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Elimination of atretic follicles from the mouse ovary: a TEM and immunohistochemical study in mice

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ABSTRACT

We examined numerous ovarian follicles from 32–35 d virgin mice by transmission electron microscopy and light microscopic immunohistochemistry. No macrophages were seen, but various stages of apoptotic granulosa cells were encountered. Presumably a granulosa cell or its debris in an advanced stage of apoptosis was destined to be phagocytosed by adjacent normal-looking granulosa cells. Other granulosa cells of normal appearance were seen in the region of the zona pellucida in contact with and apparently phagocytosing atrophic oocytes. Such granulosa cells were characterised by the presence of gap junctions with other cells and frequently contained annular gap junctions in the cytoplasm. To confirm the lack of involvement of macrophages in the process of follicular atresia and elimination, specially prepared ovarian sections were incubated with antimouse macrophage monoclonal antibodies (F4/80, Mac-1, Mac-2). None of the follicles examined showed positive immunoreactivity with these antibodies. Atretic follicles may shrink and eventually disappear from the ovary as a result of repeated apoptosis and phagocytosis by granulosa cells. There is no evidence for the presence or involvement of macrophages in the atretic follicles, at least in prereproductive mice as examined.

Key words: Ovarian follicular atresia; apoptosis; granulosa cells; macrophages.

INTRODUCTION

The majority of ovarian follicles undergo atresia, except for a few that are destined to reach maturity (Byskov, 1978). This process of atresia is currently regarded as being due to apoptosis, a genetically programmed cell death, of the granulosa cell mass and ultimately of the oocyte (Hughes & Gorospe, 1991; Tilly et al. 1991). Several authors have claimed that macrophages are active in phagocytosing apoptotic (pyknotic) granulosa cells in atretic follicles in mice (Byskov, 1974) and other animals (Peluso et al. 1980; Bukovsky et al. 1993, 1995; Kasuya, 1995, 1997).

We have shown that granulosa cells are capable of phagocytosis in the mouse ovary: gap junctional membrane parts were found to be internalised, endocytosed and digested by normal-looking granulosa cells in preovulatory mature follicles (Koike et al. 1993), and also in atretic follicles (Watanabe & Tonosaki, 1995). Acid phosphatase activity was verified in endocytosing granulosa cells (Koike et al. 1993). This phagocytotic ability of granulosa cells appeared to be involved in the course of atresia. Apoptotic granulosa cells were sometimes seen to be engulfed by granulosa cells of normal appearance (Watanabe, 1996). The purpose of this morphological study with TEM and immunohistochemistry was to obtain more convincing evidence that granulosa cells or macrophages play a pivotal role in eliminating atretic follicles from the ovary.

MATERIALS AND METHODS

Materials

Five B6C3 mice (aged 32–35 d) were supplied by our Institute for Experimental Animals. We chose these

