Histological Changes in Mouse Nipple Tissue during the Reproductive Cycle

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ABSTRACT. To obtain detailed information about the histological changes occurring in the mouse nipple during the reproductive cycle, we examined and quantified the S-phase of cell by immunohistochemical staining with bromodeoxyuridine (BrdU), and analysed histologically the subepithelial fibrous elements. The nipple markedly increased in size dramatically on days 15–18 of pregnancy. The densities of cells in the epidermis and dermis were very high during the early stages of pregnancy but low during lactation. In the epithelium of the lactiferous sinus, the densities of cells did not differ significantly among stages. The BrdU antibody labeling revealed a number of BrdU-positive cells in the basal layer of the epidermis and epithelium of the lactiferous sinus. The ratios of BrdU-positive cells to total cells in the epidermis and the epithelium of the lactiferous sinus were highest on day 15 and day 10 of pregnancy, respectively. After lactation, however, the ratios were similar to those in the virgin stage. No significant differences were detected in the dermis among all stages. The number of collagen and elastic fibers increased during lactation. These results indicate that cells in the epidermis and lactiferous sinus proliferated actively from day 10 to day 15 of pregnancy. The observation that cellular proliferation in the epithelial system of the nipple was stimulated at the early stage of pregnancy, while the dermis has two growth phases, with cellular proliferation during pregnancy and an increase in extracellular matrix during lacation, suggests that these two phenomena might be regulated by different factors. — KEY WORDS: bromodeoxyuridine, histometry, mouse, nipple, proliferation.

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The nipple is an organ that is unique to mammalian species and is indispensable for nursing pups. In reproductive biology, the nipple is usually considered to be a part of the mammary apparatus [7, 10, 11, 18]. Previously, we described histometric changes in the rat nipple as it develops into the structure used for nursing during the reproductive cycle [16]. A marked increase in size of the nipple was observed during the second half (days 15 and 20) of pregnancy and the size was the maximal during lactation. During the second half of pregnancy, the nipple increased in size and many wrinkles appeared, indicating extensive proliferation of epidermal cells. It is likely that the proliferation of the epidermal cells, as well as of the epithelial cells, of the mammary gland is induced by changes in levels of hormones in the maternal body with the progression of pregnancy [7, 15].

The use of bromodeoxyuridine (BrdU), an analogue of thymidine, and specific monoclonal antibody allows easier detection of DNA synthesized in proliferating cells than the use of ^{3}H-thymidine [4, 13, 14, 20]. In studies of cellular kinetics in the mammary gland, the use of BrdU has also become much more common [12] than that of ^{3}H-thymidine [3]. However, there have been no reports of the cellular kinetics in the nipple during the reproductive cycle, even using ^{3}H-thymidine.

In this study, to obtain precise information concerning the histological changes in the nipple during the reproductive cycle, we examined the sites and timing of cellular proliferation in the mouse nipple by immunohistochemical detection of BrdU incorporated into DNA.

MATERIALS AND METHODS

Animals: The Jcl-ICR strain mice used in this study were bred and maintained as a closed colony in our laboratory. They were all housed in an environmentally controlled air-conditioned room (temperature: 23 ± 3°C, humidity: 60 ± 10%, light-dark cycle: 12–12 hr) and given a commercial diet (MF, Oriental Yeast Co., Tokyo) and tap water *ad libitum*. A total of 33 female mice (80-day-old) were mated with males of the same age, and the day when a vaginal plug was found was determined as day 0 of pregnancy. They were divided into 11 groups; i. e. virgin, days 5, 10, 15 and 18 of pregnancy, days 5, 10, 15 and 20 of lactation, days 5 and 10 of post-weaning. Each group consisted of 3 mice. At the lactating stage, each dam was housed with her 10 pups and separated from the young for 30 min before sampling.

Administration of BrdU: BrdU (50 mg/kg b.w., Sigma, MO) was injected intraperitoneally into female mice at each reproductive stage. One hour after the injection, the nipples were excised under sodium pentobarbital anesthesia and fixed with Bouin's solution for 2 hr at room temperature. Mice have 3 pairs of thoracic and 2 pairs of abdomino-inguinal nipples, and the first left abdomino-inguinal nipple was examined. The specimens were routinely embedded in paraffin and longitudinal sections 3 μm thick were cut.

