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A Zonal Pattern of Cell Proliferation and Differentiation in the Rhesus Endometrium During the Estrogen Surge

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ABSTRACT

The cellular and tissue basis of endometrial renewal in the rhesus monkey is being investigated by radioautographic localization of proliferating cell populations. Here we report our findings on epithelial cell proliferation during the midcycle estrogen surge. Endometrial biopsies were obtained by hysterotomy at approximately 1 h after a single intravascular injection of [^{3}H] thymidine ([^{3}H]T). Light and electron microscopic radioautography was performed on 7 specimens obtained from 4 monkeys in relation to the serum estradiol (E_2) peak as follows: −2, −1, 0, +1, +2, and +3 days (± 1 day). Cell proliferation and differentiation were analyzed according to the 4 horizontal histologic endometrial zones (Bartelmez et al., 1951). Epithelial labeling indices were higher in the functionalis (Zone I, luminal epithelium, 9–12%; Zone II, uppermost gland segments, 7–14%) than in the basalis (Zone III, middle gland segments, 5–7%; and Zone IV, basal gland segments, 1–7%). Despite the large and rapid serum E_2 fluctuations during the surge from −2 days to +3 days E_2 peak, proliferating epithelial populations within Zones I, II and III remained quite uniform in size. In the basalis, the proliferative patterns of Zones III and IV were dissimilar. The labeling index of Zone III remained quite uniform (5–7%), whereas in Zone IV, it increased progressively from 1% (−2 days) to 7% (+3 days). These data establish the bipartite nature of the basalis. Radioautographic evidence indicates that endometrial cell proliferation is tightly coupled to progressive cell differentiation in the functionalis and basalis. Thus intrinsic positional differences exist in the responsiveness of the primate endometrium to common hormonal stimulation during the E_2 surge and the initial postovulatory rise of progesterone.

INTRODUCTION

The endometrium of menstruating primates provides a challenging system for study of cyclic cell growth and differentiation. It is composed of two distinct functional tissue compartments, the upper transient functionalis which is formed and shed during each cycle and the deep germinal basalis which persists from cycle to cycle (Fig. 1). The functionalis has a limited life span within a cycle, as well as during pregnancy when it becomes the maternal placenta. The cellular basis of regeneration from the basalis remains for the most part undefined. Preliminary observations indicate that preparations for postmenstrual endometrial regeneration have occurred during the middle of the previous cycle with production of progenitor cells (Padykula et al., 1983).

The proliferative response of the primate endometrium during the menstrual cycle has not yet been specifically correlated with temporal changes in the serum concentrations of estrogen and progesterone. Epithelial mitotic activity is highest during the proliferative phase in the rhesus monkey (Bensley, 1951) and

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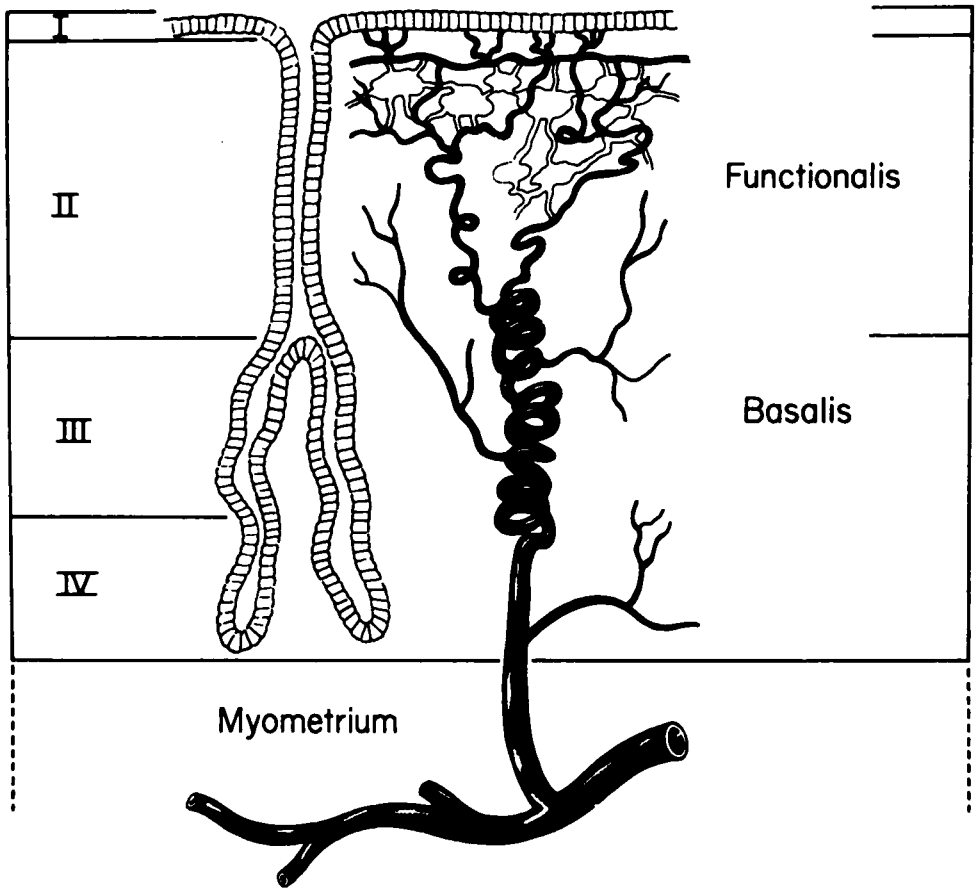


FIG. 1. Zonation in the rhesus endometrium reflects a proximodistal structural and functional gradient. Four distinct horizontal zones are recognizable on the basis of epithelial, stromal and vascular differences. The transient functionalis consists principally of Zone I (luminal epithelium and subjacent stroma) and Zone II (straight uppermost glandular segments and microvasculature). The germinal basalis is composed of Zone III (branching and coiled glandular segments) and Zone IV (glandular fundi and large blood vessels). (Modified from Blandau, 1983.)

human (Ferenczy et al., 1979). All tissue components, epithelial, stromal and vascular, are involved to varying degrees in this preovulatory hyperplasia. As the corpus luteum becomes established and plasma progesterone rises, epithelial proliferation in the functionalis begins to subside, while it continues in the stromal compartment in relation to the decidual response and expansion of the vasculature. Existing observations suggest that mitotic activity may increase in the deep basalis during the early secretory phase in the monkey (Bensley, 1951) and women (Ferenczy et al., 1979).

Current knowledge of mammalian epithelial renewal rests heavily on patterns of proliferation and differentiation which have been defined for the small intestinal mucosa, epidermis and seminiferous epithelium (Potten et al., 1979; Leblond, 1981). There is a specific locus or microenvironment within these tissues for stem cells which comprise a small population of relatively undifferentiated cells that divide slowly and are self-maintaining. Stem cells give rise to immediate progeny with high proliferative capacity which amplify the cell populations through a limited number of divisions. Such second-order cells have been referred to as transient amplifying cells (Potten et al., 1979; Lavker and Sun, 1982, 1983). Amplifying cells are viewed as progenitors for a specialized cell line or type. Postmitotic progeny arise and proceed through further differentiation until

the terminus of specialization has been attained. Cell death is a necessary component of these renewing systems. This unifying hypothesis provides a framework for interpreting the cellular mechanisms that underlie rapid tissue renewal in adult mammalian organs. It also fits the replacement system for erythrocytes and granulocytes in the bone marrow (Potten et al., 1979).

