Kinetic Analysis of Hormone-Induced Mitoses in Epithelial Cells of Mouse Uterus and Vagina

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Synopsis

The intracellular localization of $^3$H-estradiol-17β and $^3$H-progesterone to the different types of cells in the mouse uterus was investigated using autoradiographic techniques. The kinetics of cell proliferation in the surface epithelium of the uterus and in the vaginal epithelium (basal layer) are analysed by means of cumulative labeling method and mitosis chase method using $^3$H-thymidine autoradiographic procedures. The results are as follows. (1) Epithelial cell population of the uterine lumen and basal cell population of the vaginal epithelium in the ovariectomized mouse are divided into a major subpopulation of $G_0$ cells and a minor subpopulation of proliferating cells. (2) Proliferative potencies of uterine surface epithelial cells and vaginal basal cells in the ovariectomized mouse are regulated by a steroid-independent mechanisms through which the proportion of the $G_0$ cell-compartment and Tc value of the proliferating cell-compartment are determined according to their age; as the castrated mouse grows older, Tc value becomes longer and the proportion of the $G_0$ cell-compartment becomes larger. (3) If the dose levels of estrogen administered exceed the threshold value, estrogen-dependent cell proliferation will be provoked by transferring the cells in the $G_0$ cell-compartment to the proliferating cell-compartment in all or none fashion, and by reducing the Tc value of proliferating cell to 1/2-1/3 of that in the castrated mouse. (4) It is suggested that proliferating cells in the uterine surface epithelium and in the vaginal epithelium turn the cell cycle at a constant Tc value during estrous cycle, and that the tissue growth during estrous cycle is dependent on the size of the proliferating cell-compartment but not on the Tc value. (5) The results obtained from autoradiography of tritiated steroids in the mouse uterus gave a supporting clue to the kinetic data.

In the reports so far published in the literature concerning the effects of sex steroids on the cell cycle, different opinions have been expressed with regard to the possible mechanisms underlying the hormonal regulation of cell proliferation in the target tissues. According to Epifanova (1966), the generative pool in the mouse uterine epithelium contains two subpopulations of cells: one is the fraction of the proliferative cells and the other is the resting reserve or dormant fraction analogous to $G_0$ fraction (Lajtha, 1963). Under the influence of estrogens, the generation time of the uterine epithelial cells was reduced and at the same time the proliferating pool of the uterine epithelium was increased in size, which was not so far detected in nonspecific tissues. On the other hand, Galand et al. (1967) claimed that the basal layer of the vaginal epithelium and the uterine epithelium of the spayed mouse consists of a homogeneous population of cycling cells and the proliferation rate of

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those cells depends upon the presence of estrogens, the action of which is to reduce the generative cycle time.

We analysed autoradiographically the subcellular localization of $^3$H-estradiol and $^3$H-progesterone in the different types of cells in the mouse uterus to give supporting data to the cell kinetic study. Autoradiographic technique followed by injections of $^3$H-thymidine was used to analyse the kinetics of cell proliferation and to fractionize the cell-compartment of the uterine luminal epithelium and the vaginal epithelium (basal layer) in the castrated and in the estrogen-treated mice. In the present experiment, we have attempted to analyse the possible changes in the ratio between the size of the $G_0$ cell-compartment and that of the hormone-responsive generative cell-compartment, according to the time after ovariectomy.