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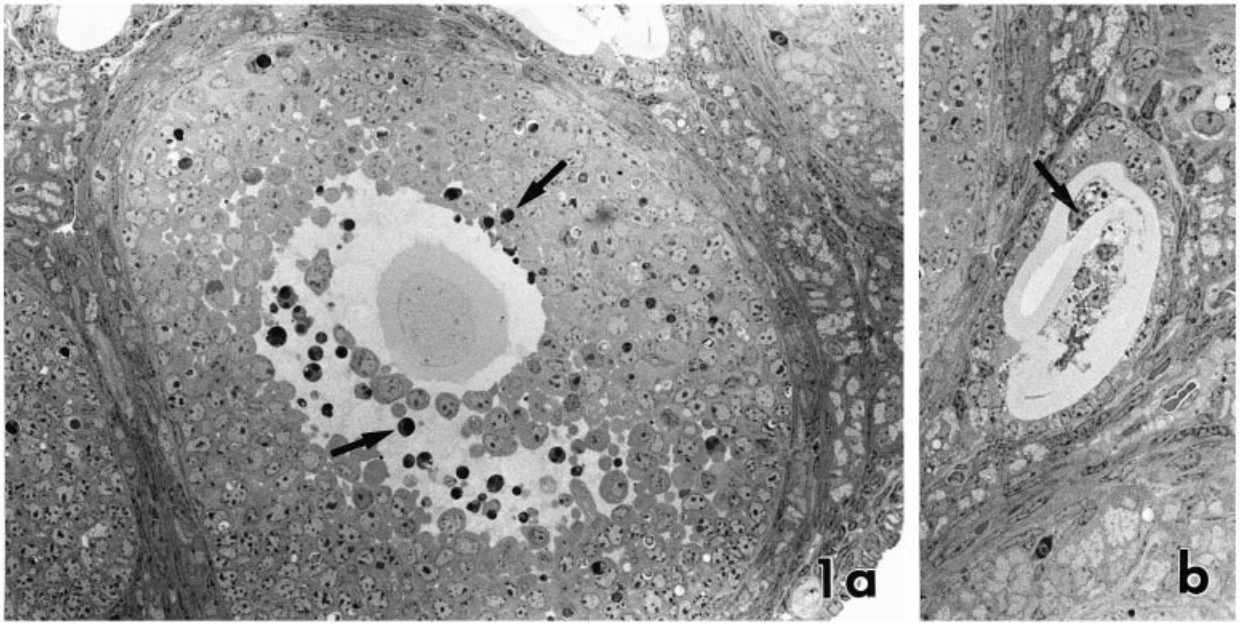

Fig. 1. Light micrographs showing early (*a*) and advanced (*b*) atretic follicles. (*a*) The granulosa layer contains apoptotic cells (arrows) characterised by darkened nuclei. This layer consists of multistratified granulosa cells. (*b*) The granulosa layer is thinner than that in *a*. The oocyte has already been abolished by this stage. The zona pellucida is coiled and appears to have been invaded by granulosa cells (arrow). ×360.

prereproductive mice, in which a maximum ratio of atretic follicles was expected (Byskov, 1978). Ovaries were isolated under anaesthesia with diethyl ether. The spleen was also isolated as a positive control for immunohistochemistry.

Light and electron microscopy

Isolated ovaries were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.4) for 2 h, and postfixed with 1% osmium tetroxide in the same buffer for 2 h. After dehydration in a graded ethanol series, specimens were placed in propylene oxide and embedded in Epon 812. Semithin sections (0.3–0.5 μm) were stained with toluidine blue for light microscopy. Ultrathin sections were contrasted with uranyl acetate and lead citrate, and examined by electron microscopy (H-700, Hitachi, Japan) at 125 kV.

Antibodies

For immunohistochemistry to examine for the presence of macrophages in atretic follicles, we used 3 types of rat anti mouse macrophage monoclonal antibodies: F4/80 (Austyn & Gordon, 1981; Hirsch et al. 1981), Mac-1 (Springer et al. 1979) and Mac-2 (Soga et al. 1997). FITC-labelled F4/80 and anti-mouse CD11b monoclonal antibody (Mac-1) were obtained commercially (Serotec, Oxford) and prepared as 1–1/50 dilutions in 0.01 M phosphate-buffered saline (PBS). The supernatant of the hybridoma clone M3/38 (Mac-2) was provided by Dr T. Itoh (Tohoku University School of Medicine, Sendai) and prepared as 1–1/25 dilutions in PBS. FITC-conjugated goat antirat IgG (H + L) antibody (Cedarlane, Ontario), prepared as a 1–1/50 dilutions in PBS, was used as the secondary antibody.

Tissue preparation for immunohistochemistry

Isolated ovaries and the spleen were fixed with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) for 1 h. After rinsing with 0.01 M PBS, they were embedded in polyacrylamide (Hausen & Dreyer, 1981) and frozen in liquid nitrogen. Frozen sections (6 μm) were cut with a cryostat at −30 °C and mounted on chrome-gelatin coated glass slides. After blocking with 3% bovine serum albumin, the sections were incubated with primary antibodies overnight at 4 °C. As a negative control, PBS was used instead of the antibodies. The slides were washed 3 times in PBS for 10 min. The slides for Mac-2 were incubated with the secondary antibody and washed 3 more times. Counterstaining was performed with haematoxylin.