Immunohistochemical staining: To detect the S-phase cells, BrdU administration and anti-BrdU immunostaining was employed. Three different sections with 9 μm intervals from one nipple were immunostained using a monoclonal

mouse anti-BrdU antibody (NeoMarkers, CA) diluted to 1 μg/m*l* with 0.01 M phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 4 μg/m*l* horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, IL) suspended in 0.1% BSA/PBS. Peroxidase activity was visualized with 0.05% diaminobenzidine hydrochloride (Sigma, MO) and 0.01% H_2O_2 in PBS. The sections were counterstained with hematoxylin. For histological observation, other sections were stained with hematoxylin and eosin, and elastica van Gieson stains.

Histometry: The size of the nipple, i.e. length, width of the middle portion [16], and the areas of epidermis, dermis and epithelium of the lactiferous sinus were measured with an image analyzer (Nikon COSMOZONE Is, Tokyo). All cells observed in each part of the nipple were counted. The numbers of cells per 1,000 μm^2 were calculated from the areas measured by morphometry, as described below. Furthermore, the numbers of cells positive for BrdU were counted and then the ratio of such cells to the total cell number of cells in each area was calculated. The data were analyzed statistically by Fisher's PLSD law after dispersion analysis for comparison.

RESULTS

Histological changes: The nipples of virgins were roughly a dull conical shape, and the nipple wall was rarely wrinkled (Fig. 1a). During the first half (days 5 and 10) of pregnancy, the nipples were similar in structure to those of virgins, but keratohyalin granules were observed in the ingrowth of the germinative layer (Fig. 5c). The nipple began to increase in size during the second half (days 15 and 18) of pregnancy (Fig. 1b). Many wrinkles appeared on the nipple wall, and the base of the nipple was surrounded by higher wrinkles. During lactation, the nipple increased in size still further, and the lactiferous sinus became very dilated (Fig. 1c). The number of wrinkles in the nipple wall continued to increase. After weaning, the nipple decreased in size and the number of wrinkles in the wall decreased. Furthermore, the epidermis of the nipple had a very thick stratum corneum (Fig. 1d). The dermis of the nipple mainly consisted of quiescent fibroblasts in the virgin stage. The number of active fibroblasts increased during the second half of pregnancy, but decreased during lactation. The number of collagen and elastic fibers increased during lactation relative to those in the virgin stage (Fig. 2).

Figure 3 shows the changes in length, width and area of the nipple. The length, width and area in virgin mice were 494.2 ± 92.9 μm (mean ± SEM), 474.2 ± 35.7 μm and 287,172.3 ± 36,462.9 μm^2, respectively. As compared to the virgin stage, there were no significant changes during the first half of pregnancy. However, all these parameters increased suddenly from the start of the second half of pregnancy. The length and area were maximal on day 5 of lactation, with increases of approximately 3.6-fold and 5.3-fold relative to those in the virgin period. The width reached the maximal value on day 10 of lactation, with an increase of approximately 2.5-fold that in the virgin period, and then decreased gradually thereafter. No significant differences were found between days 15 and 18 of pregnancy, and during lactation or the post-weaning period, respectively.

Cells in the S-phase in each part of the nipple: Figure 4 shows cell densities in each part of the nipple. In the epidermis, cells were most densely packed on days 5 and 10 of pregnancy (Fig. 4a). However, the density dropped suddenly on day 15 of pregnancy to below that in the virgin stage, and remained low in lactation and the post-weaning stages. In the dermis, the cell density reached a maximum on day 5 of pregnancy, but there was no significant difference between the density in the virgin stage and that during the first half of pregnancy (Fig. 4b). The densities after day 18 of pregnancy were significantly lower than those during the first half of pregnancy. In the epithelium of the lactiferous sinus, the density of the cells tended to increase during the second half of pregnancy but there were no significant differences among all stages (Fig. 4c).

