Pregnancy and Lactation Affect the Microvasculature of the Mammary Gland in Mice

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ABSTRACT. Microvascular changes in the mammary gland in mice during pregnancy and lactation were investigated by scanning electron microscopy (SEM) with a corrosion cast method, transmission electron microscopy (TEM) and morphometry. By SEM, duct-associated capillary plexuses were sparsely distributed to branch into adipocytes during virgin period. With advance in pregnancy, both branches from the capillary plexuses and branches from the vessels surrounding adipocytes extended further to form capillary networks. The basket-like architecture was completed by day 18 of pregnancy. These findings may indicate that angiogenesis occurs frequently during this period. During lactation, the basket-like architecture still remained and the capillaries surrounding alveoli meandered. After weaning, the regression of microvasculature followed the degeneration of alveoli. By TEM and morphometry, the density of pinocytotic vesicles (PVs) (number of PVs per μm^2 of endothelium cytoplasm) increased twofold from day 18 of pregnancy to day 5 of lactation, furthermore increased threefold from days 10 to 20 of lactation, and subsequently decreased after weaning. Marginal folds and microvillous processes gradually increased in length with advance in pregnancy, reached the maximum from days 5 to 15 of lactation, and thereafter decreased. In addition, the capillaries with thinner walls were in close contact with alveoli during the late stage of pregnancy and during lactation. Furthermore, the alveolar epithelial cells had well-developed basal infoldings during lactation. These findings suggest that the capillaries play an important role in transporting materials necessary for milk production.—KEY WORDS: corrosion cast, mammary gland capillary, microvasculature, morphometry, mouse.

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The mammary gland is an accessory gland that develops especially during pregnancy and lactation. There have been a number of reports on the endocrine factors which regulate the development of the gland according to the functional states of milk production and secretion [14, 23]. The development of blood vessels is thought to play an important role in both mammogenesis and lactogenesis, since a large quantity of blood is required to produce milk [8, 25].

The morphological relationship between the mammary parenchyma and the blood vessels has been investigated in the mouse mammary glands by light microscopy [5, 15, 21, 24]. To our knowledge, however, there is no report on the development of microvascular architecture of the mammary gland except for a report in the rat by Yasugi *et al.* [26]. Recently, a vascular corrosion casting method for scanning electron microscopy (SEM) [13] has been applied to the studies of vascular architectures in a vast variety of organs. In the present study, a vascular corrosion casting method for SEM was employed to clarify the three-dimensional development of microvasculature of the mammary gland in the mouse.

Ultrastructural changes in capillaries are reported to be involved closely in the capillary permeability [3, 4, 7, 9, 11, 16, 17, 19, 20, 27]. Although Sandborn [18], and Stirling and Chandler [22] described the fine capillary structure of the mammary gland during in non-lactating period, ultrastructural changes to be expected to occur in various physiological states have not yet been reported. In the present study, morphological changes in capillaries of the mouse mammary gland from virgin through pregnancy, lactation and post-weaning stages were examined by transmission electron microscopy (TEM). Moreover, a morphometric analysis was attempted to assess quantitative changes in the morphological features.

MATERIALS AND METHODS

Eighty five JCL-ICR female mice, bred and maintained as a colony in our laboratory, in stages of virgin (90 day-old), pregnancy (5, 10, 15 and 18 days of pregnancy), lactation (5, 10, 15 and 20 days after partum) and post-weaning (5, 10 and 15 days after weaning) were used in this study. Each stage group consisted of 7 or more animals. During lactation, each mother mouse was housed with her 8 to 10 pups. All the animals were supplied with food and water *ad libitum*.

For SEM, vascular corrosion casts were prepared according to Murakami's method [13]. In brief, the animals were perfused with Ringer's solution containing heparin sodium (4 IU/m*l*) and with Mercox CL-2R (Dainippon Ink and Chemicals, Inc., Tokyo, Japan), through the thoracic aorta. After polimerization of resin, the first abdomino-inguinal mammary glands were excised and soaked in 20% KOH solution overnight at 60°C to remove the surrounding soft tissues. Dehydrated and air-dried vascular casts were exposed to osmic vapor, coated with gold by sputtering in a vacuum evaporator and examined with a JSM-25 scanning electron microscope at 15 kV.

