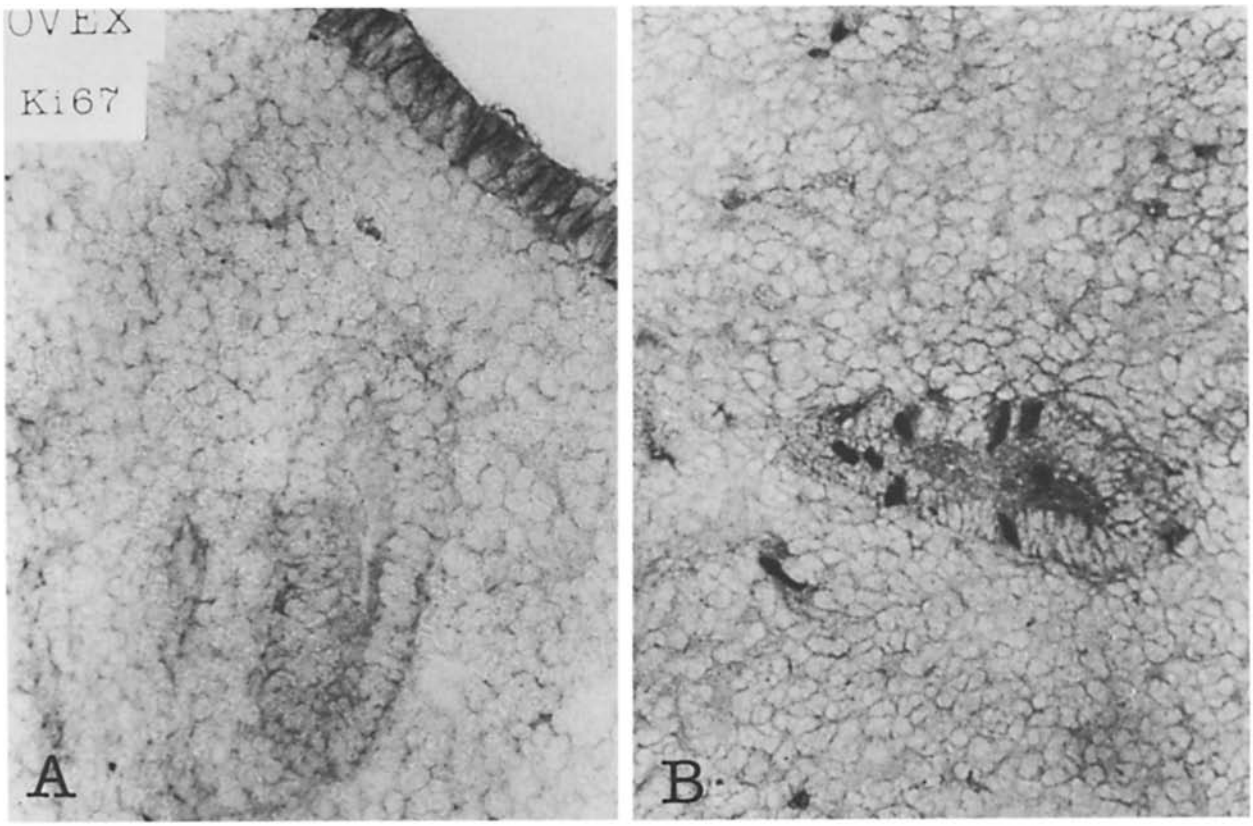

FIG. 2. Immunohistochemical analysis of the Ki67 antigen in an E-withdrawn (3 mo after ovariectomy) endometrium. A) Almost no Ki-67 immunoreactivity appears. B) A few small glands may show some staining. ×400.

I, II, and III (Fig. 1, panel d). Both stromal and epithelial cells displayed this decrease in immunoreactivity. Strong positive staining for ER remained in the glandular epithelium of zone IV. Biochemical analysis of ER showed a dramatic decrease in cytosolic and total nuclear ER between Day 13 and Day 14 (Table 2). Occupied nuclear ER was significantly decreased between Day 13 and Day 14 and remained suppressed on Days 17 and 21. Although there was a sharp reduction in cytosolic and nuclear ER binding between Days 13 and 14, the immunohistochemical profile showed little change.

In the endometrium of an estrogen-withdrawn animal (ovariectomized for 3 mo), there was almost no Ki-67 immunoreactivity (Fig. 2A), although a few small glands may show some staining (Fig. 2B). On Day 13 (peak E level), Ki-67 immunoreactivity was observed in luminal and glandular epithelial and stromal cells throughout the endometrium (Fig. 3, panel a). Twenty-four hours after the onset of rising serum P (Day 14), there was little change in epithelial or stromal cell staining (Fig. 3, panel b). On Day 17, luminal and glandular epithelia in the functionalis and upper basalis (zone III) showed sparsely stained cells, whereas epithelia in zone IV showed an apparent increase in the number of stained cells (Fig. 3, panel c). By Day 21, there were very few epithelial or stromal cells that showed positive staining for Ki-67 in zones I, II, or III (Fig. 3, panel d). In addition, the percentage of positively stained glandular epithelial cells in zone IV rose from 10.8 ± 2.2 on Day 13 to 38.0 ± 3.9 on Day 21 ($n = 4, p < 0.001$).

