Distribution of F4/80-Positive Cells in Developing Ovaries in the Mouse*

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Summary. The mature ovary contains a large number of macrophages. In the present study, the distribution of macrophages in murine ovaries at various developmental stages was immunohistochemically studied using a monoclonal antibody against F4/80, a highly specific antigen of murine macrophages. The results showed that definite F4/80-positive stains were hardly detectable in ovaries on day 0 after birth. On day 7, a few F4/80-positive cells could be identified between the developing follicles. The positive stains were irregular in shape and showed little physical contact with the primordial or primary follicles. By days 14 and 21, when the theca cell layers of growing follicles were developing, the positive cells had extended or elongated to surround the cell layer. On day 28, besides the presence of elongating positive cells surrounding the growing follicles, irregularly shaped F4/80-positive cells became apparent in the interstitium between the growing follicles and also in the capsular tissues. Thereafter, positive cells with stellate appearance were detected in the corpora lutea, which first developed around 6 weeks of age. Although the positive cells were homogenously distributed in the corpora lutea in virgin adults, only a few sporadic positive cells were found there in pregnant mice. However, the positive cells infiltrated into the corpora lutea again in the postpartum period. These results show that ovarian macrophages exhibit dramatical changes in their distribution from neonatal to postpartum periods.

Materials and Methods

Animals

Female ICR mice on day 10 of pregnancy (n=10) and at 6 weeks of age (n=24) were purchased from SLC (Shizuoka, Japan). The mice were individually housed in plastic cages under constant room temperature, humidity and 12/12 h light-dark cycle (lights on at 07.00 h) in the Laboratory Animal Center of Kagawa Medical School.

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Immunohistochemical identification of ovarian macrophages

Mice aged 0, 7, 14, 21 and 28 days, and 6, 8, 10 and 12 weeks were killed by decapitation or cervical dislocation, and their ovaries were removed and put in OCT compound (Miles Laboratories, Naperville, Ill). Ovaries were also taken from adult mice at day 15 of pregnancy and at day 5 postpartum. The organs in the OCT compound were immediately frozen in liquid nitrogen and stored at −70°C until use. Sections of 6 μm were cut with a cryostat (CM1900; Leica, Germany), dried in air, fixed in 96% ethanol for 10 min at room temperature, and then rinsed in phosphate-buffered saline (pH 7.4). The sections were incubated with normal rat serum (1:50 dilution; Vector Lab., CA), followed by incubation with rat monoclonal antibody against the F4/80 antigen (first antibody, 1:100 dilution; Serotec, Oxford) for 16 h at 4°C. After washing with phosphate-buffered saline, the sections were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-rat IgG monoclonal antibody (second antibody, 1:100 dilution; Serotec) for 1 h at room temperature. Control sections were only reacted with normal rat serum and the second antibody. The HRP-binding sites were detected in 0.05% 3,3'-diaminobenzidine 4HC1 and 0.01% H2O2 after washing the sections with phosphate-buffered saline. The F4/80-positive cells were thus identified as brown-colored cells at the light microscopic level.

Plastic sections for conventional histology

Ovaries removed from each age group as described above were immediately fixed in Bouin's solution for 3 days, dehydrated in an ethanol series, and embedded in plastic (Technovit 7100; Kulzer & Co., Germany). Sections of 5 μm were cut with a microtome (HN340E; Microme, Germany) and stained with Gill's hematoxylin III and 2% eosin Y. Cells with granulated or vacuolated cytoplasm and densely stained nuclei were identified as macrophages, as previously described (MENDIS-HANDAGAMA et al., 1987; HARDY et al., 1989; VERGOUWEN et al., 1991).

RESULTS

The plastic sections stained with haematoxylin and eosin revealed that the architecture of the ovarian tissues dramatically changed from neonatal to postpartum periods. Many primordial follicles were found on day 0 (Fig. 1a). On days 7 and 14, primary and secondary follicles appeared, respectively (Fig. 1b, c). By day 21, the interstitial tissue around the growing follicles had developed prominently (Fig. 1d). Thereafter, pre-Graafian and Graafian follicles developed, followed by formation of the corpora lutea (Fig. 1e-f). However, it was extremely difficult to discriminate macrophages from other types of cells in the interstitium, corpora lutea and follicles in the plastic sections.