For TEM, small pieces were cut out from each of the first abdomino-inguinal mammary glands. The samples were immediately fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 hr at 4°C. They were rinsed in the same buffer and post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 hr at 4°C. They were dehydrated in a graded series of ethanol and embedded in Epon 812. Thin sections were cut on an ultramicrotome, stained with uranyl acetate and lead citrate, and observed with a JEM-100C transmission electron microscope at 80 kV.

For morphometry, 3 or more animals were used for each stage. Ten or more cross sections of mammary capillaries were obtained from different animals and tissue blocks. TEM micrographs of capillaries were taken at a magnification of × 10,000. The photographs were enlarged to yield working prints of magnification × 25,000. The area of endothelium cytoplasm without the nucleus region, the length of marginal folds and the length of microvillous processes were measured by an image analyzer (Nikon Cosmozone Is). Pinocytotic vesicles (PVs) were counted on enlarged photographs and the density of PVs was calculated as the number of PVs per μm^2 of endothelial cytoplasm. The data were statistically analyzed by Student's *t*-test.

RESULTS

Virgin period: SEM revealed that duct-associated capillary plexuses ran in parallel with or encircled the ducts. These capillaries were ramified among adipocytes around the ducts (Fig. 3a).

By TEM, the blood capillaries were mainly of a continuous type (Fig. 4a), but rarely of a fenestrated type with a few fenestrations (Fig. 4b). Endothelial cells were provided with a few short marginal folds and microvillous processes. PVs scattered on the luminal and abluminal plasma membranes and in the cytoplasm.

Pregnant period: On day 5 of pregnancy, the duct-associated capillary plexuses were well developed. These capillaries were ramified to supply the buds or the alveoli. On day 10, the capillaries surrounding the ducts increased in number, while, in developing mammary lobuli, capillaries were frequently ramified and anastomosed with one another. Capillary sprouts were often observed. The capillaries, which were in close contact with the buds and alveoli, received the rami both from duct-associated capillary plexuses and from capillaries surrounding adipocytes. On day 15, as the mammary lobuli developed, the capillaries were ramified and anastomosed with one another to form a dense network (Fig. 3b). Capillary sprouts were still observed frequently (Fig. 3b). On day 18, each alveolus and duct was surrounded by a basket-like network of capillaries (Fig. 3c).

Only a few PVs were observed in the capillaries surrounding mammary ducts and buds which comprised light endothelial cells in early stage of pregnancy (Fig. 4c). The capillaries surrounding adipocytes had thinner walls and had relatively abundant PVs as compared with those surrounding buds. PVs were frequently fused with one another to form a cluster of vesicles communicating with the luminal and abluminal surfaces. On day 15, most of the capillaries were in close contact with alveoli. In the endothelial cells of such capillaries, cytoplasmic projections to stromal space, a few marginal folds and a few microvillous processes were recognizable. The endothelial cells of the capillaries surrounding the ducts had numerous polysomes. On day 18, basal infoldings appeared in the basal region of alveolar epithelial cells (Fig. 4d), especially numerous in the region facing the capillaries. The endothelial cells of the capillaries surrounding the ducts had electron dense cytoplasm. The capillaries surrounding the adipocytes had prolonged marginal folds and microvillous processes. As shown in Fig. 2, the microvillous processes significantly increased both in length and in number at any stages of pregnancy as compared with those during virgin period ($P<0.05$).

Lactation period: On day 5 of lactation, the capillaries surrounding the mammary alveoli were composed of highly ordered basket-like units. Such capillary units, each being constructed by numerous ramifications and anastomoses, were observed throughout the mammary gland. A few meandering vessels were found around the alveoli. On days 10 and 15, the microvasculatures further developed to show a complicated appearance by an increased number of meandering capillaries around the mammary alveoli and ducts (Fig. 3d). While, a partial regression of the vasculature occurred on day 20, although some capillaries still meandered (Fig. 3e).