In virgins, the numbers of BrdU-labeled cells were very low in each part of the nipple. However, a small number of positive cells were observed in the basal layer of the epidermis (Fig. 5a). From day 10 of pregnancy, the number of BrdU-positive cells increased in the epidermis and the epithelium of the lactiferous sinus. A number of positive cells in the basal layer were observed especially in the epidermal ridge and the germinative ingrowth of the epidermis (Figs. 5b, 5c). The wrinkles appeared from day 15 of pregnancy and BrdU-positive cells were found at the basal regions of the wrinkles. The epithelium of the lactiferous sinus near the teat duct contained more BrdU-positive cells than other regions of the sinus (Fig. 5d). In the epidermis and the epithelium of the lactiferous sinus, the maximal number of BrdU-positive cells was observed on day 15 of pregnancy, and the number decreased thereafter. In the dermis, BrdU-positive cells were rarely found at the virgin stage but their number tended to increase during the second half of pregnancy. In the lactating and post-weaning stages, the numbers of BrdU-positive cells were similar to that in the virgin stage (Figs. 5e, 5f). In the dermis, no significant differences were detected among the various stages. There were a few BrdU-positive cells in the skin surrounding the nipple, but no significant differences were found among the stages (data not shown).

Figure 6 shows the changes in the ratio of BrdU-positive cells to the total number of cells in each part of the nipple. As compared to the ratios in the virgin stage, significantly higher ratios were obtained in the epidermis and lactiferous sinus on days 10–18 and 10–15 of pregnancy, respectively. In the epidermis, the maximum ratio, recorded on day 15 of pregnancy, was significantly higher than that of all other stages except for day 10 of pregnancy. In the epithelium of the lactiferous sinus, the ratio on day 10 of pregnancy was significantly higher than that at all other stages examined except for day 15 of pregnancy. In the dermis, the ratio of BrdU-positive cells to total cells showed similar changes, but there were no significant differences among the various