Materials and Methods

**Autoradiography (ARG) of tritiated steroids**

6, 7-$^3$H-estradiol-17β [$^3$H-E$_2$; specific activity, 56 Ci/mM] and 1, 2, 6, 7-$^3$H-progesterone [$^3$H-P; specific activity, 81 Ci/mM] were obtained from New England Nuclear Corp. The radioactive steroids were dissolved in isotonic saline containing 2%/ ethanol (v/v). Three 8-week-old female mice of ICR strain were ovariectomized and 8 weeks after ovariectomy, two of them were injected intraperitoneally with either 100 μCi of $^3$H-E$_2$ or $^3$H-P; the animals were killed 1 hr after the injection of the labeled steroids. The remaining was primed with three consecutive injections of E$_2$ at a dose level of 100 ng/day per mouse. Subsequently, the animal was given a single intraperitoneal administration of 100 μCi of $^3$H-P. It was killed 1 hr after the injection. The uterus was excised and the small pieces were frozen with CO$_2$ at -20°C. Frozen sections of 7-8 μm in thickness were cut in a Cryostat (FS/FCS type, Bright Instrument Co., Ltd., England) and dried immediately on a hot plate at 60°C for 30 seconds. Dry emulsion (Sakura NR-M2, Konishiroku Photo Ind., Co. Ltd.) was mounted on the dry sections by the wireloop method (Nagata, 1970). After 3 months of autoradiographic exposure, the slides were photographically processed and stained with hematoxylin and eosin (H-E).

**Autoradiography of $^3$H-thymidine**

A: Mitosis Chase Method

Sixteen cycling mice at different stages of estrous cycle and 10 ovariectomized mice, 4 weeks after operation, were used. Each animal received a single intraperitoneal injection of 4 μCi/g body weight of $^3$H-thymidine ($^3$H-TdR; specific activity, 5.0 Ci/mM, New England Nuclear Corp.) dissolved in physiological saline. The cycling mice were sacrificed by decapitation 1 to 20 hr and the ovariectomized mice were sacrificed 1 to 30 hr after the injection of $^3$H-TdR. Uterus and vagina were removed, fixed in Carnoy's fluid, embedded in paraffin, cross-sectioned at 4-5 μm, and coated with Sakura NR-M2 nuclear track emulsion. After autoradiographic exposure for 4 weeks, the slides were photographically processed and stained with H-E. All metaphase figures in the uterine surface epithelial cells of the middle region of the uterine horn and the vaginal basal cells of the middle part of the vagina present in several alternate sections were counted (over 100 mitotic figures for each animal) to assess the fraction of the labeled mitoses according to the method of Quastler and Sherman (1959).

B: Cumulative Labeling Method

In the first experiment, ovariectomized mice, 4 weeks after operation, received multiple injections of 2 μCi/g body weight of $^3$H-TdR separated at 3-hr intervals from 0 to 24 hr. The animals were sacrificed by decapitation 30 min after the first (0 hr), the second (3 hr), the fourth (9 hr), the seventh (18 hr), and the ninth (24 hr) injection of $^3$H-TdR. Ovariectomized mice, 6 weeks after the operation, received multiple injections of 1 μCi/g body weight of $^3$H-TdR separated at 5-hr intervals from 0 to 70 hr and were killed by decapitation 30 min after the first (0 hr), the sixth (25 hr), the tenth (45 hr), and the fifteenth (70 hr) injection of $^3$H-TdR.

In the second experiment, ovariectomized mice, 6 weeks after operation, were used. The animals were injected subcutaneously with 50 ng of E$_2$ dissolved in 0.1 ml saline containing 2% ethanol (v/v) at 12-hr intervals repeatedly. One group received injections of 1 μCi/g body weight of $^3$H-TdR separated by 5 hr interval (and received the additional injections of $^3$H-TdR at 12 hr and 17 hr) from 0 hr (the time of the first injection of E$_2$) to 30 hr. Autopsies were performed at 1/2, 5.5, 10.5, 12.5, 15.5, 17.5, 20.5, 25.5 and 30.5 hr. The other group received similar injections of $^3$H-TdR from 50 hr to 70 hr, where the animals were given sc injections of 50 ng of E$_2$ separated at 12-hr intervals. These animals were sacrificed at 50.5, 55.5, 60.5, and 70.5 hr. ARGs were made by the same technique described above. At least 1,000 uterine surface epithelial cells and vaginal basal cells per mouse were counted to estimate the percentage of labeled cell by the method of Quastler and Sherman (1959).
of Fujita (1962). Outlines of cumulative labeling with \(^3\)H-TdR are shown in Fig. 1.