By TEM, basal infoldings of alveolar epithelial cells increased in number on day 5 of lactation (Fig. 4e). The capillary wall became thinner, closely in contact with alveolar epithelial cells. Numerous PVs scattered in the endothelial cytoplasm and significantly increased in number as compared with those on day 18 of pregnancy (Fig. 1). They tended to fuse with each other and to form a cluster. Marginal folds and microvillous processes grew longer (Fig. 2). These features were especially prominent in the capillaries around alveolar epithelial cells with well-developed basal infoldings. On day 10, PVs reached the maximum in number and plateaued until day 20 (Fig. 1). Marginal folds and microvillous processes, which were occasionally curved and/or were ring-like in appearance, became the longest (Figs. 2, 3f). On day 15, lysosome-like structures were observed in the endothelial cytoplasm. On day 20, convoluted basal laminae were found in some capillaries facing the alveolar epithelial cells (Fig. 4g). As shown in Fig. 2, microvillous processes were significantly longer at any stages of lactation than at any stages in pregnancy.

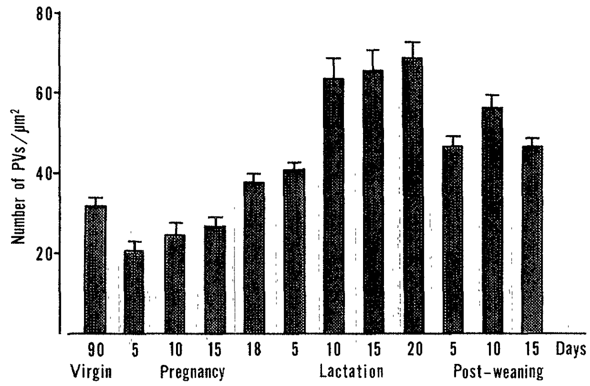

Fig. 1. Changes in density of pinocytotic vesicles (PVs) of endothelial cells of blood capillaries supplying to the parenchyma of the mouse mammary gland from virgin through pregnancy, lactation and post-weaning. Each value shows mean ± SEM.

Post-weaning period: On day 5 after weaning, the basket-like networks seen during lactation period decreased in number to a greater degree, due to the regression of mammary alveoli and to the increased number of adipocytes (Fig. 3f). Especially, the capillary network surrounding each lobule was regressive. The capillaries surrounding the alveoli meandered to a lesser degree as compared with those on day 20 of lactation. After day 10, the vasculature recovered to a degree similar to that during virgin period, although the capillary net-

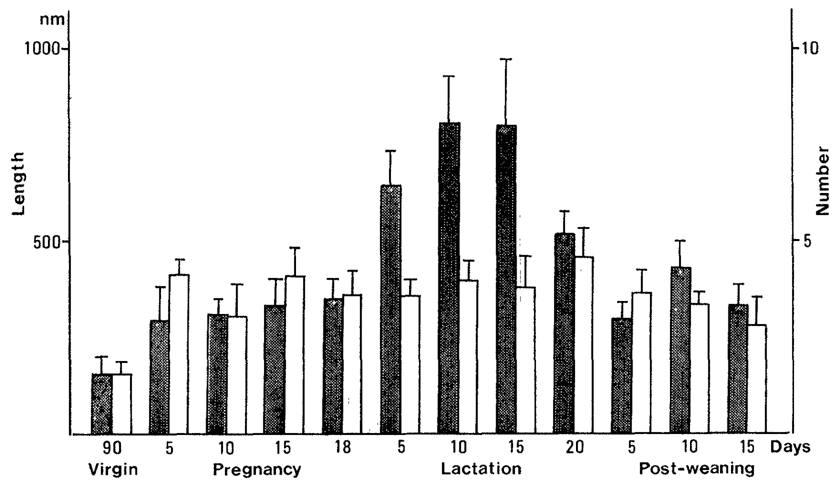

Fig. 2. Changes in number: □, and length; ▦ of microvillous processes of endothelial cells of blood capillaries supplying to the parenchyma of the mouse mammary gland from virgin through pregnancy, lactation and post-weaning. Each value shows mean ± SEM.