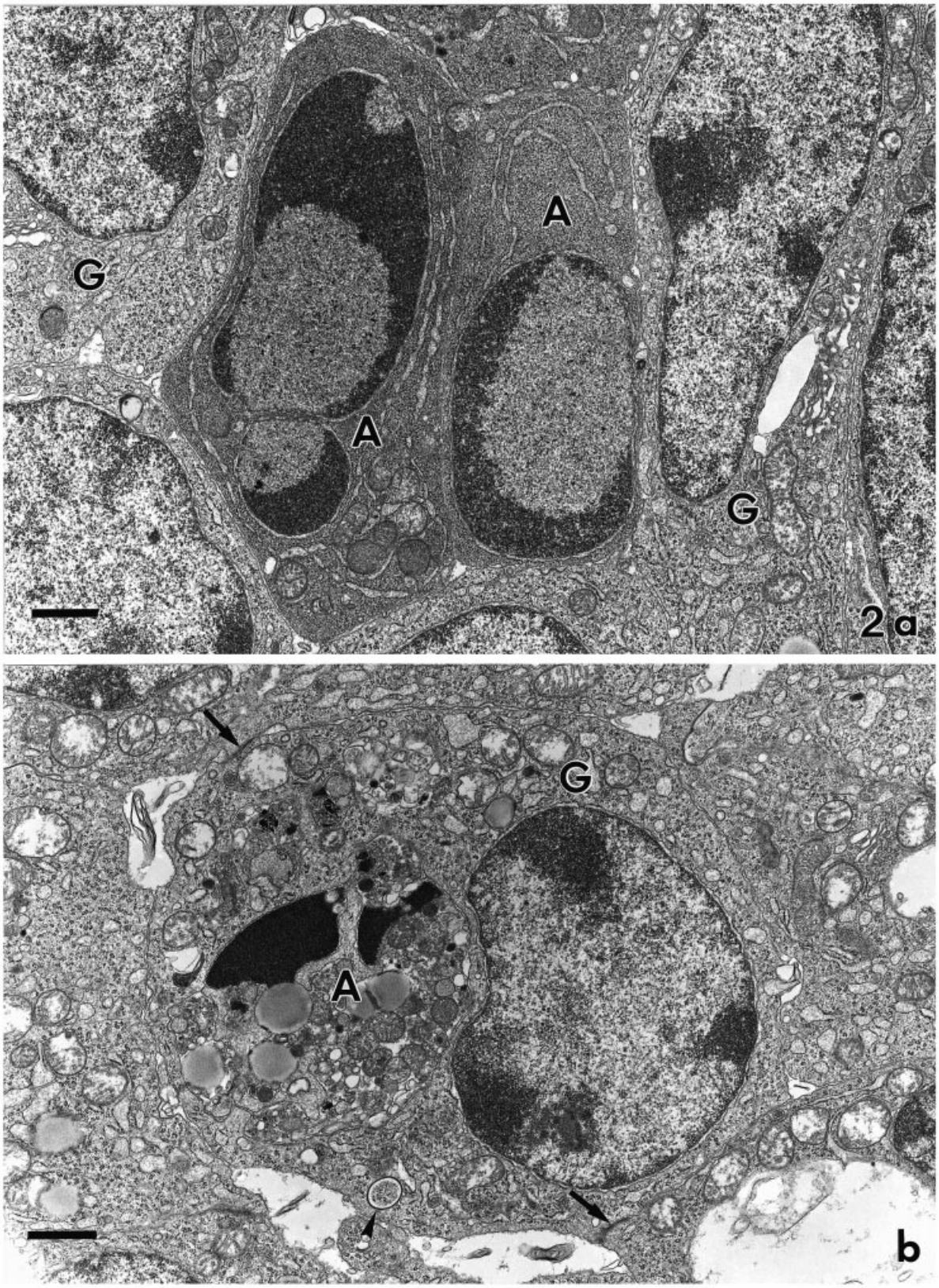

Fig. 2. Electron micrographs showing apoptotic granulosa cells. (*a*) In contrast to normal granulosa cells (G), apoptotic granulosa cells (A) are characterised by condensation of chromatin granules in the nucleoplasm and increased cytoplasmic density. (*b*) An apoptotic cell (A) can be seen to have been phagocytosed by a normal-looking granulosa cell (G) which has formed gap junctions (arrows) and contains an annular gap junction (arrowhead). Bar, 1 μm.