The hormonal stimulus for the increase in proliferation in zone IV epithelia was assessed by removal of all E implants on Day 13 and maintenance of normal serum P level through Day 21. In the absence of basal E levels during the secretory phase, zone IV proliferation continued unabated on cycle Day 21 (percentage of positively stained glandular epithelial cells was 35.3 ± 2.8, n = 4; Fig. 4). These data show that the stimulus for zone IV proliferation of glandular epithelia is independent of secretory serum E.

DISCUSSION

The mechanisms by which P regulates uterine function in order to provide an appropriate milieu for implantation in mammalian species are not clearly understood. The redirection and modulation of uterine response to E, however, does appear to play an important role in this process. One mechanism by which P modulates E action in the uterus is the down-regulation of ER in uterine target cells, leading to a heterologous desensitization to E. This general mechanism of P action has been shown to occur in the human

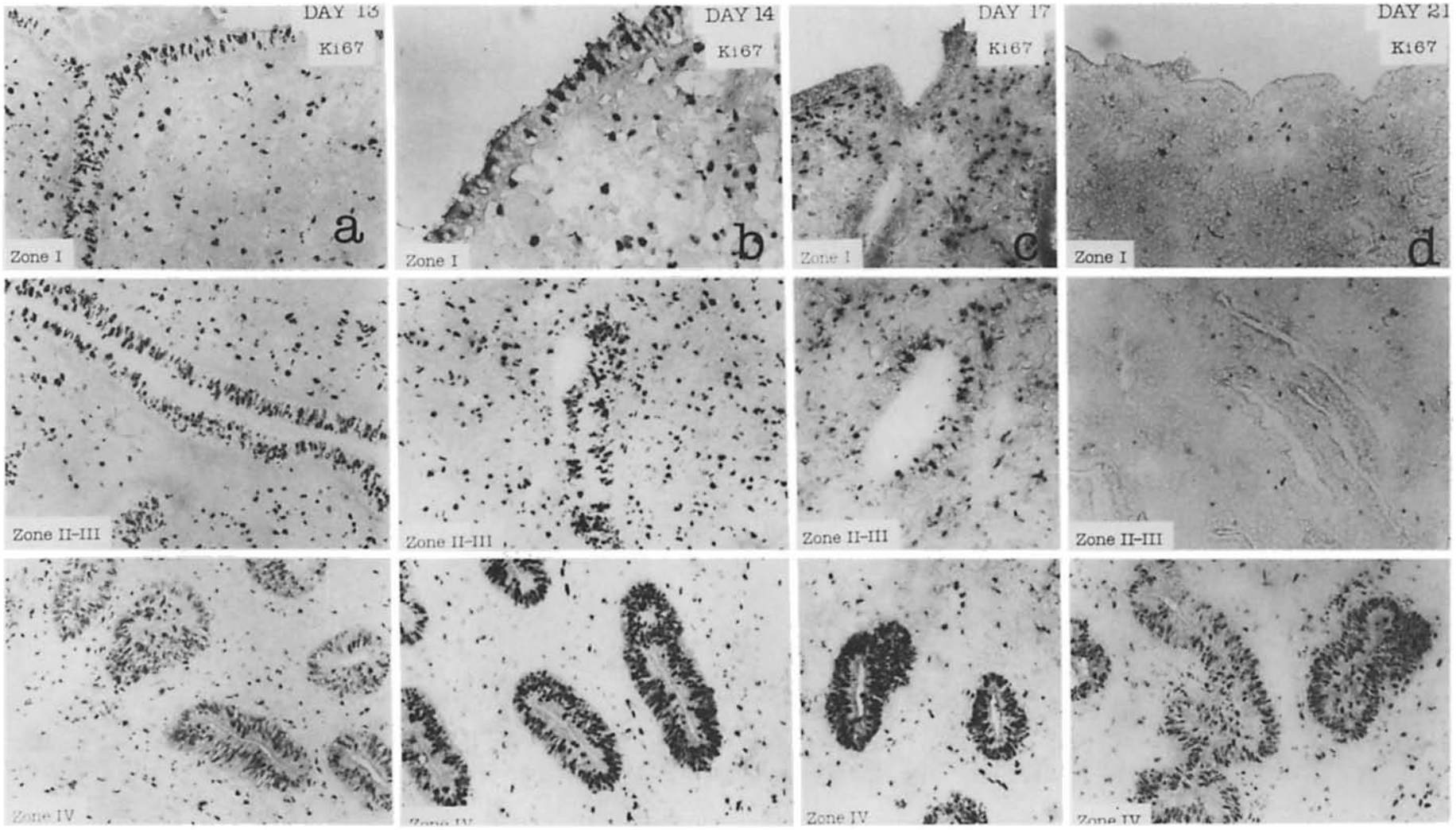

FIG. 3. Immunohistochemical analysis of Ki67 antigen (proliferation antigen) on Days 13, 14, 17, and 21 during artificial menstrual cycles in the rhesus monkey. Panels a, b, c, and d: Endometrial immunoreactivity on cycle Days 13, 14, 17, and 21, respectively, for zones I, II-III, and IV. ×200, except for zone I, panel b, ×400.

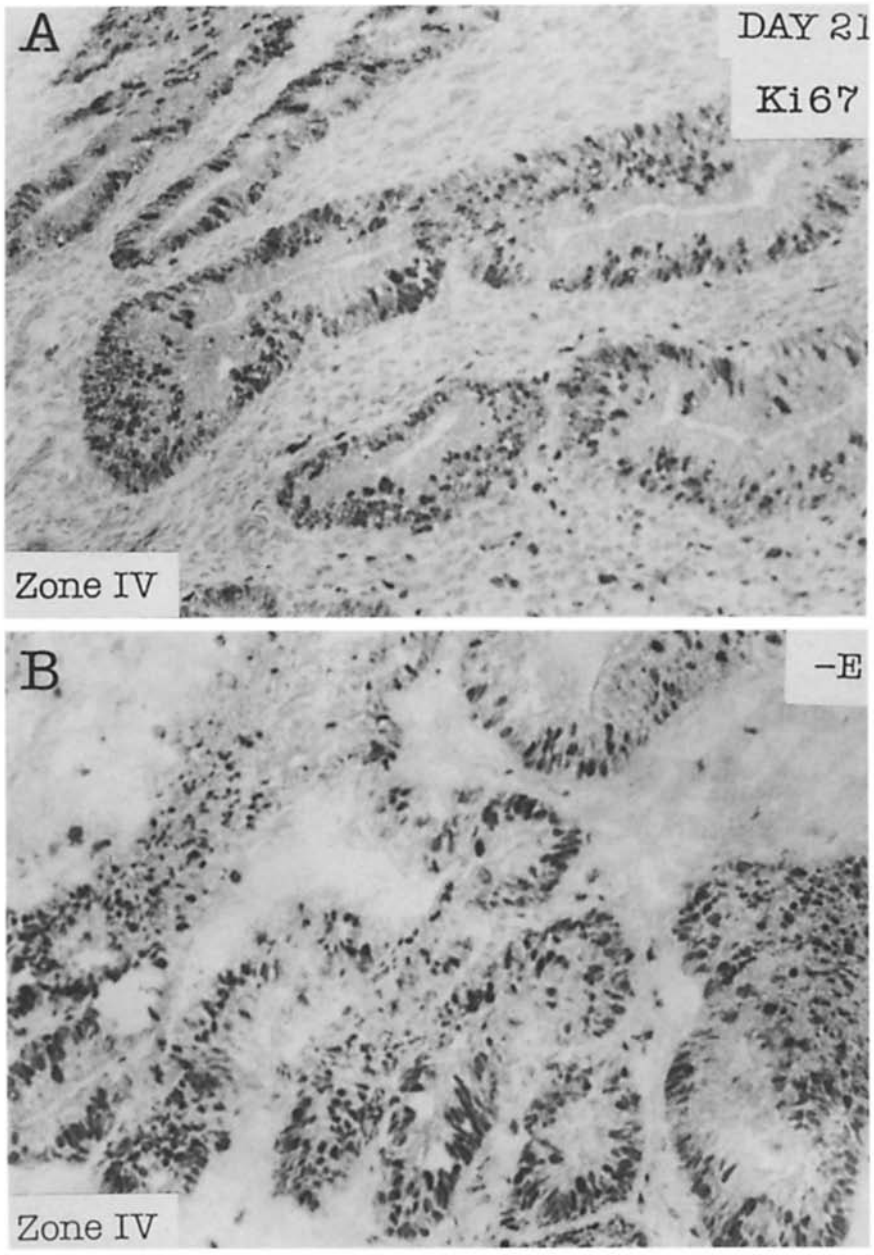

FIG. 4. A: Immunohistochemical analysis of Ki67 antigen on Day 21 of a manipulated artificial cycle (B: absence of E during the secretory phase). Endometrial immunoreactivity is shown for zone IV of the basalis. ×400.