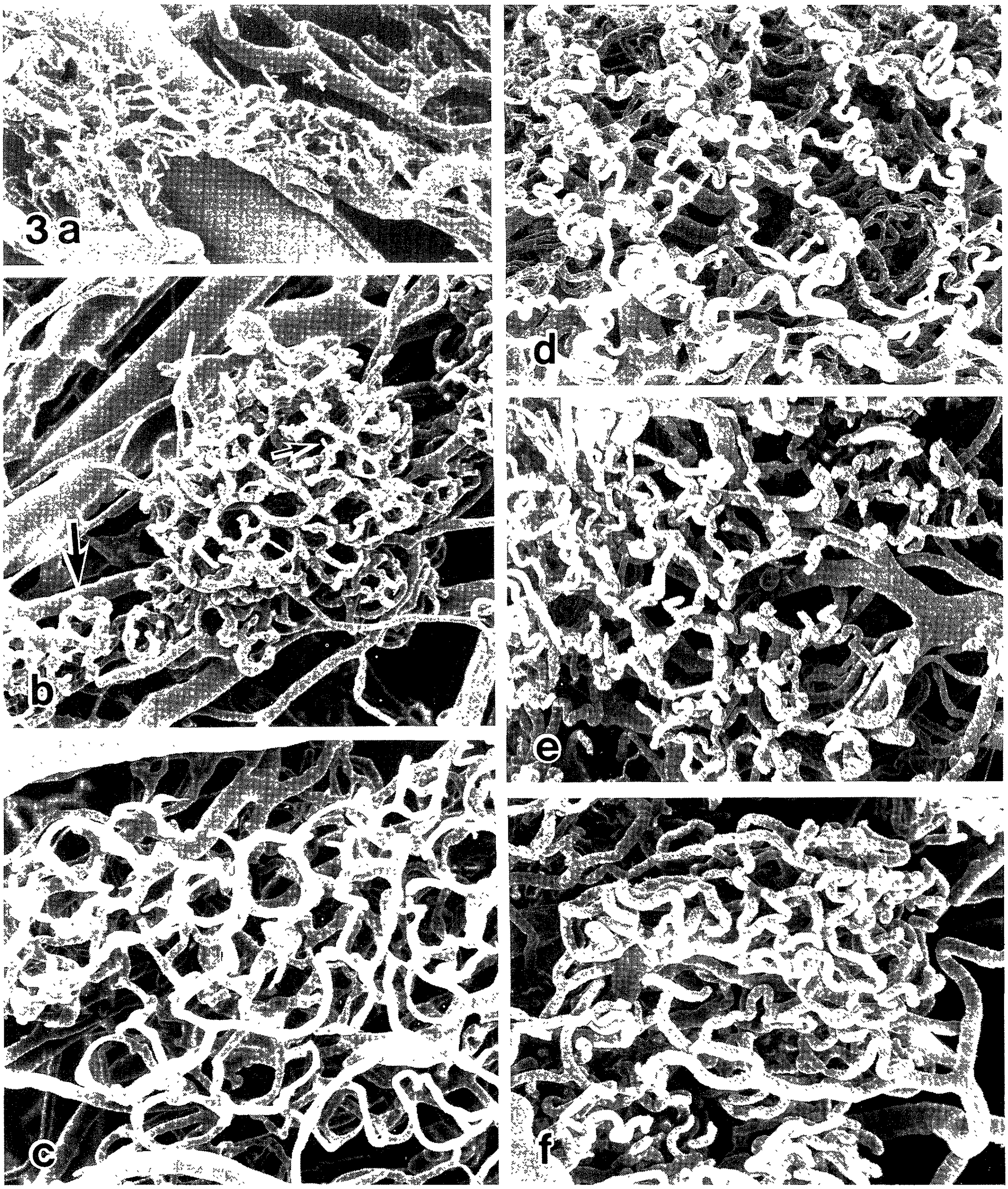

Fig. 3. Scanning electron micrographs of the vascular corrosion casts of the mammary gland. a) Duct-associated capillary plexus in virgin mouse. × 200. b) Vascular bed of lobuli on day 15 of pregnancy. A duct-associated capillary plexus (large arrow) is connected with it. Small arrow; capillary sprout. × 300. c) Capillary network surrounding an individual alveolus on day 18 of pregnancy. × 400. d) Alveolar capillary networks on day 10 of lactation. Note markedly meandering capillaries. × 150. e) Alveolar capillary networks on day 20 of lactation. Freeze fracture surface of the resin cast. × 400. f) Capillary networks of lobuli on day 5 after weaning. Apparent reduction of basket-like networks is seen in the upper part of the figure. × 500.