To determine the applicability of this hypothesis to cyclic endometrial renewal in menstruating primates, this initial study is directed toward radioautographic qualitative and quantitative analysis of cell proliferation during the mitogenic stimulus of the estrogen surge. To map the endometrial mitogenic response in the rhesus monkey during the estrogen surge, a single intravascular injection of [^{3}H] thymidine ([^{3}H] T) has been made in vivo on various days during midcycle. Our radioautographic analyses localize cells in the S-phase in relation to the horizontal division of the rhesus endometrium into 4 zones in which the functionalis is composed of Zones I and II and the basalis consists of Zones III and IV (Fig. 1) (Bartelmez et al., 1951).

We have determined that epithelial cell proliferation in the functionalis and basalis varies zonally and is tightly coupled to progressive cellular differentiation. The zonal epithelial labeling indices remain quite uniform during the E_2 surge except for that of the deep basalis (Zone IV) which increases with time. Cell replacement in the primate endometrium resembles most closely the process for production of erythrocytes and granulocytes in the bone marrow.

MATERIALS AND METHODS

Determination of Cyclicity

Rhesus monkeys (proven breeders) were housed at the New England Regional Primate Research Center in Southboro, MA. Cyclic activity was followed by daily vaginal smears as well as by radioimmunoassay (RIA) determination of serum estradiol (E_2) (Longcope et al., 1974) and progesterone (P) (Abraham et al., 1971), using celite chromatography for separation. Blood samples were taken at 1- to 3-day intervals during the menstrual cycle. Using the average length of the menstrual cycle, previous RIA cyclic patterns of serum estrogen and progesterone concentrations for each monkey, and the onset of menses, we estimated the period of the estrogen surge for timing the administration of each single intravascular injection of [^{3}H] thymidine ([^{3}H] T) to label cells entering the cell cycle.

Seven endometrial biopsies were taken from 4 different monkeys by laparotomy and hysterotomy at 1 h after [^{3}H] T injection. The accuracy of our temporal estimate of the E_2 surge was determined through gross examination of both ovaries for development of the dominant follicle (e.g., 8 mm follicle at −1 day) or corpus luteum (e.g., corpus hemorrhagicum at +3 days) as well as the color of the fimbria and sex skin. Retrospectively, the RIA patterns of serum E_2 and P, as well as the histological organization of the endometrium, contributed to identification of the day of the cycle. Our cumulative estimates varied by ±1 day and were determined by correlation of the above parameters. In addition, one biopsy was obtained at 1 mo following ovariectomy.

Labeling of Cells in the S-Phase of the Cell Cycle

This investigation centered on light and electron microscopic radioautographic analysis of 8 endometrial biopsies obtained from 4 monkeys approximately 1 h after a single injection of methyl-[^{3}H] T (5 or 10 mCi per kg BW; spec. act. 50–80 Ci/mmol, New England Nuclear Co. Boston, MA) into the femoral vein. These injections were made on the following cycle days in relation to the E_2 peak: −2, −1, −1, 0, +1, +2 and +3 days. Nowakowski and Rakic (1974) demonstrated that in pregnant monkeys after such a single injection, most of the [^{3}H] T is cleared from the circulation by 10 min. This rapid removal presents the advantage of a relatively short pulse labeling of DNA. An interval of at least 7 mo intervened between injections of [^{3}H] T into the same monkey. Sufficient clearance from cellular compartments had occurred since the previous injection.

Surgical and Biopsy Procedures

Preoperative procedure included sedation by ketamine hydrochloride (10–20 mg/kg BW), intramuscular atropine (1/120 grain), and the insertion of an endotracheal tube. After injection of the [^{3}H] T, the animal was prepared for surgery. Anesthesia was induced and maintained by halothane/oxygen given in a closed system. Sterile saline (0.9% NaCl) was administered at a rate of 25 ml/h during surgery. After laparotomy via a midline incision through the abdominal body wall, the endometrium was first exposed through an anterior-posterior incision, usually along the midventral aspect of the uterine corpus. Because of the tautness and thickness of the myometrium, an additional transverse incision at right angles to the longitudinal incision was sometimes made to facilitate visualization of the endometrial-myometrial junction.

The biopsy specimens were isolated with a scapel via 2 techniques. *1*) A horizontal slice was made just below the endometrial-myometrial junction. The myometrial end was grasped by forceps to provide a myometrial tab to avoid handling the delicate endometrium, as well as to provide orientation for determination of the plane that passes longitudinally through the uterine glands. *2*) A vertical slice was removed that included the entire thickness of the uterine wall. Excessive contraction of the myometrial component of the biopsy may lead to distortion of the endometrium. Later cellular analysis depends heavily on the degree of precision in the identification of the longitudinal plane through the endometrial glands during the first trimming of the biopsy. Longitudinal sections through the endometrial glands were necessary for zonal interpretation of differentiation and prolifer-

ation. The oriented specimen was divided vertically into at least three parts for structural preservation in 3 fixatives that contained 0.1 M phosphate buffer: *1*) 2% formaldehyde, 2.5% glutaraldehyede and 0.1% picric acid; *2*) 4% glutaraldehyde and 2% formaldehyde; and *3*) 3% glutaraldehyde. Because zonal differences in epithelial glycogen storage exist, 0.1 M phosphate buffer was used to enhance glycogen preservation. Also for this same reason, en bloc staining with uranyl acetate was omitted for most blocks. The use of several fixatives was prudent because, as with other rapidly differentiating systems, the response of the endometrium to a given fixative may vary in accordance with the extant physiologic state.

After osmication and dehydration, the blocks were flat embedded in Epon for light and electron microscopic analysis. The first goal was the identification of well-preserved blocks in which the plane-of-section passed largely along the longitudinal axes of the glands. This was determined with semithin sections (0.75 μm) stained with toluidine blue. Blocks that included all 4 endometrial zones (see *Results*) were preferentially selected for light microscopic radioautography. Camera lucida drawings (Fig. 2) of radioautographs were prepared as maps to zonal identification of glandular profiles during determinations of the labeling indices, as well as for zonal transmission electron microscopic analysis of ultrathin sections mounted on 200-mesh grids and formvar-coated one-slot grids. Such maps were essential for zonal analysis of labeling indices at the light microscopic levels and for accurate zonal ultrastructural characterization of cells in the S-phase of the cell cycle.

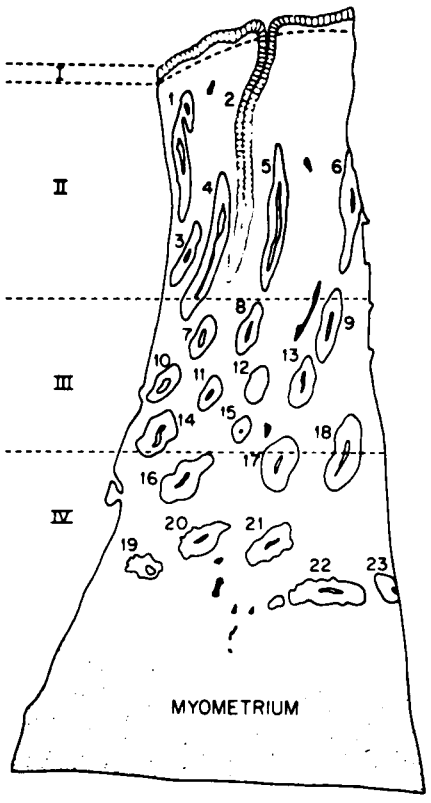


FIG. 2. Camera lucida tracing of a 2-μm Epon section of rhesus endometrium (−2 days E_2 peak). Zonal boundaries are determined by microscopic analysis. The endometrial glands are tubular and coiled to varying degrees. Hence, preparation of a 2- μm section produced pieces or "profiles" of glands. The gland profiles are numbered to create a structural map. Such tracings are necessary guides for determining zonal labeling indices and for specific identification of ultrastructural zonal characteristics.