We examined the slides with a BX50 fluorescence microscope (Olympus, Tokyo).

RESULTS

Light and electron microscopy

Nearly half of total number of follicles (about 20 per section) were represented by primordial and primary ($\leqslant$ type 5; Pedersen & Peters, 1968) follicles with only a few if any apoptotic granulosa cells. In contrast, more than 70% of type 6 and type 7 secondary follicles were regarded as being atretic. In the early stage of atresia, apoptotic cells were seen in the multistratified granulosa cell layer (Fig. 1*a*). At more advanced stages of atresia, the thickness of the granulosa layer decreased and the former types of follicles were not longer distinguishable. The zona pellucida was atrophic and coiled (Fig. 1*b*).

Apoptotic granulosa cells were characterised by several structural features including condensation of chromatin granules in the nucleoplasm and an increase in cytoplasmic electron density (Fig. 2*a*). Apoptotic granulosa cells or their cytoplasmic remnants were found to be engulfed by granulosa cells of normal appearance. The latter were in contact with neighbouring granulosa cells with gap junctions and frequently contained internalised annular gap junctions in the cytoplasm (Fig. 2*b*).

In nonatretic follicles, granulosa cells and oocytes possessed cytoplasmic processes and microvilli (Fig. 3). Occasionally, gap junctions were observed between these various cell processes. No such cytoplasmic processes or microvilli were present in atretic folliculi (Figs 4, 5). Granulosa cells became longer and thinner with elongated pseudopodia and lysosomal inclusions. Basal lamina and gap junctions were retained by granulosa cells even in follicles showing advanced atretica.

Around the perimeter of degenerating oocytes, a number of cells invaded the region of the zona pellucida and made contact with the degenerating oocytes. Such invading cells also connected by gap junctions with neighbouring granulosa cells (Fig. 5*a*). In further advanced atretic follicles, the oocyte was completely abolished and its debris appeared to be digested by invading cells (Fig. 5*b*).