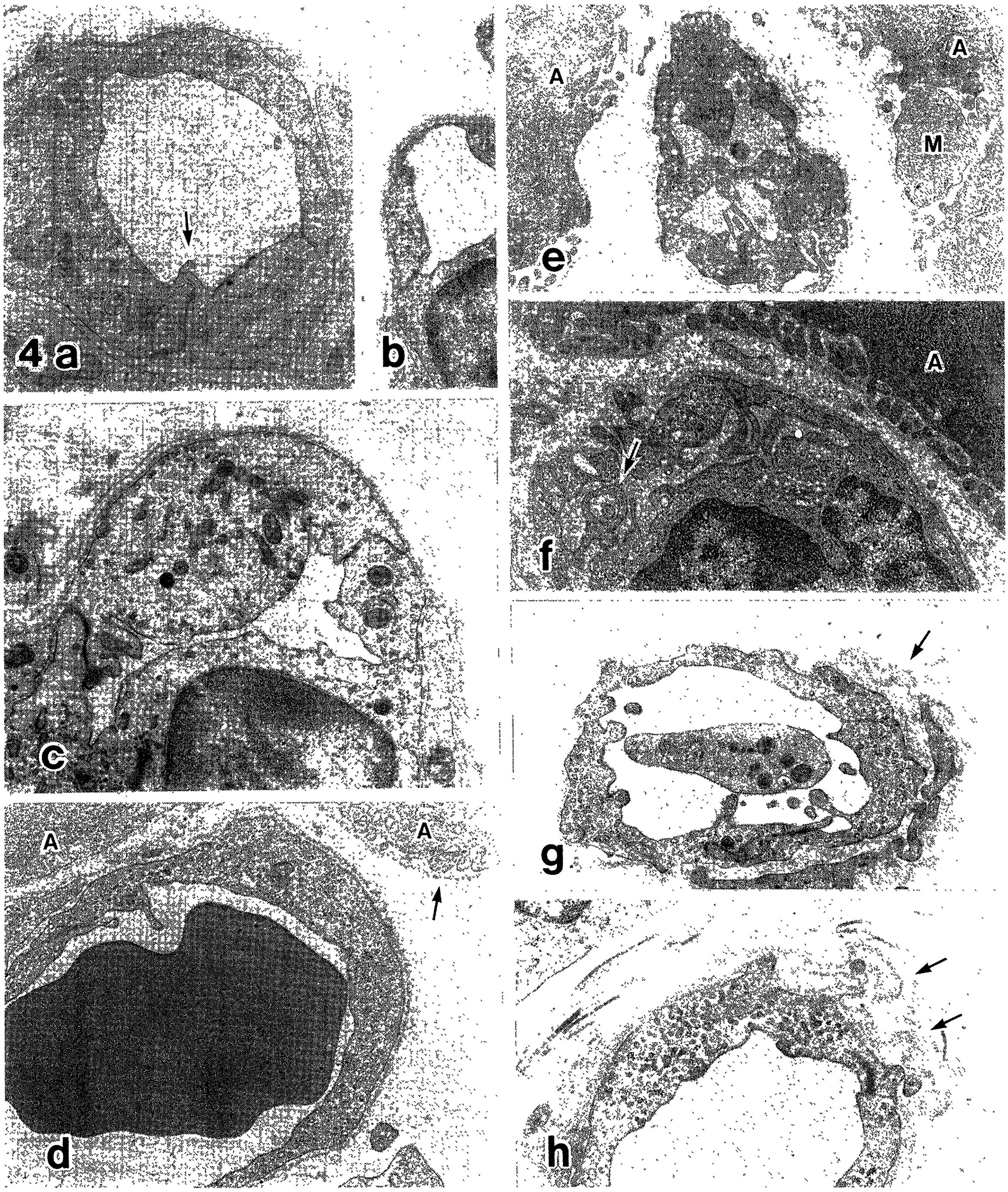

Fig. 4. Transmission electron micrographs of the capillaries surrounding the parenchyma. A; alveolar epithelial cell. M; myoepithelial cell. a) Continuous capillary in virgin mouse. Arrow; short marginal folds. × 12,000. b) Fenestrated capillary with a few fenestrations surrounding a main duct of the mammary gland. × 12,000. c) Capillary surrounding a bud on day 10 of pregnancy. × 12,000. d) Capillary beneath alveolar cells at 18 day of pregnancy. Basal infoldings (arrow) are seen in the basal portions of alveolar epithelial cells. × 12,000. e) Capillary between alveoli on day 5 of lactation. Long marginal folds and microvillous processes are seen. Note the well-developed basal infoldings. × 12,000. f) Capillary between alveoli on day 10 of lactation. Arrow; ring-formed microvillous process. × 12,000. g) Capillary surrounding an alveolus on day 20 of lactation. Arrow; convoluted basal lamina of capillary. × 12,000. h) Capillary around an involuting alveolus on day 5 after weaning. Arrows; convoluted basal lamina of capillary. × 14,000.

works surrounding the lobuli were still encountered.