For light microscopic radioautography, semithin Epon sections (2 μm) were prestained with the periodic acid-Schiff reaction and hematoxylin. These sections were coated with Kodak NTB2 emulsion (Kopriwa and Leblond, 1962) at 75% humidity and 27°C and exposed at 4°C. Determination of background in the emulsion was based on counts of 10 areas equal to 1000 μm^2. The labeling index (% of labeled nuclei in the S-phase of the cell cycle within the total population counted) was determined zonally. Usually, at least 2000 cells were counted in 3 different blocks for each of the 4 endometrial zones, with specific reference to the numbered gland profiles in camera lucida maps (Fig. 2). A labeled cell had 5 or more grains over the nucleus.

For electron microscopic radioautography, the flat substrate or dipping method was used (Kopriwa, 1973). Sections were placed on celloidin-coated slides which were then dipped in Ilford L4 emulsion diluted 1:3 (emulsion:distilled H_2O). After appropriate exposure, the slides were developed at 20°C in D-19 diluted 1:10 (D-19:distilled water) for 1 min, rinsed in distilled water for 30 sec, and fixed in sodium thiosulfate for 2 min. The celloidin film-section-emulsion sandwich was then stripped onto distilled water. Grids were placed on top of the sections and picked up with parafilm. Prior to poststaining, grids were immersed in glacial acetic acid for 2 min to make the celloidin film porous. The grids were then stained in 2.5% aqueous uranyl acetate for 3 min and Reynolds lead citrate for 5 min.

RESULTS

Endometrial Zonation

A proximodistal functional polarity exists in rhesus endometrium which is reflected histologically by 4 horizontal zones (Bartelmez et al., 1951) (Fig. 1). The distribution of epithelial proliferative activity (labeling index) during the E_2 surge was analyzed by radioautography with respect to this zonation. The functionalis, which is reformed and later shed during each cycle, is composed of Zones I and II. Zone I consists of a simple columnar luminal epithelium and the immediate subjacent connective tissue which usually has a more diverse population of stromal cells. Zone II consists of the upper straight portions of the glands and the microvasculature. The transition from the

functionalis to the basalis occurs at the junction of Zones II and III and is marked by branching and coiling of the glands. Zone IV contains the fundi of the glands, some of which may extend partly into the adjacent myometrium. The larger portions of the coiled arteries are located in Zones III and IV; they terminate in the upper zones as a network of arterioles and large diameter capillaries.

Although the functional activities of the 4 zones are not precisely known, they may reflect the proximodistal physiologic gradient created by the distribution of the vascular blood supply. The epithelial cells of Zones I-IV differ in ultrastructure and in cell height. Ciliated cells are sparsely interspersed among the secretory cells in Zones II-IV. Zonal stromal variation consists of differences in the relative proportions of the collagen and proteoglycan components in the extracellular matrix, as well as in the degree of leukocytic infiltration. After the menses, the endometrium consists of Zones III and IV and possibly irregular portions of Zone II (Bartelmez et al., 1951).

Determination of Epithelial Labeling Indices at Midcycle

The E_2 surge is the major mitogenic stimulus during the menstrual cycle. Zonal distribution of mitotic activity was determined in endometrial biopsies at 7 different time points during the E_2 surge (Fig. 3). The [^{3}H]T labeling indices of the functionalis (Zones I and II) were remarkably uniform from −2 and +3 days E_2 peak (I, 9−12%; II, 7−14%) (Fig. 4), despite the progressively changing serum E_2 concentrations. This proliferating population in the functionalis was approximately twice that of the basalis. Within the basalis, the labeling index for Zone III over this 6-day period also remained quite uniform but at a lower level (5−7%). However, Zone IV differed in that the labeling index rose steadily from 1% (−2 days) to 7% (+3 days) (Fig. 4), while those in zones I−III remained at their respective levels. Progressive change in proliferation with time sets Zone IV apart from the upper 3 zones in which epithelial labeling indices remained steady during this period. The magnitude of the mitogenic stimulus of the E_2 surge could be estimated by comparison with endometrial zonal labeling indices at 1 mo after ovariectomy (OVEX) (Fig. 4). The existence of zonal differences in proliferation in the OVEX monkey suggested that zonation was an intrinsic property of the primate endometrium.

This comparison demonstrated that the rapid rise and then fall in serum E_2 levels did

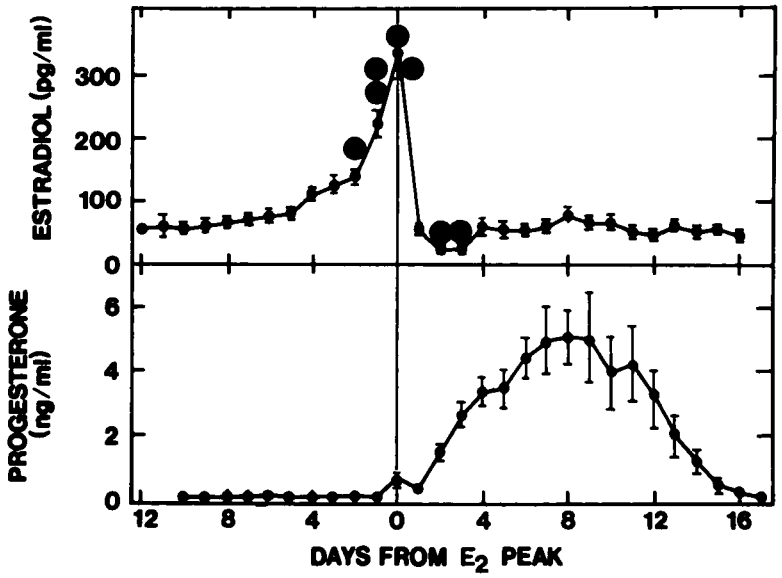


FIG. 3. Endometrial biopsies obtained during the E_2 surge. Seven endometrial biopsies were obtained at approximately 1 h after a single intracascular injection of [^{3}H]T into 4 monkeys at midcycle. The day of the biopsy was determined retrospectively by radioimmunoassay of serum estradiol and progesterone as well as gross and histologic criteria. These biopsies were taken on −2, −1 (2), E_2 peak, +1, +2 and +3 days. These time points are displayed against the normalized serum levels of estrogen and progesterone as presented by Knobil (1974).

Midcycle Zonal Distribution of Mitotic Activity Rhesus Monkey Endometrium Epithelial Labeling-Index[1]

Day of Cycle	Zone I	II	III	IV
-2	11% (841)	10% (5023)	6% (3969)	1% (3293)
-1	10% (1630)	10% (4897)	5% (4522)	2% (3564)
-1	------	14% (2945)	7% (4279)	3% (4383)
0	11% (856)	7% (3005)	5% (2947)	4% (2974)
+1	11% (2103)	11% (8579)	5% (2020)	5% (1806)
+2	12% (12055)	13% (19870)	7% (2746)	4% (11785)
+3	9% (1429)	11% (5141)	6% (4878)	7% (4472)
OVEX[2]	5% (2122)	2% (2702)	1% (2157)	1% (2024)

[1] Endometrial biopsies taken at 1 hr. after ^{3}H-thymidine injection. A minimum of 2,000 cells/zone/specimen was used to determine L.I.

