Zygote **12** (August), pp. 269–276. © 2004 Cambridge University Press
DOI:10.1017/S0967199404002825 Printed in the United Kingdom

Characteristics of invasive cells found in between zona pellucida and oocyte during follicular atresia in mice

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Date submitted: 15.03.04. Date accepted: 9.07.04

Summary

During the process of follicular atresia, cells are observed to invade the zona pellucida (invasive cells) where they presumably play an important role in eliminating degraded oocytes. Although our preliminary studies have suggested that these cells may originate from granulosa cells and not from macrophages, a detailed morphological analysis of the cells has not been conducted. The objective of this study was to characterize the cells more precisely by electron microscopy and immunohistochemistry, using sexually immature mice. The results show that the invasive cells were first observed within advanced primary (non-antral) atretic follicles. The cells frequently contained cytoplasmic lysosome-like granules after passing through the zona pellucida. F4/80 and Mac-1, reported as macrophage-specific antibodies, were reactive with the cells in most cases, but some immunonegative invasive cells were also observed. The ultrastructural features of the invasive cells were quite similar to those of granulosa cells, not macrophages. Gap junctions, which are typical cytoplasmic structures of epithelial cells, were frequently identified between neighbouring cells. Although direct evidence indicating a contribution by the cells to the elimination of degenerated oocytes was not obtained, our results strongly suggest that the invasive cells originated from granulosa cells surrounding the zona pellucida, and that they may have a macrophage-like cell function for the elimination of oocytes from atretic follicles in mice.

Keywords: Follicular atresia, Granulosa cell, Macrophage, Mouse, Oocyte

Introduction

In mammals, cyclic ovulation occurs during the reproductive period; the ovarian cycle is a recurring expression of the synchronized interaction between the hormones of the hypothalamus-pituitary-ovarian system (Khan-Dawood, 2003). It is well known, however, that the mammalian female has far more gametes than will ever be ovulated. Indeed, vast numbers of oogonia are observed in the ovary but the majority of these oocytes (approximately 77% in mice and greater than 99% in women) are lost when the follicles in which they are enclosed undergo a degenerative process called follicular atresia (Greenwald & Roy, 1994; Araki, 2003). Although the molecular mechanisms involved in follicular atresia have been reported to be somewhat different among mammalian species (Evans, 2003), follicular atresia is generally observed in both prepubertal and adult individuals. Thus, a follicle can become atretic at any stage during its growth and development (Greenwald & Roy, 1994; Hubbard & Oxberry, 1991; Araki, 2003).

In general, atretic changes in follicles appear primarily on granulosa cells, not oocytes. One of the earliest morphological signs of atresia seen by light

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microscopy is the presence of darkly stained pyknotic nuclei, an apoptotic change that is observed in granulosa cells. These cells are eliminated by phagocytic cells; thus atresia of the follicle is completed mainly by cyclic cellular events, such as phagocytosis, followed by apoptosis of the follicular granulosa cells. The characteristics of the phagocytic cells that eliminate the apoptotic granulosa cells have been well documented, and the evidence accumulated to date strongly suggests that the granulosa cells themselves play a major role as phagocytic cells in follicular atresia (Byskov, 1974; Greenwald & Roy, 1994; Inoue *et al*., 2000; Irving-Rodgers *et al*., 2001; Fenwick & Hurst, 2002; Pedersen *et al*., 2003; Araki, 2003). The involvement of tissue macrophages, typical phagocytic cells located in the ovary, has also been reported in some mammalian species (Petrovska *et al*., 1996; Kasuya, 1997; Katabuchi *et al*., 1997; Gaytan *et al*., 1998). Despite these experimental advances, considerable controversy remains regarding the origin of the phagocytic cells.

Obvious morphological changes, such as those seen in granulosa cells, have not been noticed in oocytes at the beginning of follicular atresia (Greenwald & Roy, 1994; Irving-Rodgers *et al*., 2001, Petersen *et al*., 2003). However, atretic degeneration (for example, fragmentation of the cytoplasm) is usually observed at the advanced stage of atresia. The cytophysical change in the oocyte is thought to be related to apoptosis (Van Blerkom & Davis, 2001; Depalo *et al*., 2003), or to cell death that does not involve classically described apoptosis (Devine *et al*., 2000). By the final stage of atresia, the oocyte has morphologically disappeared from the follicle, whereas the folded (degenerated) zona pellucida remains in the follicle. However, many details are unclear with regard to the degeneration of the oocyte and the cellular mechanism for the elimination of oocytes from atretic follicles.

Previous ultrastructural and immunohistochemical studies by our research group, based on immature mice, produced no evidence for the presence or involvement of macrophages in atretic follicles (Inoue *et al*., 2000). In the atretic follicles, we further showed that the cells that invaded the zona pellucida (invasive cells) had gap junctions. We therefore preliminarily concluded that the invasive cells were of epithelial (granulosa) origin and were not macrophages (Inoue *et al*., 2000). The aim of this study was to characterize the invasive cells more precisely, on the presumption that they are phagocytic cells with an important role in eliminating degenerated oocytes from atretic follicles. We carried out ultrastructural and immunohistochemical studies using antibodies reported to be macrophage-specific. The present study shows further convincing evidence that granulosa cells play a critical role in eliminating oocytes from primary atretic follicles, at least in mice.