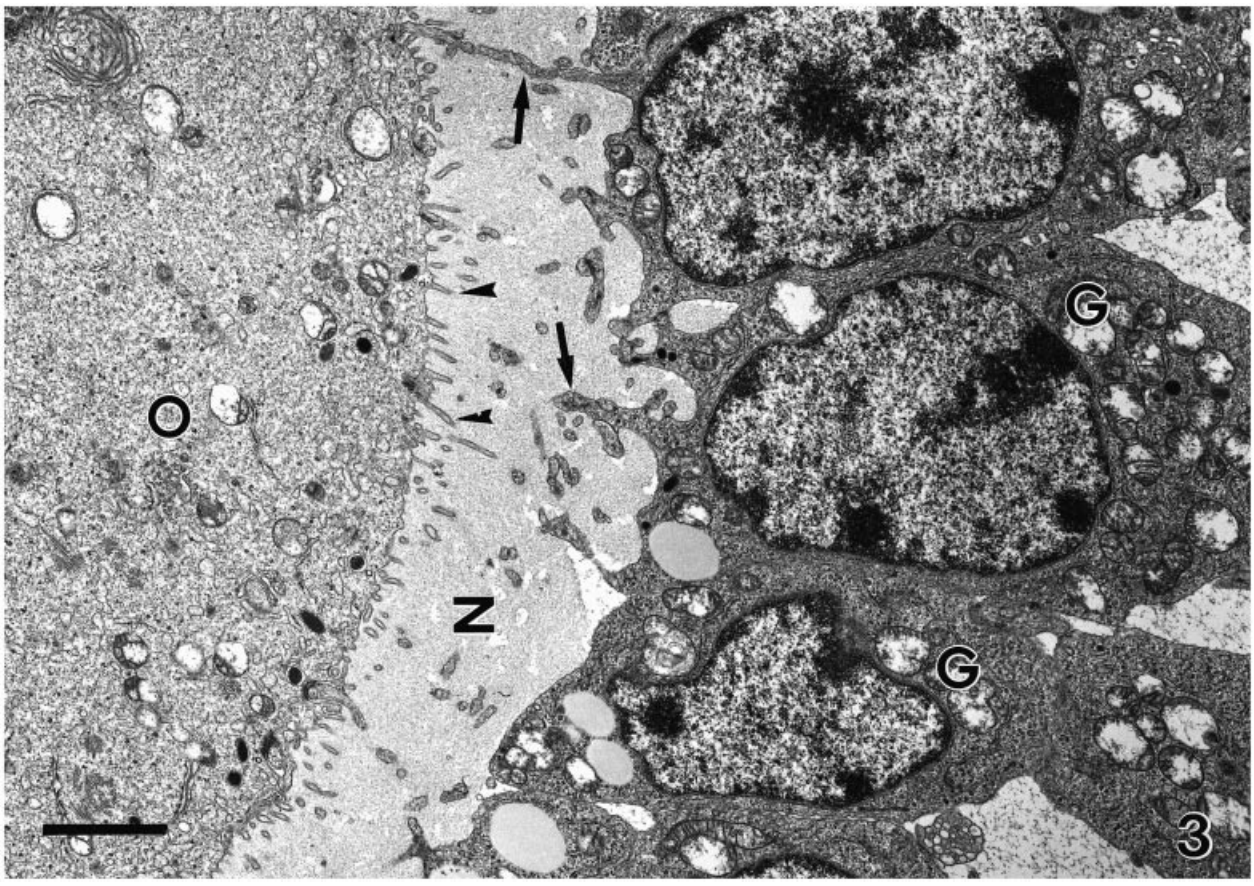

Fig. 3. Electron micrograph showing a nonatretic follicles. Granulosa cells (G) are spherical, their cytoplasmic processes (arrows) appearing to have traversed the zona pellucida (Z). An oocyte (O) possesses microvilli (arrowheads). Bar, 2 μm.