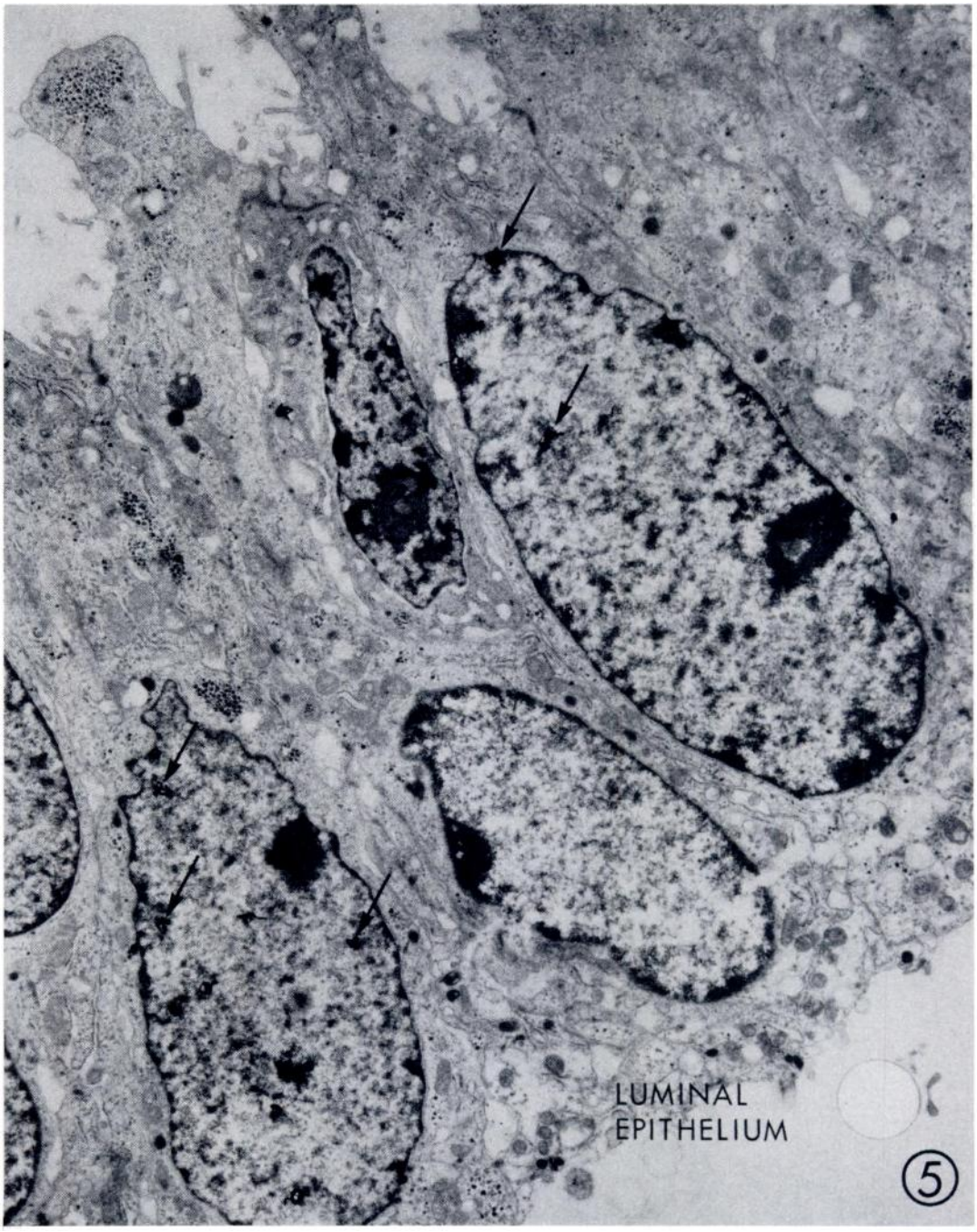
[2] Endometrial biopsy obtained at 1 month after removal of ovaries.

FIG. 4. Epithelial labeling indices (LI) of 7 endometrial biopsies obtained from 4 cyclic monkeys during the E_2 surge and 1 biopsy from an OVEX monkey at 1 mo following removal of ovaries. Labeling indices were determined at 1 h after [^{3}H]T injection. Determination of the day of cycle at which the biopsy was taken varies ±1 day. The *numbers in parentheses* indicate the total number of nuclei counted. Standard deviations of zonal labeling indices (n=3) of late proliferative specimens (−2, −1, −1 day) were: II, 11% (±2); III, 6% (±1); IV, 2% (±1); and for early luteal specimens (+1, +2, and +3 days) were: I, 11% (±1); II, 12% (±1); III, 6% (±1) and IV, 5% (±1).

not noticeably affect the proliferative activity of Zones I, II and III. Also, during the early postovulatory rise in serum progesterone, the sizes of the proliferating pool in Zones I–III remained quite constant, whereas in Zone IV the labeling index rose from 1% (−2 days) to 7% (+3 days). As serum progesterone began to rise from +2 to +3 days, the proliferating pool doubled in Zone IV. Thus, positional differences existed in proliferative response of endometrial cells to the same hormonal stimuli.

Coupling of Endometrial Cell Proliferation and Differentiation at Midcycle

To determine the nature of cells labeled in the S-phase of the cell cycle, ultrastructural



FIGS. 5–10. Electron microscopic radioautographs prepared from endometrial biopsies obtained 1 h following a single intravenous injection of [^{3}H]T. Figures 5, 8 and 9 were taken from a biopsy obtained at +2 day E_2 peak. The biopsy in Fig. 8 was obtained at +1 day E_2 peak. Figures 7 and 10 originated from a biopsy obtained at the E_2 peak (Day 0).

FIG. 5. Luminal epithelial, Zone I. The *arrows* indicate silver grains that mark 2 luminal epithelial cells in the S-phase. The specializations are distinct apical projections, glycogen storage, and cell polarity with respect to the distribution of the membrane systems. X7700.