In ovarian sections treated with the anti-F4/80 monoclonal antibody, a few stains were found among and around primordial follicles on day 0 after birth, although these were very weak and indefinite (Fig. 2a). On day 7, some positive stains were visible among primary follicles (Fig. 2b). The stained cells were irregular in shape and showed no intimate contact with the follicular cells. On day 14, F4/80-positive cells were found to have changed in shape from irregular to elongate. They surrounded the theca cell layers of growing follicles (Fig. 2c). Compared with that on day 14, the interstitium on day 21 was more developed (Fig. 1f, g), and the presence of irregular-shaped F4/80-positive cells had become conspicuous in this region (Fig. 2d). On day 28, when pre-Graafian follicles had developed (Fig. 1e), irregular-shaped F4/80-positive cells became more concentrated in the interstitium, in addition to the presence of elongating F4/80-positive cells around the theca cell layer (Fig. 3a, b). Many F4/80-positive cells were also found in the capsular tissues on day 28 (Fig. 3c). Most of the granulosa cell layers in growing follicles were free of F4/80-positive cells, although they occasionally contained one or several F4/80-positive cells (Fig. 3a, c). Control ovarian sections did not exhibit any significant staining (Fig. 3d).

From 6 weeks after birth, F4/80-positive cells became a major cellular component of the ovarian interstitium (Fig. 4a, c, e). Positive cells usually had long cell processes and exhibited physical contact with adjacent F4/80-positive cells, forming a macrophage network in the interstitium and theca cell layers.

Fig. 1. Plastic sections of ovaries stained with haematoxylin and eosin. The sections were obtained from mice aged 0 (a), 7 (b), 14 (c), 21 (d) and 28 (e) days, and 6 (f), 8 (g), and 10 (h) weeks. B blood vessel, C corpus luteum, I interstitial tissue, G granulosa cell layer, T theca cell layer. a–h: ×70
Fig. 1. Legend on the opposite page.
Moreover, F4/80-positive cells appeared in the corpora lutea from 6 weeks of age (Fig. 4b, d, f). They were distributed homogeneously with a stellate appearance. It was noted that F4/80-positive cells were more densely present in the corpora lutea of 10 week-old mice than in the newly developed corpora lutea of 6 week-old mice (Fig. 4b, f). F4/80-positive cells were also present in atretic follicles, although their cell shape was round in contrast with those in the interstitium, theca cell layers and corpora lutea (Fig. 4a). As seen in the ovaries on day 28, a few F4/80-positive cells were occasionally detected in the granulosa cell layer of growing follicles after 6 weeks of age (Fig. 4e).

In pregnant mice, the size of the corpora lutea became larger than that in virgin adult mice. In this stage, only a few F4/80-positive cells with short cell processes were sporadically and randomly found in the corpora lutea (Fig. 5a). In the postpartum period, the corpora lutea were again found to be infiltrated with F4/80-positive cells with long cell processes (Fig. 5b). Control sections of immature mice, pregnant mice and mice in the postpartum period were also negative for immunostaining as seen in Figure 3d (not shown). Five experiments were performed for each age group, with all yielding similar results.

**DISCUSSION**

In the present study, ovarian macrophages in developing mice were immunohistochemically studied using a rat monoclonal antibody against macrophage-specific antigen F4/80. This is the first demonstration of postnatal change in the distribution of ovarian macrophages in the mouse. A few F4/80-positive cells free of follicular cells appeared between days 0 and 7 after birth. From 2 weeks of age, the positive cells became elongate and surrounded the follicles. From 3 weeks of age, irregular-shaped positive cells became conspicuous in the interstitium. From 4 weeks of age, irregular-shaped positive cells became conspicuous in the interstitium. From 4 weeks of age, irregular-shaped positive cells became conspicuous in the interstitium. From 4 weeks of age, irregular-shaped positive cells became conspicuous in the interstitium. From 4 weeks of age, irregular-shaped positive cells became conspicuous in the interstitium.
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stium and theca layers. Thus, the distribution of ovarian macrophages showed very dynamic changes during the period of postnatal maturation.

The F4/80 antigen is a pan-macrophage marker which is expressed in monocyte-macrophage lineages at all differentiation stages (AUSTYN and GORDON, 1981). Different from BM8, a differentiated macrophage antigen (MALORYN et al., 1986), F4/80 is expressed on immature macrophages on day 0 after birth (LI et al., 1998). We did not examine F4/80-positive cells during the prenatal period of the mouse in the present study. However, the inability to observe any definite stains on day 0 gives evidence of the poor development of macrophages during gestation.