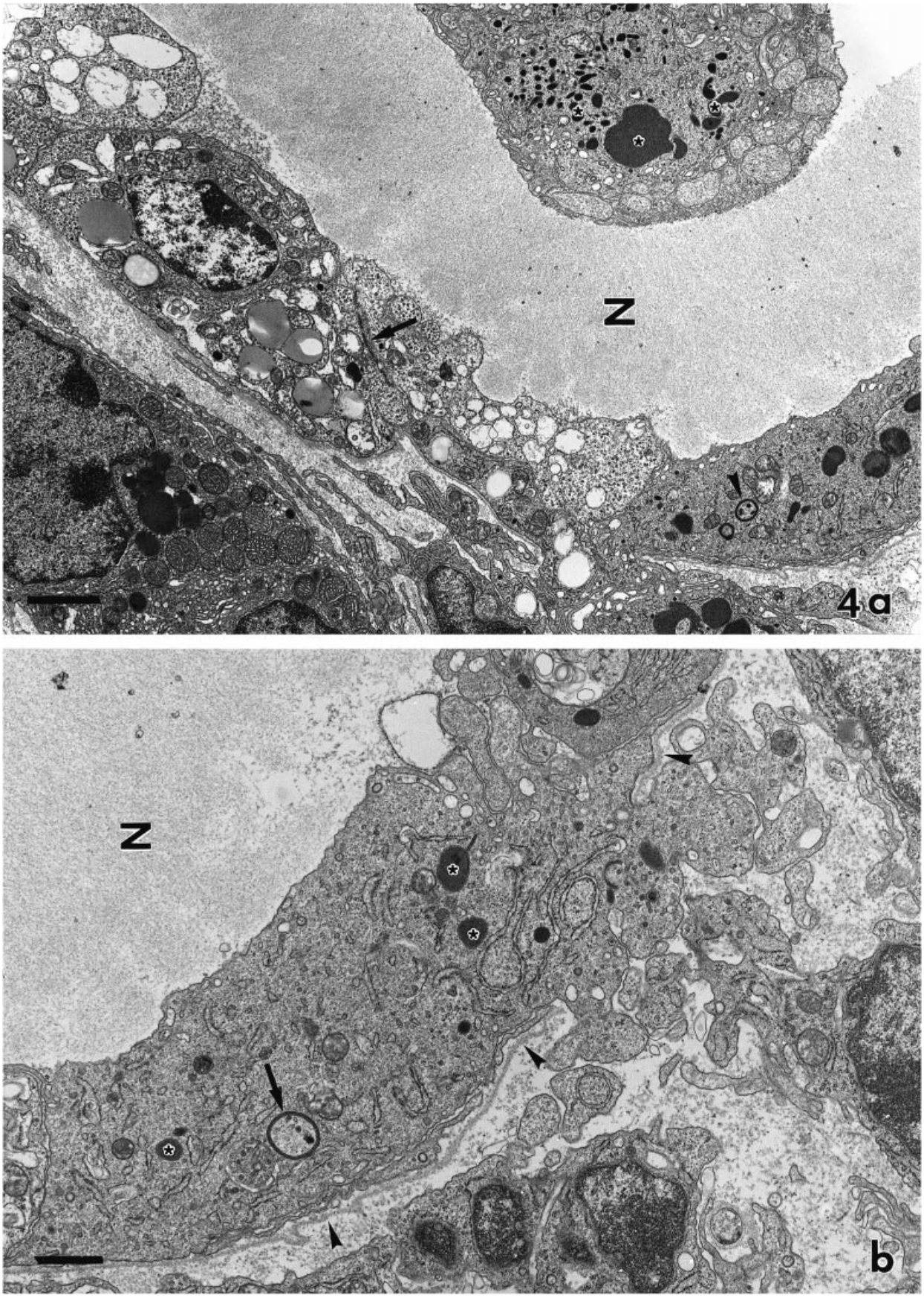

Fig. 4. For legend see page 109.