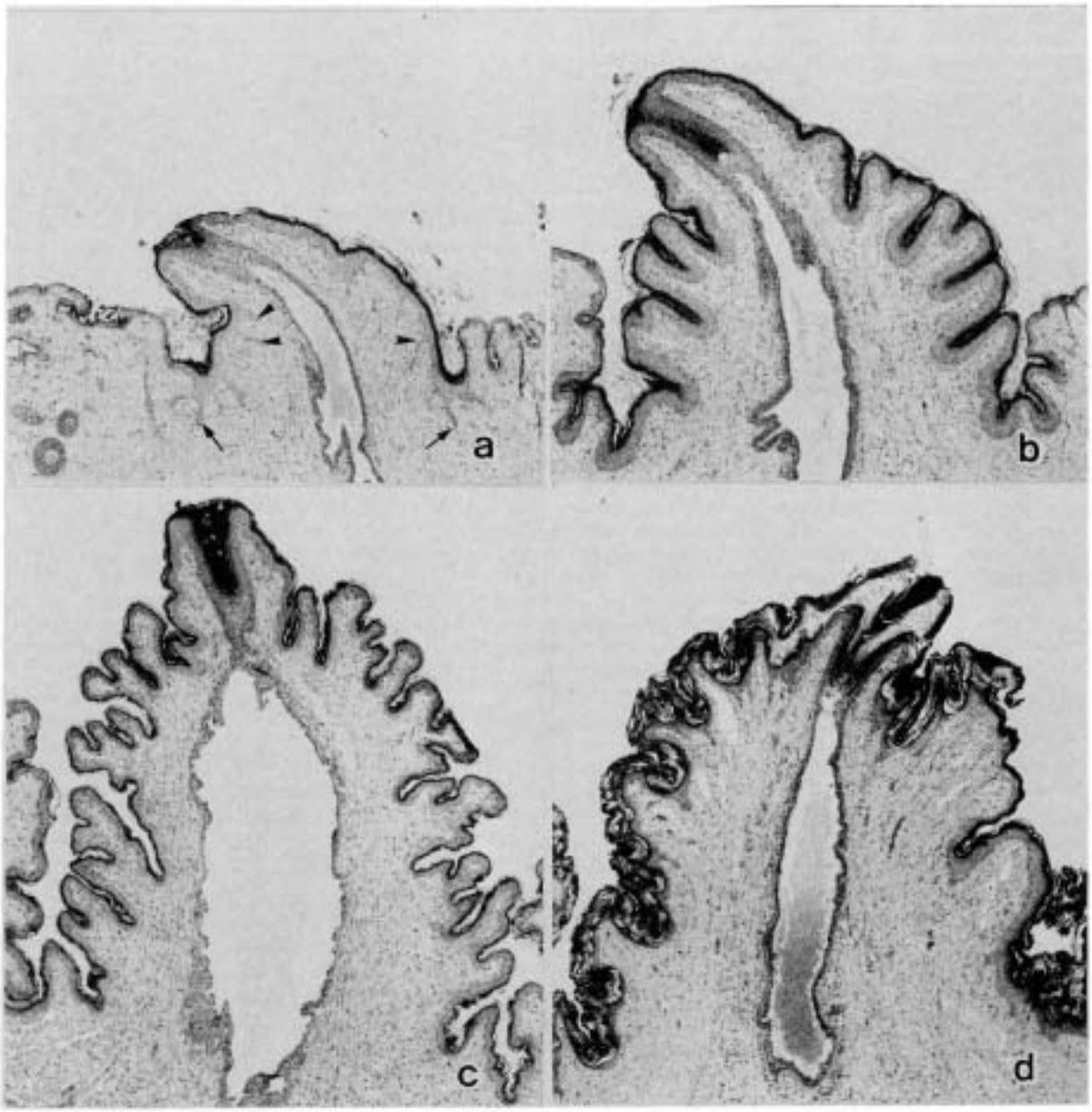

Fig. 1. Longitudinal sections of nipples from a virgin (a), and from mice on day 15 of pregnancy (b), day 5 of lactation (c) and day 10 after weaning (d). The epidermal ridge (arrowheads) and the germinative ingrowth (arrows) are observed in the virgin state (a) and during the first half of pregnancy. Hematoxylin and eosin stain. × 80.

stages.

DISCUSSION

In our previous study, we confirmed that the rat nipple begins to increase considerably in size during the second half of pregnancy [16]. In the present study, we observed similar morphological and histological changes in the mouse nipple. The nipple rapidly grew and many wrinkles appeared during the second half of pregnancy. The size of the nipple reached a maximum during lactation. These changes in the nipple prepare an appropriate structure for nursing pups. To characterize the histological changes in the nipple during the reproductive cycle at the cellular level, we performed immunohistochemical labeling with BrdU to identify nipple cells that synthesize DNA actively during pregnancy.

The densities of the cells in the epidermis and dermis of the nipple were maximal on day 5 of pregnancy and then decreased during the second half of pregnancy and lactation.

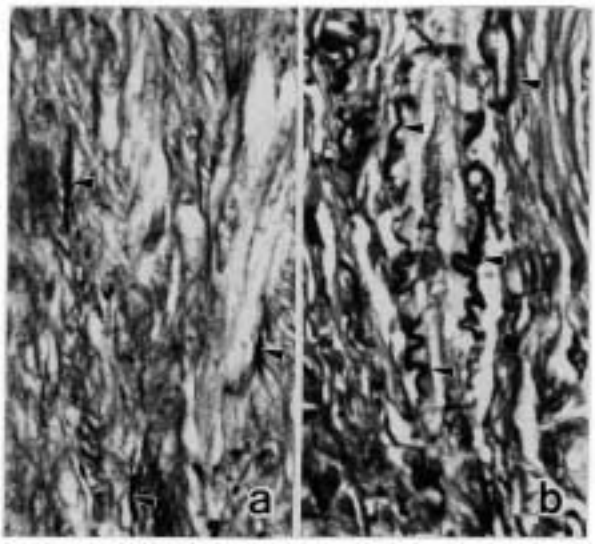

Fig. 2. Light micrographs of nipples from a virgin (a) and from a mouse on day 10 of lactation (b). The numbers of collagen and elastic (arrowheads) fibers increase during lactation. Elastica van Gieson stain. × 800.

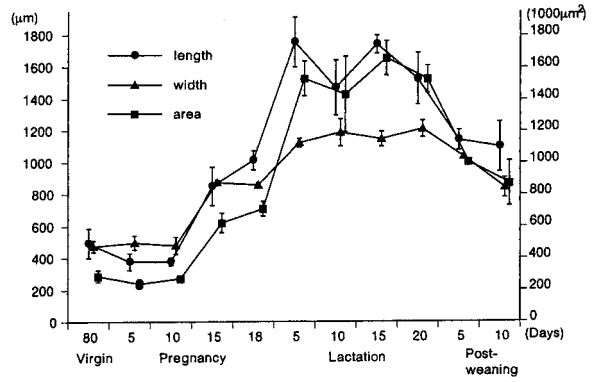

Fig. 3. Changes in length, width and area of the nipple. As compared to the nipple in the virgin stage, there are significant differences in length after day 18 of pregnancy. The width after day 15 of pregnancy and the area after day 5 of lactation are significantly greater than those at the virgin stage. There are no significant differences in the respective parameters between the first and the second half of pregnancy. There are also no significant changes in the respective parameters during lactation, and during the post-weaning stage (p<0.01).