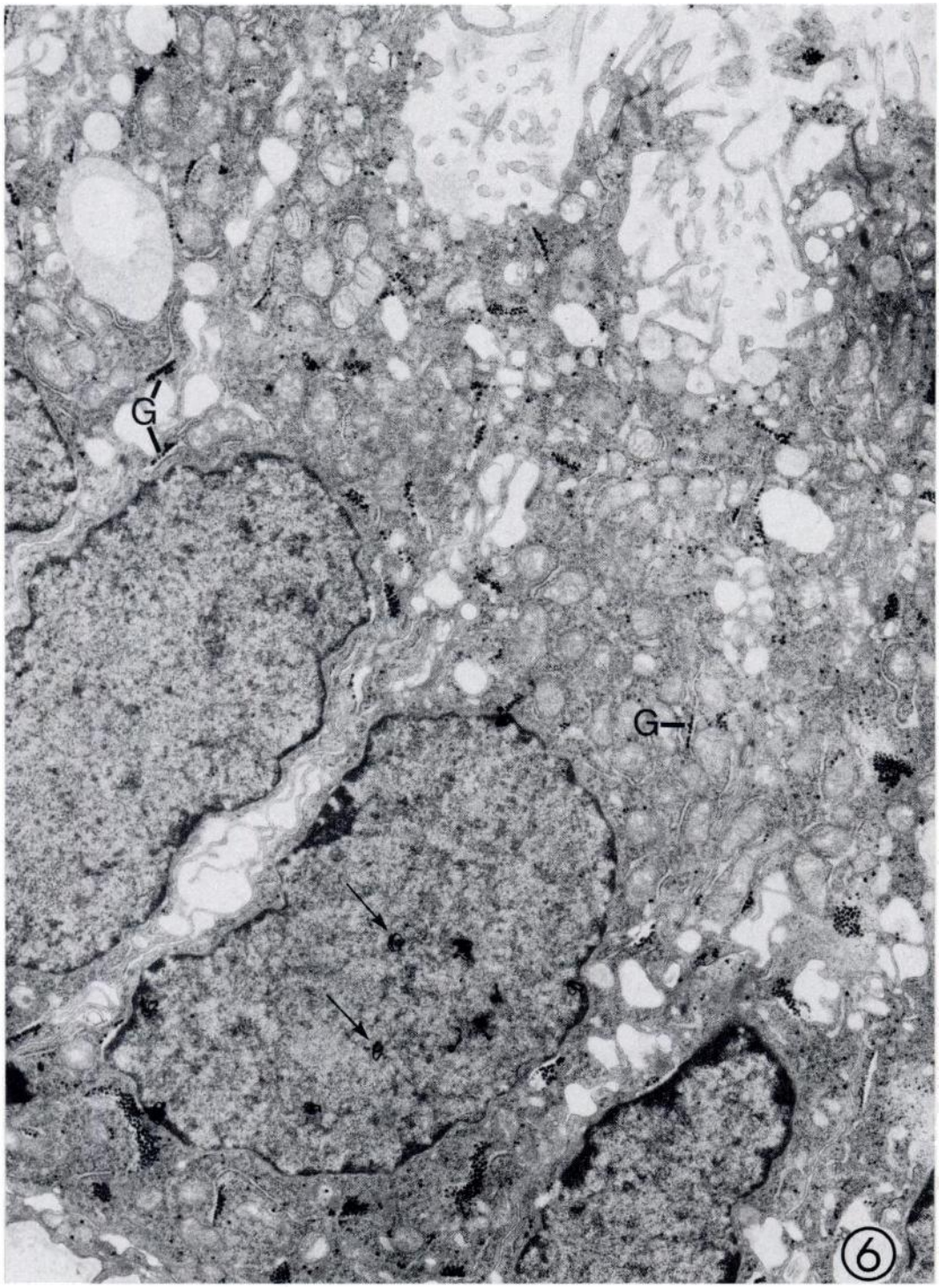


FIG. 6. Glandular epithelium, Zone II. *Arrows* indicate silver grains over a nucleus. Note the pleomorphic cell surface and subjacent vacuoles indicating endocytosis or exocytosis. The dilated intercellular spaces may reflect fluid and ionic transport. Glycogen deposits (*G*) occur intermittently along certain cytoplasmic surfaces of the endoplasmic reticulum. ×10,200.

radioautographs were analyzed. It is important to state that at the electron microscopic level all labeled and unlabeled cells observed possessed distinctive structural features in all zones of the endometrium. In other words, division of cells accompanied cytoplasmic differentiation during the E_2 surge and initial rise of serum progesterone. Undifferentiated uterine luminal or glandular epithelial cells were not observed, except for developing ciliated cells which are relatively rich in free polysomes and sparse in endoplasmic reticulum.

Ultrastructural features of S-phase endometrial cells at 1 h after [^{3}H]T injection are illustrated in Figs. 5–10: luminal epithelial cell (Fig. 5), secretory epithelial cell (Figs. 6 and 7), fibroblast (Fig. 8), endometrial granular cell (Fig. 9), and an intraepithelial monocyte-macrophage (Fig. 10). It is evident that these cell types possessed distinctive structural characteristics. Special features of these cell types are indicated in the legends to the figures. Further comment is, however, necessary for Figs. 7 and 10. Figure 7 represents a secretory glandular cell (Zone II) involved in DNA synthesis while remodeling of the cytoplasm was proceeding. Autophagic regression was evident in the immediate supranuclear position. The smooth membrane of the autophagic vacuoles enclosed remnants of mitochondria and small opaque granules that may be glycogen or polysomes. Figure 10 challenges interpretation because an intraepithelial monocyte-macrophage was in the process of DNA synthesis. Approximately 1 h elapsed between the injection of [^{3}H]T and excision of this biopsy; whether the cell was labeled in its intraepithelial tissue location, or within the blood or bone marrow compartment is unknown. Intraepithelial monocytes and macrophages have been identified in rodent uteri (Padykula and Campbell, 1976; Tansey and Padykula, 1978), where they may be involved in removal of effete epithelial cells (Sandow et al., 1979).

DISCUSSION

The endometrium of primates possesses distinct proximodistal physiologic polarity which reflects the distribution of the vascular supply as well as cyclic regenerative potential (Padykula, 1980). This polarity is further defined here through studies on the variation in epithelial proliferative patterns. At midcycle in rhesus monkeys and women, the proliferating epithelial cell population in the functionalis is larger than that of the basalis (Bensley, 1951; Ferenczy et al., 1979). This important difference is here verified and further specified by data on zonal [^{3}H]T incorporation in the rhesus endometrium during the estrogen surge.

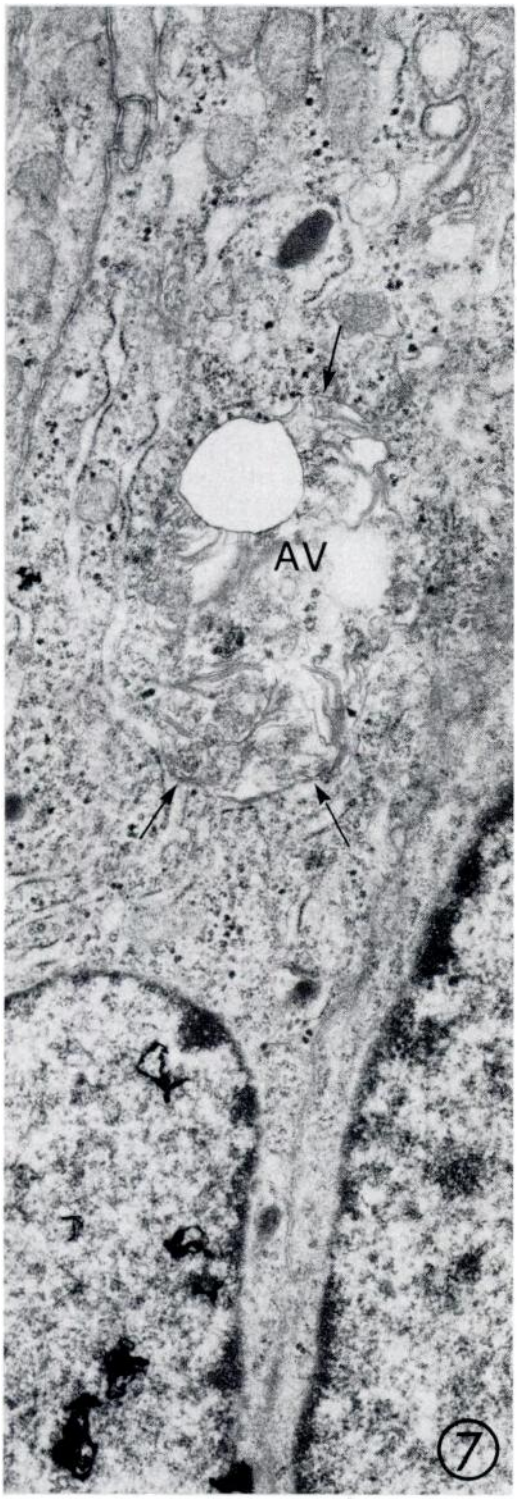
Zonation of Proliferative Activity

Our analysis of the distribution of endometrial epithelial proliferative activity provides functional evidence in support of zonal differences. During the E_2 surge, the average labeling indices of the rhesus functionalis (10%) are twice those of the basalis (5%). Within Zones I, II and III the magnitude of the mitogenic response remains relatively constant during the E_2 surge. This uniformity in mitotic activity across time unites the upper germinal basalis (III) with the transient functionalis. On the other hand, the smaller size of the proliferating epithelial population in the upper basalis sets it apart from the functionalis. Notably different is the lower germinal basalis (IV), in which the size of the proliferating pool enlarges over this 6-day period from 1% to 7%. Of potential importance is the approximate doubling of the epithelial cells entering the proliferating pool in Zone IV from +2 to +3 days, because at this time serum E_2 concentration has fallen while serum progesterone is rising. These striking zonal differences in mitotic response to the same systemic hormonal milieu point toward the existence of zonal variation in the stimulation of intracellular control mechanisms by estrogen and progesterone.