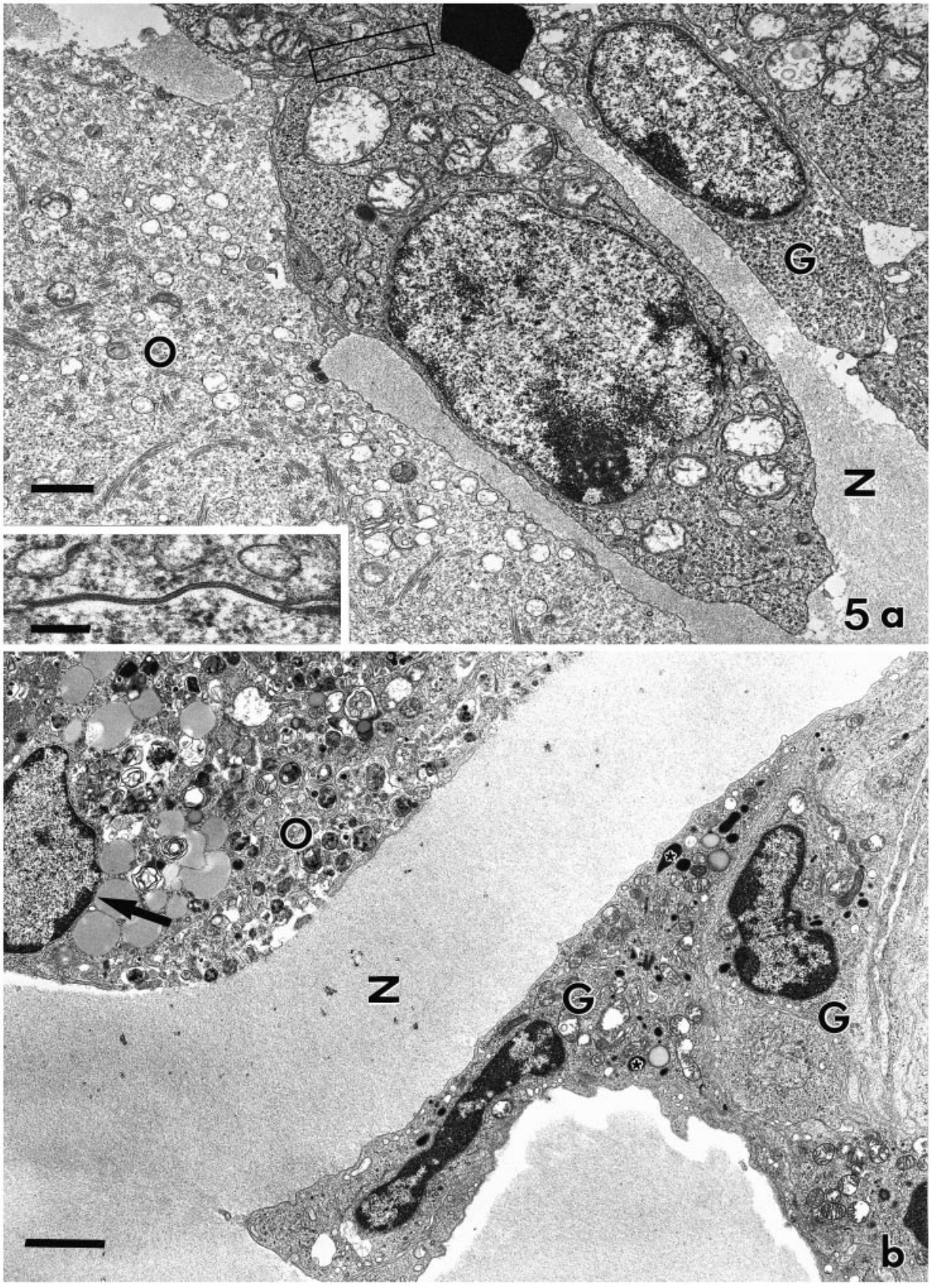

Fig. 5. For legend see opposite.

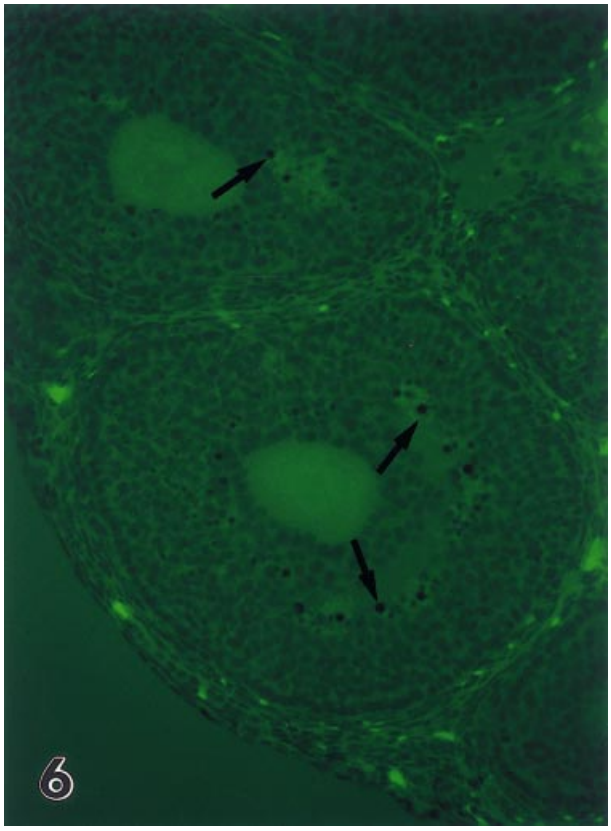

Fig. 6. Fluorescence micrograph showing a frozen section of the mouse ovary. Note the true positive fluorescence against Mac-2 is limited to the interstitial space between the follicles. The specimen was stained with haematoxylin to give contrast to the apoptotic nuclei (arrows) of granulosa cells. ×185.