Proliferative cycle (C) was separated into G1 phase, S phase, G2 phase, and M phase following the "gap" nomenclature (Howard and Pelc, 1953) and the time taken for cells to transit in each phase is expressed as TG1, TS, TG2, and TM, respectively. TC is the time for cells to pass through an entire proliferative cycle. Pf means the percentage of proliferative cells in the generative cell zone and it was assumed in our study that the generative cell zone is equivalent to the uterine surface epithelial layer or the vaginal basal layer.

Results

ARG of tritiated steroids;

Autoradiograms obtained from the uterus of the ovariectomized mouse, prepared 1 hr after the administration of \(^3\)H-E2, showed nuclear concentration of radioactivity in the cells of uterine surface epithelia, glandular epithelia, substantia propria, and muscularis (Fig. 2). ARGs of \(^3\)H-P obtained from the uterus in the ovariectomized mouse rarely showed cellular accumulation of the radioactivity (Fig. 3). ARGs of \(^3\)H-P obtained from the uterus in the estrogen-primed mouse, however, showed the tendency of the nuclear concentration in the uterine surface epithelia, glandular epithelia as well as the stromal cells and the muscular cells. Sparse radioactive labeling was also noted in the cytoplasm and the extracellular spaces (Fig. 4).

Mitosis Chase Method;

FLM curves describing the fraction of labeled metaphases in the uterine surface epithelium and in the vaginal epithelium expressed as a function of time, were prepared using data obtained from mice killed at different stages of estrous cycle (Fig. 7). Labeled metaphases in both cell types were first observed 1 hr after the injection of \(^3\)H-TdR. Shortly thereafter, the fraction of labeled cell rapidly rose, and approached to 0.8-0.9 by 5 hr post-injection, continuing level for 2-4 hr, then fell approximately to 0.2 by 12 hr post-injection. The labeled cells entered the second cycle and the fraction of labeled cells in both cell types rapidly increased again, and approached to 0.75-0.85 by 20 hr.

FLM curve for the uterine surface epithelium in the castrated mice was shown in Fig. 8. The fraction of labeled cell sharply increased from 2 hr post-injection and approached to 0.75 by 6 hr, continuing this level by 12 hr, falling to 0.15 by 18 hr, then rose again to 0.75 by 26 hr. FLM curve for the vaginal basal cells in the castrated mice could not be prepared because of the severe atrophic cell pattern and the difficulty to determine definitely cells in the labeled metaphase. It is possible to determine the parameters of the cell cycle from these FLM curves by locating the intersections of the curve with 0.5 level of FLM (Mendelsohn and Takahashi, 1971). This gave successively, TG2+0.5M, TS, TG3+M+G2, etc (Table 1).

Cumulative Labeling Method;

In the first experiment, the percentage of labeled cells in the uterine surface epithelium and in the vaginal epithelium...
Fig. 2. Autoradiograph showing distribution of $^3$H-estradiol-17$\beta$ in the endometrium of the castrated mouse. $^3$H-estradiol-17$\beta$ is concentrated in the nuclei of the luminal epithelium (lu), the glandular epithelium (gl), and the stromal cells (st).

Fig. 3. Autoradiograph showing distribution of $^3$H-progesterone in the endometrium of the castrated mouse. It shows sparse radioactivity in the endometrium. (lu): luminal epithelium, (st): stromal cells.
Fig. 4. Autoradiograph showing distribution of $^3$H-progesterone in the endometrium of the estrogen-primed mouse. $^3$H-progesterone or its metabolites tend to be concentrated in the nuclei of the luminal epithelium (lu), the glandular epithelium (gl), as well as the stromal cells (st). Exposure time, 3 months. Stained with hematoxylin and eosin. ×800 (Figs. 2-4).

Fig. 5. A burst-like appearance of mitotic figures is shown in the uterine surface epithelium 20 hr after the injection of estradiol-17β. Stained with gallocyanin chrome alum. ×800.