[18], nonhuman primate [19, 20], rodent [21, 22] and avian [23] female reproductive tract. Although the down-regulation of ER by P has been well documented in several species, the biochemical assays primarily used for these studies do not permit an analysis of differential cell-type response to P action. Several studies have shown that differential cell-type response to the same hormonal stimuli occurs in the mammalian uterus [3, 4].

In the study reported here, we used both biochemical assays and immunohistochemical analyses to complement our understanding of P down-regulation of ER in the nonhuman primate uterus. Our results show that biochemically assayable ER (cytosolic, total, and occupied nuclear ER) are significantly suppressed during the secretory phase compared to the late proliferative phase. Despite these dramatic changes in ER determined by steroid binding assays, our immunohistochemical data show that the down-regulation of ER is cell-type sensitive, zone-dependent, and temporally related to the rising titers of serum P during the secretory phase in the rhesus monkey.

On Day 14, stromal fibroblasts in zone IV appeared to be the first cells to respond to P down-regulation of ER. Although the biochemical data show a sharp decrease in ER between Day 13 and Day 14, the immunohistochemical

data do not reflect this striking decrease. One explanation for this disparity is that the antibody may recognize nonbinding forms of the ER and that loss of ligand binding is an initial step in P action during the changeover from an E- to P-dominant endometrium. Glandular epithelia retained ER immunostaining through Day 17 and exhibited a temporal lag in down-regulation compared to stromal cells. Although a significant decrease in glandular ER was observed by immunohistochemical analysis on Day 21, biochemical assay of ER showed no further decrease. It is possible that the increase in proliferating cell population and/or increases in ER level in zone IV glandular epithelia offset the immunohistochemical decreases in ER.

The down-regulation of stromal ER by P was clearly observable in zones I, II, and III on Days 17 and 21 as serum P titers reached their maximal level. Indeed, the loss of stromal ER was coincident with the loss of luminal and glandular epithelial proliferation in these zones on Day 17. Previous studies by Cunha and associates have shown that stroma can profoundly influence the response and differentiation of adjacent epithelia [24]. Whether a stromal-epithelial interaction may play a role in the regulation of proliferation by P in endometrial epithelia is unclear at present.

Although ER was dramatically down-regulated in zones I, II, and III during peak serum P levels, strong positive immunoreactive staining for ER was retained in the glandular epithelium of zone IV. Strong positive staining for the P receptor has also been also observed in zone IV glandular epithelia during P dominance on Day 23 [13]. These results show that there is a zone-dependent regulation of ER by P in the rhesus uterus and provide further support for a cell-type-specific and zone-dependent regulation of ER by P. Similar zone-dependent changes in ER during the late luteal phase in the human [25–28] and nonhuman primate [29, 30] have been described previously. However, studies in the human that have addressed stromal versus epithelial cell response [27, 28] to P have not shown the temporal sensitivity of stromal cells to P down-regulation of ER as shown in our studies. This may in part be attributable to the difficulty in precise dating of human endometrium samples. ER staining was, however, shown to be absent in stroma of the functionalis during the luteal phase (Day 26) in the baboon while some glandular epithelia cells continued to show positive staining for ER [30]. The latter study in the baboon also showed an absence of stromal ER staining in the basalis with the maintenance of strong positive staining in the glandular epithelium.

Different zonal patterns of cellular proliferation have been observed during E or P dominance in the natural menstrual cycle of the rhesus monkey [5, 6]. These studies, using [^{3}H]thymidine uptake in vivo, have shown that during P dominance epithelial proliferation is strongly inhibited in endometrial zones I, II, and III, whereas mitotic activity in zone IV is maintained and increased 10-fold compared to late proliferative phase endometrium. The results of our present study, using immunohistochemical analyses of the Ki-67 antigen and cell counting, show a similar pattern of change in endometrial proliferation during P dominance. These data show that P down-regulation of both ER and Ki-67 is a zone-dependent and cell-type-specific response.

The maintenance of strong positive immunoreactive staining for ER in zone IV of the basalis in combination with the maintenance of cellular proliferation might suggest that E continues to exert a proliferative stimulus on glandular epithelial cells in this endometrial zone. Although glandular ER in zone IV escape P down-regulation, no direct evidence to date supports a role for E as the mitogenic stimulus in this zone. Proliferation of glandular epithelia in zone IV increases during P dominance, and this proliferation is independent of secretory E. These data suggest a potential role for P either directly or indirectly as a mitogen for glandular epithelia in zone IV. Alternatively, recent studies in the rabbit have suggested that the glandular epithelium in the basalis of the endometrium may possess a regenerative capacity not wholly dependent on E or P [31]. Further studies are required to determine the precise mitogenic stimuli that regulate zone IV proliferation during the primate secretory phase.

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