Support for the intrinsic nature of zonation is evident in the endometrial labeling indices of the OVEX rhesus (Fig. 4). At 1 mo following ovariectomy, the proliferating pool in the functionalis remains greater than that of the basalis. The OVEX uterus may represent a steady-state basal condition in which the number of dividing cells equals the number of cells dying.

Limitation of the Midcycle Proliferative Response in the Rhesus Endometrium

To the target cells the midcycle E_2 surge presents a strong hormonal stimulus which, after the E_2 peak, is withdrawn abruptly. It is puzzling that, despite large serum hormonal fluctuations during the surge, the proliferating pools of epithelial cells in Zones I, II and III of the rhesus endometrium remain remarkably uniform in size. After the abrupt decline of



serum E_2, the intracellular effects of estrogen binding by uterine target cells may remain for awhile through activation of intracellular mechanisms related to cell growth. Our evidence indicates that the proliferative pools in the rhesus functionalis and upper basalis are maintained through the 3rd day after the E_2 peak (Fig. 4). In normal human and rhesus endometria, the total concentration of estrogen and progesterone receptors is highest during the later proliferative stage (Bayard et al., 1978; Baulieu et al., 1980; Kreitmann-Gimbal et al., 1980). During the early luteal phase, it is likely that estrogenic stimulation persists to some degree in the target cells while progesterone action is commencing. This interpretation fits well with in vitro radioautographic evidence (Ferenczy et al., 1979) which indicates that the uptake of [^{3}H]T by the upper third of the normal human functionalis during the first postovulatory days is similar to that of the late proliferative stage.

Our data on the rhesus endometrium suggest that maximal in vivo rates of epithelial divisions may be achieved at levels of E_2 stimulation considerably lower than those extant during the E_2 peak. In other words, there appears to be a "ceiling" on the magnitude of the mitotic response within normal endometrial epithelial cells in Zones I, II and III to continuous in vivo estrogenization. These data present the possibility that the full excursion of the E_2 surge may be primarily a signaling system to the hypothalamus for the LH surge. It will be important to determine experimentally the magnitude and duration of E_2 stimulation that will produce an epithelial labeling index of 10% in the functionalis of an OVEX monkey.

The data presented in Fig. 4 represent the first attempt at in vivo determination of zonal labeling indices in the primate uterus. Obtaining carefully timed and well-oriented endometrial biopsies has been difficult to execute. Thus, while recognizing the preliminary nature of our observations, we were impressed by the uniformity of the labeling indices across time in

FIG. 7. Glandular epithelium, Zone II. This cell with a distinctly labeled nucleus was involved in DNA synthesis while undergoing cytoplasmic reorganization, as indicated by the supranuclear autophagic vacuole (*AV*). *Arrows* mark the limiting membrane of the vacuole. X17,100.

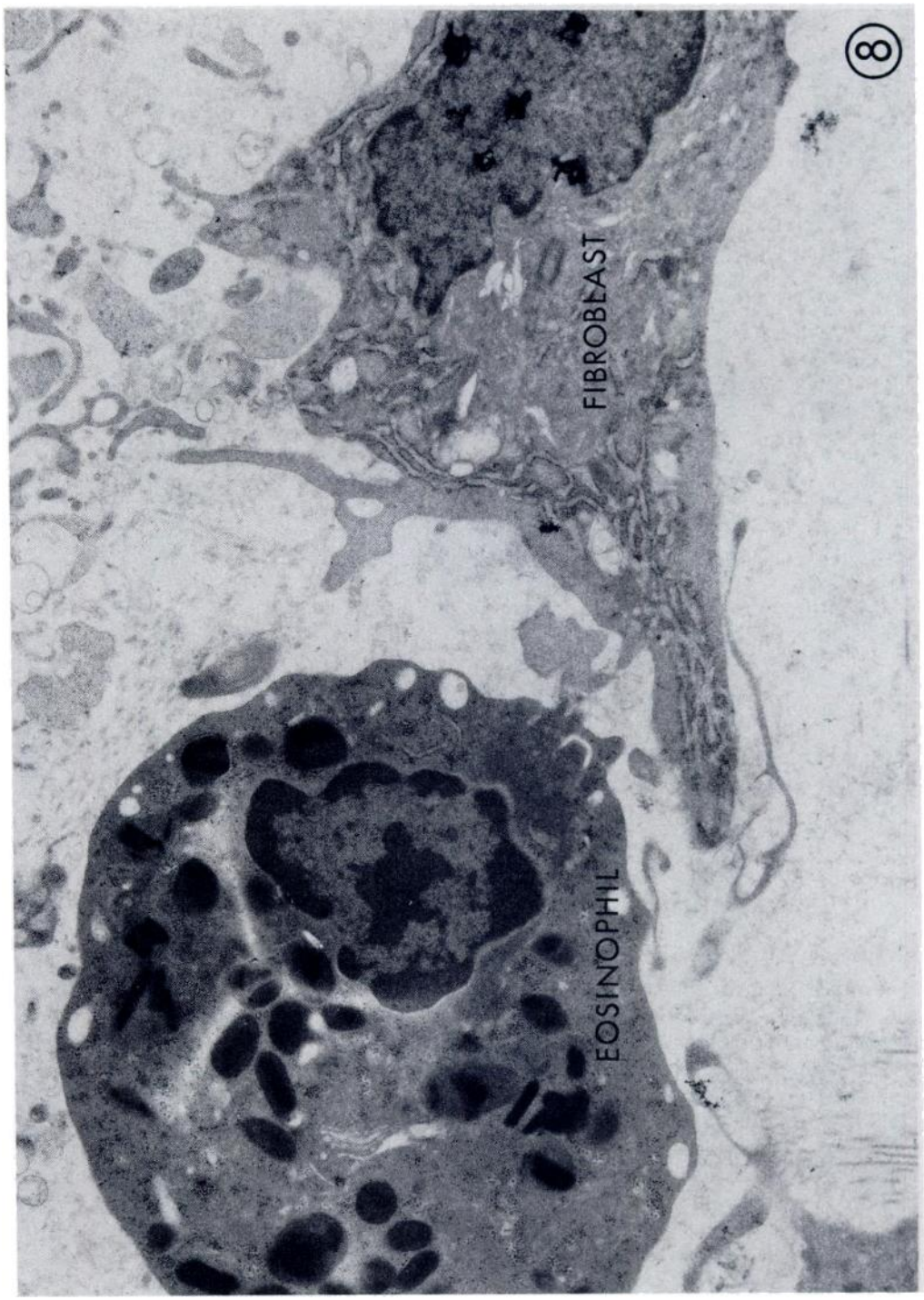


FIG. 8. Endometrial stromal cells. Note the nucleus in a well-differentiated fibroblast with abundant rough endoplasmic reticulum and the unlabeled tissue eosinophil. ×13,600.