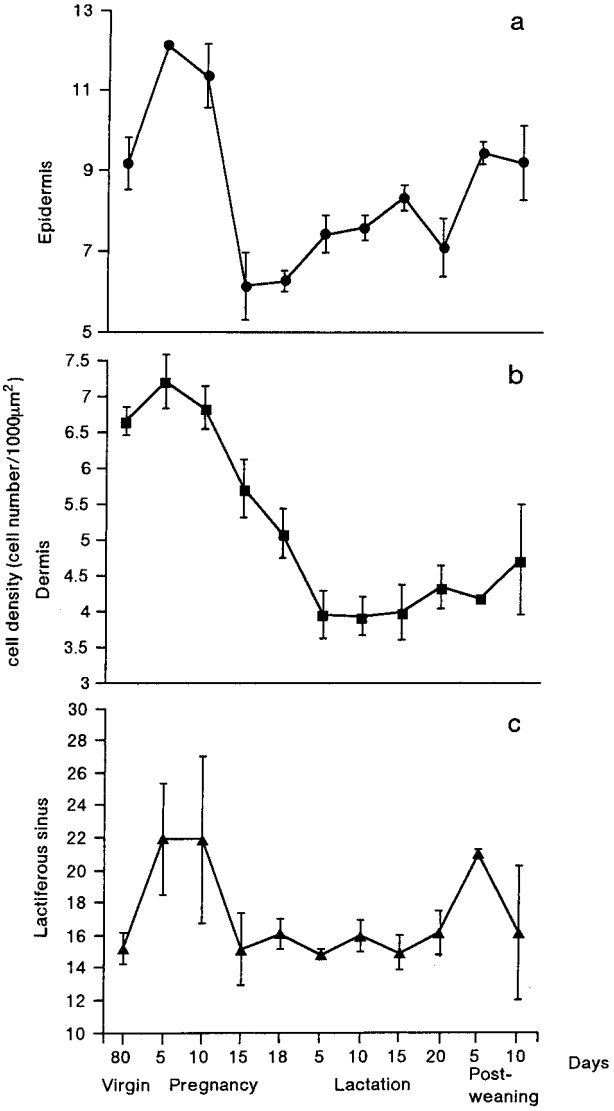

Fig. 4. Changes in the cell density (cell number/1,000 μm^2) in the epidermis (a), dermis (b) and epithelium of the lactiferous sinus (c) of the nipple. The cell densities in the epidermis and dermis are maximal on day 5 of pregnancy. As compared to day 5 of pregnancy, the other stages with the exception of day 10 of pregnancy have significantly lower cell densities in the epidermis. The cell density in the dermis after day 18 of pregnancy is significantly lower than that on day 5 of pregnancy. Dispersion analysis indicates no significant differences the cell density of the lactiferous sinus epithelium among all stages examined (p<0.01).

Furthermore, the densities of collagen and elastic fibers in the dermis, which may be synthesized by active fibroblasts observed during the second half of pregnancy, were higher during lactation than in the virgin stage. Thus, the marked increase in the area of dermis from the start of the second half of pregnancy to lactation seemed to occur not only as a result of cell proliferation but also as a result of increases in amount of extracellular substances such as collagen and elastic fibers. On the other hand, although the density of cells tended to increase during the second half of pregnancy, no significant changes in cell density were observed during the reproductive cycle in the epithelium of the lactiferous sinus. This suggests that the increase in epithelial area was due only to cell proliferation.