Fig. 6. Autoradiograph of $^3$H-thymidine obtained from the castrated mouse 20 hr after the injection of estradiol-17β. All the luminal epithelial cells are labeled with $^3$H-thymidine. Stained with hematoxylin and eosin. ×800.
Fig. 7. Fraction-of-labeled-mitoses curve in the uterine surface epithelium (△—△) and in the basal layer of the vaginal epithelium (●—●) of the cycling mice.

Fig. 8. Fraction-of-labeled-mitoses curve in the uterine surface epithelium (●—●) of the castrated mice.

Table 1. The parameters of the mitotic cycle and the proliferating fraction in the luminal and vaginal epithelium obtained by the mitosis chase and cumulative labeling methods.

<table>
<thead>
<tr>
<th>Parameters of the mitotic cycle (hr)</th>
<th>Proliferating fraction (%)</th>
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<tbody>
<tr>
<td>TC</td>
<td>TS</td>
</tr>
<tr>
<td>Vaginal Basal cell</td>
<td></td>
</tr>
<tr>
<td>(Cycling mouse)*</td>
<td>15.0</td>
</tr>
<tr>
<td>(4 weeks after ovariectomy)**</td>
<td>26</td>
</tr>
<tr>
<td>(6 weeks after ovariectomy)**</td>
<td>53</td>
</tr>
<tr>
<td>(Estrogen treated)**</td>
<td>15</td>
</tr>
<tr>
<td>Uterine surface epithelium</td>
<td></td>
</tr>
<tr>
<td>(Cycling mouse)*</td>
<td>13.5</td>
</tr>
<tr>
<td>(4 weeks after ovariectomy)*</td>
<td>17.2</td>
</tr>
<tr>
<td>(4 weeks after ovariectomy)**</td>
<td>26</td>
</tr>
<tr>
<td>(6 weeks after ovariectomy)**</td>
<td>53</td>
</tr>
<tr>
<td>(Estrogen-treated)*</td>
<td>28</td>
</tr>
</tbody>
</table>

* data obtained by the mitosis chase method  ** data obtained by the cumulative labeling method.
Fig. 9. The increment of the percentage of labeled cells in the uterine surface epithelium (△—△) and in the basal layer of the vaginal epithelium (●—●) during the time of cumulative injections of \(^3\)H-thymidine. (A): ovariectomized mice (4 weeks after operation), (B): ovariectomized mice (6 weeks after operation).

Fig. 10. A schematic representation of the percentage of labeled cells versus duration of cumulative labeling with \(^3\)H-thymidine. If the percentage of labeled cells increases linearly to (b) hr and it becomes to be flat thereafter, it can be calculated that \(T_S\), \(T_C\), and \(P_f\) are (a) hr, (a+b) hr, and (p) percent, respectively.

Fig. 11. The initiation of epithelial proliferation after the administration of estradiol-17\(\beta\). (△—△): uterine surface epithelium, (●—●): basal layer of the vaginal epithelium. Each symbol expresses one animal.

Fig. 12. The increment of the percentage of labeled cells after the administration of estradiol-17\(\beta\). \(^3\)H-thymidine was injected to the mice from 50 hr after the first administration of estradiol-17\(\beta\). (△—△): uterine surface epithelium, (○—○): basal layer of the vaginal epithelium.

by 15 hr, then rose rapidly to 100\% by 20 hr (Fig. 11). A burst-like appearance was observed in the mitotic figures of both cell types from 17 hr to 20 hr (Figs. 5 and 6).

For the second period, the percentage of labeled cells in the uterine surface
epithelium and in the vaginal epithelium increased lineally and reached 100% by 70 hr, and 60 hr, respectively (Fig. 12). Thus, TC, TS and Pf of the uterine surface epithelial cells and vaginal basal cells in the ovariectomized and estrogen-treated mice were determined by the cumulative labeling method. The results were summarized on Table 1.