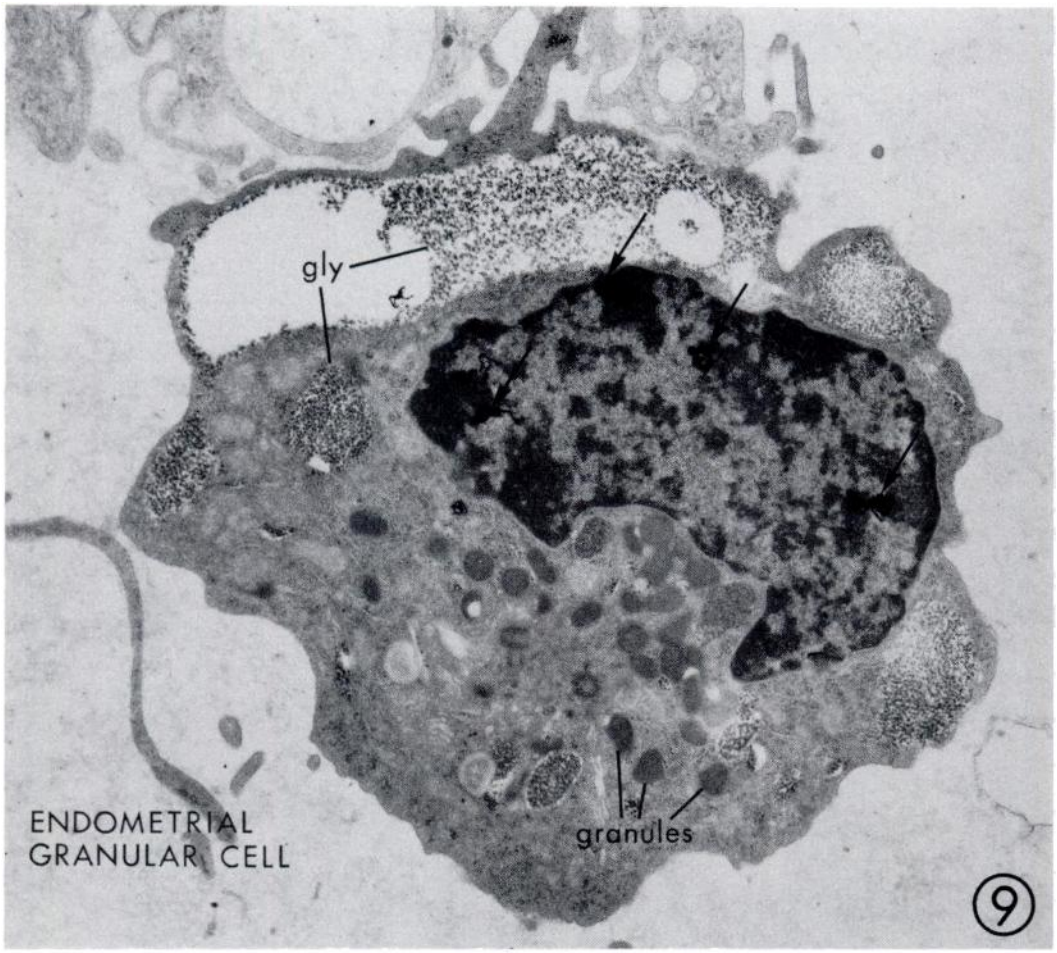


FIG. 9. Endometrial granular cell. This specialized cell with a labeled nucleus (*arrows*) possesses electron-opaque granules and rich glycogen stores. X11,600.

Zones I to III as well as the difference in magnitude across time between Zone III and that of Zones I and II. Viewed against such uniformity, the progressive increase in the size of the proliferating pool in Zone IV enhanced the credibility of the intrinsic nature of these differences. Our recent observations on 2 biopsies at +5 days indicate that the labeling index of Zone IV rises from 7 to 9% between +3 and +5 days (Padykula et al., 1984).

Endometrial growth potential in the OVEX rhesus monkey was explored variously by Hisaw (1950), and Hisaw and Hisaw (1961). After 10–14 days of daily injections of estrogen, the endometrium resembled closely that of the late proliferative phase. However, prolongation of E_2 treatment beyond that period resulted in unresponsiveness. For example, the mitotic rate of the luminal epithelium increased steadily from 0 to 10 days, but thereafter declined steadily. Thus in cyclic and OVEX rhesus endometria, the mitotic response to estrogen levels may include zonal intracellular control mechanisms that regulate recruitment of epithelial cells into the proliferating pool.

Previous experimental studies on rodent endometrial growth have also indicated that estrogen-stimulated proliferation is followed by a refractoriness or desensitization marked by inhibition of hyperplasia (Epifanova, 1966; Stormshak et al., 1976; Finn et al., 1979; Murai et al., 1980). For example, in immature or OVEX

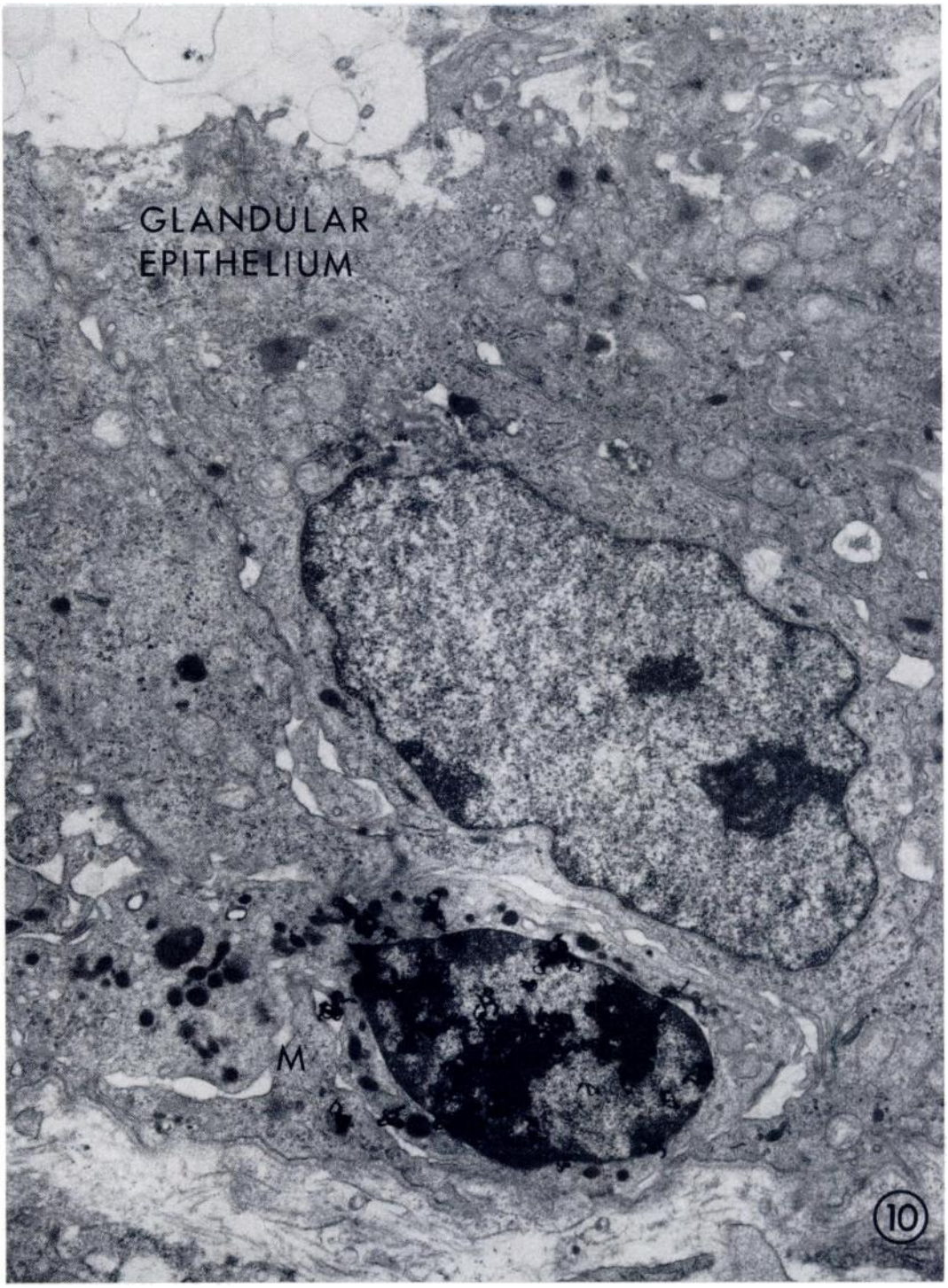


FIG. 10. Intraepithelial monocyte-macrophage, Zone II. A monocyte-macrophage with a heavily labeled nucleus is located in the basal intercellular epithelial compartment of a gland. X12,500.

rodent uteri, maximal DNA synthesis occurs at 12–16 h after an injection of E_2 but following 3 additional daily injections it becomes depressed to the control level (Stormshak et al., 1976).