In the epidermis, the majority of BrdU-positive cells were found in the epidermal ridge and the germinative ingrowth at the base of the nipple on day 10 of pregnancy, and of the base of wrinkles that protruded from the nipple wall on day

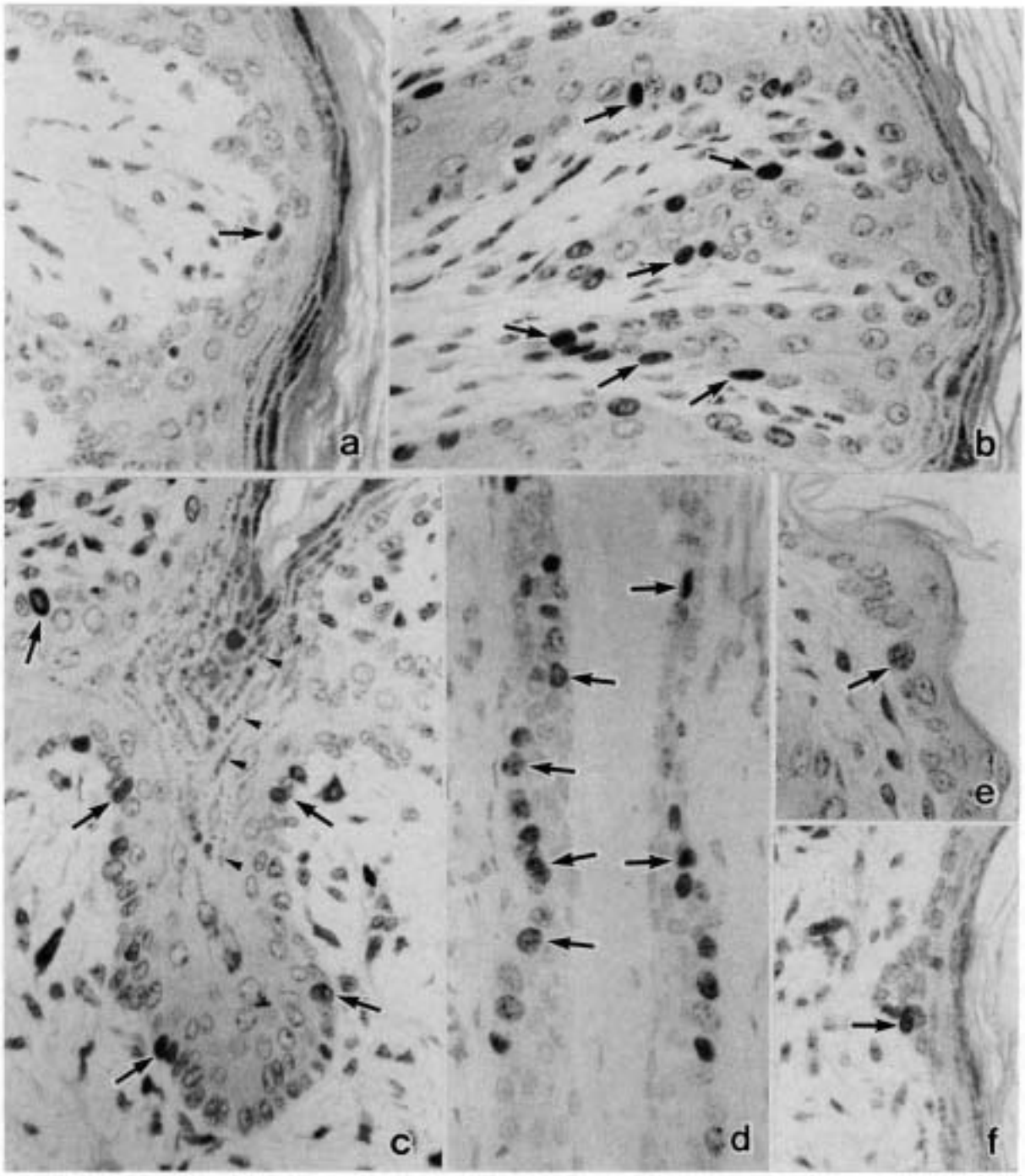

Fig. 5. Immunohistochemical staining of BrdU in nipples at the virgin stage (a), day 10 of pregnancy (b-d), day 10 of lactation and day 5 of post-weaning. a, b, e and f; epidermis, c; basal region of epidermis, d; epithelium of lactiferous sinus near the apex. Arrows indicate BrdU-positive cells. The keratohyalin granules (arrowheads) are observed in the germinative ingrowth. × 560