On day 5 after weaning, the endothelial cells of the capillaries surrounding alveoli had a lesser amount of PVs (Fig. 1), shortened marginal folds and microvillous processes (Fig. 2), thinned walls, and convoluted basal lamina (Fig. 4h). While, the endothelial cells of the capillaries surrounding adipocytes still had numerous PVs, especially in their walls facing the adipocytes. After day 10, the fine structure of the capillaries was almost similar to that during virgin period, except the presence of a large amount of PVs (Fig. 1).

DISCUSSION

In the present study, changes in the microvasculature of the mouse mammary gland during the reproductive cycle were three-dimensionally observed by a SEM corrosion cast method. The findings during pregnancy and lactation were well in agreement with those in the rat mammary gland by Yasugi *et al.* [26]. However, duct-associated capillary plexuses appeared from the virgin period to the middle stage of pregnancy and branched into adipocytes. With advance in pregnancy, that is, in formation of mammary lobuli and alveoli, both branches from the cpaillary plexuses and branches from the vessels surrounding adipocytes extended to form capillary networks. These findings support the descriptions by Dabelow [5], Soemarwoto and Bern [21], and Nishinakagawa [15]. Furthermore, it is assumed that angiogenesis occurs frequently during this period.

The capillaries in mammary alveoli during the middle and late stages of pregnancy frequently ramified and anastomosed with one another to form basket-like networks. Each alveolus was surrounded by 3 or more branches of capillaries. Moreover, during lactation, the capillaries surrounding alveoli meandered. Such meandering capillaries may be advantageous in increasing the contact area to the alveoli. The morphology of capillaries seems to alter with the expansion and contraction of alveoli. In addition, the most marked meandering of capillaries was observed on days 10 to 15 of lactation, a period corresponding to the maximal milk production time. These findings are consistent with the fact that a large quantity of blood is supplied to mammary alveoli for milk production in this period [8, 25]. Therefore, it is suggested that the capillary meandering is an adaptation to the active state of the mammary gland. After weaning, the microvasculature of each alveolus was preserved by day 5 in spite of degeneration of alveoli.

By TEM, the mammary gland capillaries were mainly of a continuous type during any reproductive cycles, even in the middle stage of lactation when the permeability of capillaries was estimated to reach the maximum. Sandborn [18] described the endothelium of mammary capillary as thickness type, while Stirling and Chandler [22] reported as skeletal type. Therefore, the mammary gland capillaries of mice should be mainly composed of a continuous type. In addition, the capillary walls became thinner at the late stage of pregnancy, showing the same appearance from lactation period to day 10 after weaning. From these findings, the morphological changes in capillaries seem to occur prior to the functional changes in mammary alveoli accompanying with mammogenesis and lactogenesis.

To our knowledge, morphological changes in marginal folds and microvillous processes of the mammary gland capillaries during the reproductive cycle have not yet been examined by TEM. In the present observations, marginal folds and microvillous processes increased in length to become slender from the late stage of pregnancy to the early stage of lactation, and grew longer to a greater degree during the middle stage of lactation. Therefore, there is a possibility that these marginal folds and microvillous processes may serve as a device to take in fluid and particles, and/or as a barrier to slow down the blood velocity. Ehrenbrand [6] described the active transport by PVs in the endothelial cells of mammary gland capillaries during lactation. As PVs have been observed in large numbers during lactation, especially in the middle stage, it is assumed that pumping transport by PVs is closely related to the supply of a large quantity of materials necessary for milk production.

The basal infoldings, which appeared at the late stage of pregnancy and were constantly present during lactation, were the most marked in the alveolar epithelial cells. These structures have been thought to play an important role in taking up specific components necessary for milk production [1, 2, 10, 12]. In the present study, they were also changeable according to the functional state of alveolar epithelial cells. Therefore, the basal infoldings may act to expand the surface area for absorption. Furthermore, it is suggested that the morphological changes in basal infoldings are closely related to the density of PVs and the length of marginal folds and microvillous processes during lactation.

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