Immunohistochemistry

As controls, frozen sections of the spleen were positively stained with all monoclonal antibodies. In contrast, those of atretic ovarian follicles demonstrated no positive immunofluorescence regardless of their stage of atresia. Only a small number of cells, apparently belonging to the theca layer or stromal in nature, were stained with Mac-2 (Fig. 6).

DISCUSSION

Apoptosis is regarded as an important mechanism involved in follicular atresia (Hughes & Gorospe, 1991; Tilly et al. 1991). Some early studies suggested that macrophages were actively involved in phagocytosis of apoptotic granulosa cells and the subsequent elimination of atretic follicles (Byskov, 1974; Peluso et al. 1980). Bukovsky et al. identified macrophages immunohistochemically in rat (1993) and human (1995) ovaries. Kasuya (1995) reported that apoptotic granulosa cells were phagocytosed by 'large round cells', which were identified as macrophages on the basis of acid phosphatase positivity in rabbits and positive immunoreactivity with an antimacrophage monoclonal antibody (MR-1) in guinea pigs (Kasuya, 1997).

On the other hand, based on the results of quantitative light microscopic analysis, Logothetopoulos et al. (1995) presumed that dead granulosa cells would be extirpated after autolysis, because there were no inflammatory cells such as granulocytes, lymphocytes or monocytes in the atretic follicules although 'fibroblastoid looking cells' were seen. Moreover, the phenomenon of atresia, in which healthy granulosa cells phagocytose dead granulosa cells, has been reported in sheep (Hay et al. 1976), dogs (Spanel-Borowski, 1981), mice (Byskov, 1979; Kuryszko, 1983; Kuryszko et al. 1987; Watanabe, 1996) and rats (Peluso et al. 1980; Takeo et al. 1995). The phagocytic cells have frequently been considered to be granulosa cells despite their variable appearance. These previous studies, however, mostly failed to provide morphological evidence for their recognition as granulosa cells.

In the present study on the mouse ovary, phagocytosing cells in atretic follicles were clearly identified as granulosa cells because of their characteristic gap junctions. The involvement of macrophages in this process therefore seems unlikely, at least in prereproductive-age mouse ovaries. This conclusion was further supported by the lack of immunoreactivity of these phagocytosing cells as examined with antimacrophage monoclonal antibodies. We have demonstrated the phagocytotic ability of granulosa cells (Koike et al. 1993; Watanabe & Tonosaki, 1995). This ability will be even more enhanced by the fact that

Fig. 4. Electron micrographs showing advanced atretic follicle. Neither cytoplasmic processes nor microvilli are detectable in the zona pellucida region (Z). (*a*) Cells of the granulosa layer are flattened, formed a gap junction (arrow) and containing an annular gap junction (arrowhead). Numerous lysosomal bodies and debris of the degenerating oocyte are present in the remaining intrazonal space. Bar, 2 μm. (*b*) Higher magnification view of the granulosa layer. These cells, containing lysosomal bodies and extending pseudopodia, resemble macrophages, but note the annular gap junction (arrow) in the cytoplasm and the basal lamina (arrowheads), neither of which are features of macrophages. Stars, lysosomal bodies. Bar, 1 μm.

Fig. 5. Electron micrographs showing a degenerating oocyte (O) and surrounding granulosa cells (G). (*a*) A small number of cells possess gap junctions (boxed area) and appear to have invaded the zone pellucida (Z) and made contact with the degenerating oocyte. Bar, 1 μm. Inset shows a higher magnification view of the boxed area. Bar, 0.2 μm. (*b*) Debris of a degenerating oocyte and nucleus of a cell of granulosa type (arrow) within the region of the zona pellucida. Granulosa cells are varied in shape and contain numerous lysosomal bodies. Stars, lysosomal bodies. Bar, 2 μm.

granulosa cells invade the zona pellucida and phagocytose degenerating oocytes.

Granulosa cells seem to be responsible for elimination of atretic follicles by phagocytosis of apoptotic granulosa cells and also degenerating oocytes.

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