15 of pregnancy. Many keratohyalin granules were found in the germinative ingrowth at the base of the nipple on day 10 of pregnancy. These keratohyalin granules may have contributed to the sudden elongation of the nipple which occurred during the second half of pregnancy because they contribute to keratinization and disjunction of keratinocytes. On the other hand, no keratohyalin granules were observed in the epidermal ridge, on day 10 of pregnancy. This suggested that the wrinkles of the nipple wall are formed as a result of protrusion of the epidermis and dermis (dermal

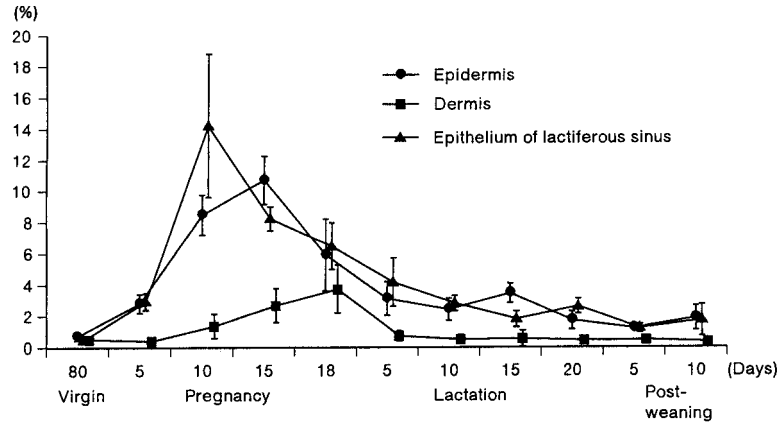

Fig. 6. Changes in percentages of BrdU-positive cells in the epidermis, dermis and epithelium of the lactiferous sinus of the nipple. As compared to the virgin stage, the percentages in the epidermis and the lactiferous sinus epithelium are significantly higher on days 10 to 18 of pregnancy, and on days 10 and 15 of pregnancy, respectively. Dispersion analysis shows no significant differences in the percentage of BrdU-positive cells in the dermis among all stages examined ($p<0.01$).

papillae). However, there were very few BrdU-positive cells in the dermis, and therefore the development of the dermis may be associated with an increase of the extracellular matrix. The epithelium of the lactiferous sinus contained a site of cellular proliferation localized near the teat duct which suggests that the elongation of the duct in the nipple growth occurs locally in this site. The ratio of BrdU-positive cells to total cells was highest in the epidermis and epithelium of the lactiferous sinus on day 15 and day 10 of pregnancy, respectively; in the dermis, the ratio tended to increase during pregnancy and reached a maximum on day 18 of pregnancy. It seems that cell proliferation occurs at a different time in each part of the nipple during the reproductive cycle.

The mammary gland changes histologically during the reproductive cycle, and this process is regulated by many growth factors [7, 5, 15]. It has been reported that the role of proliferation of mammary epithelial cells increases bimodally; the highest relative number of dividing cells is found at an early stage (day 4) of pregnancy with a smaller peak on day 12 [1, 17]. These reports indicated that the period of maximal cell proliferation during pregnancy might be correlated with the periods of high concentrations of progesterone and prolactin or placental lactogen. It has been reported that estrogen increases the relative number of epidermal cells in mitosis [2]. The morphological changes in the nipple during the reproductive cycle are probably also regulated by these growth factors.

Sherwood and co-workers reported that rat relaxin, secreted by the corpus luteum during the second half of pregnancy, promoted the growth of the nipple at this stage [6, 8, 9]. Unemori and Amento [19] reported that relaxin induced significant turnover of collagen both by stimulating expression of collagenase and by down-modulating synthesis and secretion of collagen. In the present study, the numbers of BrdU-positive cells tended to increase and many active fibroblasts were observed during the second half of pregnancy. Therefore, it is possible that relaxin modulates the activity of fibroblasts in the dermis during nipple development.

In summary, our study revealed the sites and timing of cell proliferation in the mouse nipple during the reproductive cycle. It was suggested that the growth patterns vary in among the different parts of the nipple; the epidermis and epithelium of the lactiferous sinus grow mainly as a result of cell proliferation during pregnancy, while the dermis has two growth phases, with cellular proliferation during pregnancy and an increase in extracellular matrix during lactation.

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