There are several reports noting that tissue sections stained with toluidine blue or haematoxylin, testicular macrophages are light microscopically identifiable from their granulated and vacuolated cytoplasm and densely stained nuclei (MENDIS-

HANDAGAMA et al., 1987; HARDY et al., 1989; VERGOUWEN et al., 1991). However, we encountered much difficulty in identifying ovarian macrophages using the morphological criteria described above. Therefore, immunohistochemical studies involving an antimacrophage antibody are needed to determine the distribution of ovarian macrophages.

HUME et al. (1984) were the first to detect immunohistochemically ovarian macrophages by using a monoclonal antibody against the F4/80 antigen. They showed that the ovarian interstitium, thecal cell layers, atretic follicles and corpora lutea are the major anatomical locations of F4/80-positive cells in adults. We confirmed these findings but additionally noted that many F4/80-positive cells accumulated in the capsular tissues when the first ovulation was about to occur. Although the functions of these cells remain unclear, they might be involved in the repair of ruptured sites of the capsular tissues after ovulation.

**Fig. 3.** F4/80 antigen-positive cells in ovarian sections obtained from 28-day old mice. **a.** Macrophages surrounding follicles. **b.** Macrophages in the interstitium of the medulla. **c.** Macrophages in the capsular tissues. **d.** A control section only reacts with normal rat serum and the HRP-conjugated rabbit anti-rat IgG mAb. **B** blood vessel, **CT** capsular tissue, **I** interstitial tissue. Asterisks indicate growing follicles. The arrow indicates a F4/80-positive cell in the granulosa layer. a-d: ×70
SIMON et al. (1994), on the other hand, applied a monoclonal antibody against the Mac-1 antigen (a marker of macrophages, neutrophils and mast cells) to stain ovarian macrophages in the mouse. They also showed a similar distribution, but stated that Mac-1-positive macrophages were never detected within growing follicles. However, the present study clarified the presence of F4/80-positive cells in the granulosa cell layers of developing follicles, although the incidence was very low. It has been reported that rats and guinea pigs also have macrophages in the granulosa layers (FUKUMATSU et al., 1992; KASUYA, 1997). These papers reported that they may participate in promoting the proliferation of granulosa cells or eliminating apoptotic granulosa cells.

PETROVSKA et al. (1996) found that Mac-1-positive
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Macrophages in the interstitium, theca cell layers, corpora lutea and atretic follicles of adult mice change their numbers over the course of the estrous cycle: The macrophage density was significantly higher in proestrus and metaestrus, when compared with that in diestrus and estrus. Most recently, Cohen et al. (1997) also reported a decrease in the number of F4/80-positive cells through the estrous cycle in ovaries of osteopetrosis (op/op) mice, which have reproductive failure. These two studies indicate that the ovarian macrophages play a significant endocrinological role in the tissue and their distribution is still dynamic in adults.

It has been accepted that the most basic function of macrophages is phagocytosis. In fact, previous studies showed that ovarian macrophages phagocytose luteal cells, granulosa cells and atretic follicles in guinea pigs and mice (PaaVola, 1979; Kuryszko and Adamski, 1987; Kasuya, 1997). These observations lead to the speculation that the ovarian macrophages contribute to the luteolysis and atresia of follicles through their phagocytic activity. Alternatively, it has been demonstrated in the rat that the co-cultivation of ovarian macrophages with luteal cells leads to enhanced progesterone secretion from the luteal cells in vitro, indicating a paracrine function of ovarian macrophages (Kirsch et al., 1983). It is also well known that luteal cells undergo hypertrophy with the augmented secretion of progesterone during pregnancy. However, we demonstrated that the macrophages in the corpora lutea were few in number and their cell size diminished during pregnancy. Therefore, we suppose that the macrophages in the corpora lutea during pregnancy are less active for stimulating luteal cells. Progesterone may inhibit the accumulation of macrophages in the corpora lutea, or the decrease in number of degenerating luteal cells during pregnancy may not attract phagocytic macrophages. We also showed that macrophages again infiltrated into the corpora lutea in the postpartum period. This also indicates that the macrophages are involved in the regression of the corpora lutea.

Previously, the physical contact of macrophages with such steroid-producing cells as luteal and theca cells attracted much attention for the consideration of the paracrine functions of ovarian macrophages (Kirsch et al., 1981; Halme et al., 1985; Bagavandoss et al., 1988; Adashi, 1990; Mori, 1990; Fukumatsu et al., 1992; Stern and Coulam, 1992). We have clarified the presence of macrophage-macrophage contact through the long cell processes of great numbers of F4/80-positive cells in the interstitium outside the corpora lutea and follicles. The network of macrophages develops rapidly when the mouse ovary reaches maturity. However, it remains unknown whether or not the network formation in the interstitium plays an important role in the ovary.

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