Most of these experimental analyses have centered on the effects of a single injection of E_2 or several daily injections. The phenomenon under discussion here in the rhesus endometrium, however, reflects the effect of continuous E_2 stimulation over approximately 5 days. In OVEX mice, the effect of continuous estrogenization for 25 days on the uterus has been examined (Lee, 1972). On Days 2 and 3, a wave of proliferation occurred in the luminal epithelium but declined to control level on Days 4 and 5. Cell proliferation remained depressed for about a week and then a second wave of mitotic activity occurred. The intervening mitotic refractoriness to estrogen was time but not dose dependent. A desensitization mechanism in uterine cells of intact rabbits under constant estrogenization has been suggested by Conti et al. (1981). Alterations in receptors may be components of an intracellular regulatory device which influences cell cycle. Interpretation of the apparent midcycle desensitization to estrogen in the rhesus uterus for now is limited by the absence of experimental data.

The E_2 surge rises from the basal level of serum estrogen (approx. 50 pg/ml) to reach a peak of at least 300 pg/ml in a normalized menstrual cycle (Knobil, 1974). The relationship of this basal stimulation to endometrial cell proliferation is unknown. The surge may be viewed as a large injection of E_2 whose effects may last approximately 5 days. A well-documented effect of E_2 on uterine epithelial cells is shortening of the length of the cell cycle (Epifanova, 1966; Galand et al., 1971; Das, 1972). The greater labeling indices of the rhesus functionalis (I and II) in the present study may reflect shortened cell cycles as well as the existence of intracellular hormonal control mechanisms over mitosis which differ from those of the basalis, such as prolonged influence of elevated nuclear estrogen receptor complex.

It is likely that developmental differences in intracellular genetic expression exist among the epithelial cell lines of the 4 zones. These cells differ in position, ultrastructure, height, and most likely in function and possibly in origin. It will be important to determine whether the concentrations of estrogen and progesterone receptors are different in the functionalis and basalis.

Germinal Nature of the Basalis

Anatomically the basalis is that endometrial region which persists after menses to give rise to the new functionalis. This fundamental regenerative role implies that stem cells and/or progenitors of all constitutive cell types for both zones of the functionalis are present in the basalis. The remarkable regenerative potential of the rhesus uterus was best demonstrated by Carl Hartman (1944) who removed the endometrium as an intact sac by dissecting it out with a scalpel, followed by wiping the uterine cavity clean with a cotton swab. Even after such drastic removal, the endometrium regenerated within 16–19 days. One monkey conceived 13 days following removal of the entire endometrium, and pregnancy commenced 22 days after hysterotomy. In Hartman's numerous experiments, the entire endometrium arose from remnants of the deepest portion of Zone IV, which forms an irregular interface along the myometrium. Thus the stem cells for the entire endometrium are located at the endometrial-myometrial junction. The regenerative potential of the endometrium is comparable to that of the epidermis which can originate from a small number of stem cells (Rheinwald and Green, 1975).

Our findings on cell proliferation establish the bipartite nature of the rhesus basalis as reflected by Zones III and IV. Zone IV is the only epithelial region in which the number of dividing cells increases steadily from −2 to +3 days E_2 peak. This phenomenon indicates the existence of a distinctly different intracellular hormonal control mechanism in the deep basalis. This increasing proliferative activity coincides closely with the initial rise in serum progesterone levels during the early postovulatory period. In early luteal rhesus endometrium, Bensley (1951) observed that mitotic activity had "shifted to the basal zone" (p. 95). Thus progesterone may be a stimulus of epithelial proliferation in the deep basalis and, moreover, may exert influence over the production of stem cells or progenitor amplifying cells. Preliminary observations indicate that during midcycle progenitor cells originate in the basalis which will give rise to the new functionalis in the subsequent cycle (Padykula et la., 1983).

Coupling of Epithelial Proliferation with Progressive Differentiation in the Rhesus Endometrium

Cyclicity in the endometrium of menstruating primates is manifested by a high rate of cell replacement and cell loss. Menstruation is a drastic form of endometrial regression peculiar to women and Old World Monkeys in which only the basalis survives. Thus it can be assumed that the stem cells for renewal of constitutive epithelial, stromal and vascular cells of the functionalis are present in the basalis at the close of a cycle.

Our radioautographic evidence at midcycle indicates that proliferating epithelial and stromal cells are distinctly differentiated and committed to certain cell lines (Figs. 5–10). This observation fits in well with current emerging hypotheses concerning rapidly renewing mammalian systems. Potten et al. (1979) have proposed a model for rapid cell replacement which unites studies performed on diverse adult organ systems such as bone marrow, testes, intestine, and epidermal and lingual epithelia. This model assumes that stem cells consist of a very small population of long-lived cells located in a particular microenvironment at the origin of cell migration. Stem cells are self-maintaining, transplantable, and can repopulate. Stem cells give rise to immediate progeny comprised of short-lived transit amplifying cells of limited life span which have a high labeling index. The amplifying cells can form a colony in cultures but cannot be transferred serially because growth ceases eventually.

From this model the interpretation may be made that practically all endometrial cells in the S-phase identified in Figs. 5–10 are components of the system of transit amplifying cells. These labeled cell lines consist of at least 5 structurally different epithelial cells (luminal I; secretory II, III and IV; and ciliated cells) and 3 different stromal cells (fibroblasts; endometrial granular cells; and monocyte-macrophages). The regeneration and expansion of the endometrial vasculature adds proliferating endothelial, stromal, and possibly smooth muscle cells to this list of cell lines.

This hypothesis indicates a finite life span for the amplifying cells because they eventually enter into terminal differentiation, which is followed by cell shedding or death. In the case of the primate endometrium, the possibility exists that the cells and tissue of the functionalis may be incompletely differentiated at the time of menses. The principal basis for this interpretation is a biologic one, i.e., the raison d'etre of the functionalis during the luteal phase is to provide an implantation site for the blastocyst and then to become converted into the maternal placenta. Decidualization of the human functionalis is an example of further differentiation that might occur as transit amplifying cells enter into terminal differentiation.

This fragmentary characterization of primate endometrial renewal is unsatisying because much remains unknown about the vertical pattern of regeneration of this quadripartite endometrium. As our evidence demonstrates, the endometrium at midcycle differs from intestinal, epidermal, and lingual epithelia by possessing 4 zones of epithelial proliferative activity, each marked by distinct progressive differentiation of cell types. At midcycle spatial segregation of postmitotic epithelial populations is not evident, as in the upper epidermal layers or along the intestinal villi. Instead, the widespread distribution of dividing cells throughout the endometrium resembles more closely the process of cell replacement in the bone marrow where cell division and differentiation are tightly coupled during the formation of blood erythrocytes and granulocytes. Deciphering the nature of vertical migration and differentiation will depend on gaining greater understanding of the origin and function of these endometrial zones.

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