**Discussion**

There remains a major problem to be solved; namely the problem concerning the existence of the resting cell- (G₀ cell-) compartment in the uterine surface epithelium and in the vaginal epithelium in the spayed mice. Ladinsky and Peckham (1965) examined the generative cell-compartment of the rat vaginal epithelium by hourly injections of ³H-TdR for 48 hr. They concluded that the increase in labeling index associated with estrogen stimulation was caused by shortening the generative cycle time rather than by activation of a resting population. Galand et al. (1967) also reported that the basal layer of the vaginal epithelium and the uterine epithelium of the spayed mouse represent homogeneous populations of cycling cells, because a total labeling of the cell populations was attained by 98 hr.

On the other hand, Epifanova (1966) reported that the labeling index of the uterine epithelium in the castrated mouse was 8.3% at 45 hr, when total labeling could be expected if the generative cell-compartment of the uterine luminal epithelium was homogeneous. From this result, she stated that the generative pool in mouse uterine epithelium contained two subpopulations of cells, the proliferating cell pool and the resting reserve such as G₀ cells.

In the present study, it was demonstrated that the percentage of labeled cells in the uterine surface epithelium and in the vaginal epithelium in the ovariectomized mice reached a plateau at 6% by 45 hr and the levels were unchanged to 70 hr. Thus, it can be concluded that both epithelial cells in the castrated mouse consisted of two cell-compartment, one is the proliferating cell-compartment and the other is the non-proliferating cell-compartment. The results obtained by Galand et al. should be carefully discussed, because reactive proliferation due to hourly intraperitoneal injection of ³H-TdR could be expected. After the administration of E₂, the percentage of labeled cells in the uterine surface epithelium and in the vaginal epithelium reached to 100% by 20 hr resulting a burst-like appearance of the labeled mitotic figures. Perrotta (1962) obtained similar results following a single injection of estradiol benzoate to the castrated mouse. This suggests that non-proliferating cells synchronously synthesize DNA after 12 hr to 15 hr under the influence of estrogen and are transferred to the proliferating cell-compartment, then undergo mitosis. The present study indicates that the non-proliferating cells in the castrated mice are G₀ cells which can be activated by estrogen.

Subcellular localization of ³H-estradiol and ³H-progesterone in the target tissues has been clearly demonstrated using dry-mount autoradiography (Stumpf and Roth, 1966 and 1969; Sar and Stumpf, 1974). Our results of the autoradiograms of ³H-estradiol in the mouse uterus agree with the data obtained previously in the rat and rabbit uteri (Stumpf and Roth, 1966). Jensen et al. (1968) proposed a two-step mechanism in the action of estrogen. The results of ARG of ³H-E₂ gave a supporting clue to the kinetic data; namely, ARG of ³H-E₂ revealed that almost all the uterine luminal cells which mainly consisted of G₀ cells are labeled with ³H-E₂. In other words, it might be claimed that G₀ cells in the luminal epithelium react
with estrogens strongly enough to provoke specific cellular reactions. In the experiment with \(^3\)H-progesterone, it was suggested that nuclear uptake of \(^3\)H-progesterone and its metabolites to the proliferative cells in the luminal epithelium was enhanced with estrogen pretreatment.

It is suggested from these results that proliferative potencies of uterine surface epithelial cells and vaginal basal cells in the ovariectomized mouse were regulated by a steroid-independent mechanism through which the proportion of the G\(_0\) cell-compartment and the Tc value of the proliferating cell-compartment are determined according to their age; as the castrated mouse grows older, Tc value becomes longer and the proportion of the G\(_0\) cell-compartment becomes larger. It can be also proposed that if the dose levels of estrogen administered exceeds the threshold value, estrogen-dependent cell proliferation will be provoked by transferring cells in the G\(_0\) cell-compartment to the proliferating cell-compartment in all or none fashion, and by reducing Tc of the proliferative cell to 1/2-1/3 of the value in the castrated mouse.

Additionally, it seems to be interesting that FLM curves prepared from mice at different stages of estrous cycle gave isocyclic waves in both types of the cells. This suggests that proliferating cells in the uterine surface epithelium and in the vaginal epithelium turn the cell cycle at a constant Tc value during estrous cycle, and that tissue growth during estrous cycle is dependent on the size of the proliferating cell-compartment rather than the changes in the Tc value.

References