Materials and methods

Animals and reagents

Female mice (Jcl/ICR, immature: 3–4 weeks old; mature: 10 weeks old) were obtained from Japan Clea (Tokyo, Japan). They were maintained at the Animal Center in Yamagata University under 12L:12D conditions and given free access to food and water. For sexually mature animals, the oestrous cycle was monitored by the vaginal smear test. The studies described in this paper adhered to Guide for the Care and Use of Laboratory Animals, Yamagata University.

Embedding medium for frozen tissue specimens (OCT compound, Tissue-Tek) was purchased from Miles, Elkhart, IN, USA. Biotinylated anti-rat IgG F(ab)'2 fragment was from Seikagaku, Tokyo Japan. Anti-macrophage rat monoclonal antibodies, Mac-1 (Springer *et al*., 1979) and F4/80 (Austyn & Gordon, 1981) were either obtained from Chemicon International (Temecula, CA, USA) or Serotec (Oxford, UK). All other chemicals were purchased commercially and were of the highest purity available.

Morphological studies by light and transmission electron microscopy

The ovaries from either immature (3–5 weeks old) or mature (10 weeks old) mice were fixed in 100 mM sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde for 1.5 h at 22 °C, then further fixed in 1% osmium tetroxide in the same buffer for 2 h and block-stained with 2% uranyl acetate. After dehydration in a graded ethanol series, specimens were placed in propylene oxide and subsequently embedded in Epon 812 (TAAB Laboratories, Berkshire, UK). Semithin sections (0.45 μm) were stained with toluidine blue for light microscopic studies. A study with serial semithin section was carried out to examine the number of invasive cells per oocyte. Ultra-thin sections (60–80 nm) were stained with uranyl acetate and lead citrate, and examined in a Hitachi H-7100 electron microscope (Hitachinaka, Japan).

Immunohistochemistry

The fresh ovaries isolated from Jcl/ICR mice were embedded in OCT compound, then frozen in liquid nitrogen immediately. Immunohistochemical localization of either Mac-1 (Chemicon International, Temecula, CA, USA) or F4/80 (Serotec, Oxford, UK) reactive antigen was examined in 6 μm thick frozen sections, essentially according to the method described by Maeda *et al*. (1992, 1995) as follows: air-dried sections were fixed with cold acetone, then treated with phosphate-buffered saline (PBS; pH 7.4) containing periodic acid dihydrate (1.44 mg/ml) to inactivate

endogenous peroxidase. After blocking with 3% bovine serum albumin (BSA) in PBS, the sections were incubated with Mac-1 (10 μg/ml) or F4/80 (10 μg/ml) for 1.5 h at 22 °C. The secondary antibody employed was biotinylated mouse anti-rat IgG F(ab)'2 fragment (Seikagaku, Tokyo, Japan) for 1 h incubation at 22 °C. Immunoreactivity was visualized using a Vectastain peroxidase-ABC kit (Vector Laboratories, Burlingame, CA, USA) with 3,3'-diaminobenzidine 4HCl (Dojindo Laboratories, Kumamoto, Japan) as a substrate. The colour reaction was stopped by washing with distilled water several times, then the sections were counterstained with 1% methylgreen. Sections of lymph nodes isolated from the mice were used as a positive control, and control sections received the same treatment except that the primary antibody was omitted. For further characterization of immunoreactive cells, serial cross-sections stained with hematoxylin–eosin (H-E) were prepared for light microscopic observation.

Results

Morphological characteristics of the cells invading the zona pellucida of the atretic primary follicle

Light microscopy findings

In preliminary experiments, some apoptotic cells were observed in the granulosa cell layer of follicles in the primary stage of atresia, whereas no morphological changes, such as condensation of cytoplasm, were noted in the oocyte (data not shown). In addition, the basal lamina of the follicle usually kept its normal shape at this stage of atresia. As the atretic changes advanced, oocytes became irregular in shape, and invasive cells were frequently observed (Fig. 1a). In some cases, phagocytic cells involved in the elimination of the degraded (apoptotic) cells were observed (Fig. 1a). At this stage of the atretic process, oocytes were sometimes fragmented and/or the zonae pellucidae were folded (Fig. 1b, c); the number of invasive cells present ranged from 4 to 100 (mean 37.8, SD 32.9, $n = 5$), as analysed by light microscopy of serial semi-thin sections. Invasion by the cells occurred either from a specific region, or from multiple regions of the zona pellucida simultaneously (Fig. 1). After invasion, the cells lay in close contact with the oocyte and exhibited typical spindle or round shapes (Fig. 1). The degradative changes in oocytes, and the invasive cells, were frequently observed in atretic follicles without a follicular antrum and were scarcely seen in growing antral follicles from sexually mature mice (data not shown). Therefore, we focused on the invasive cells in atretic primary follicles (non-antral follicles) from immature mice in the following experiments.

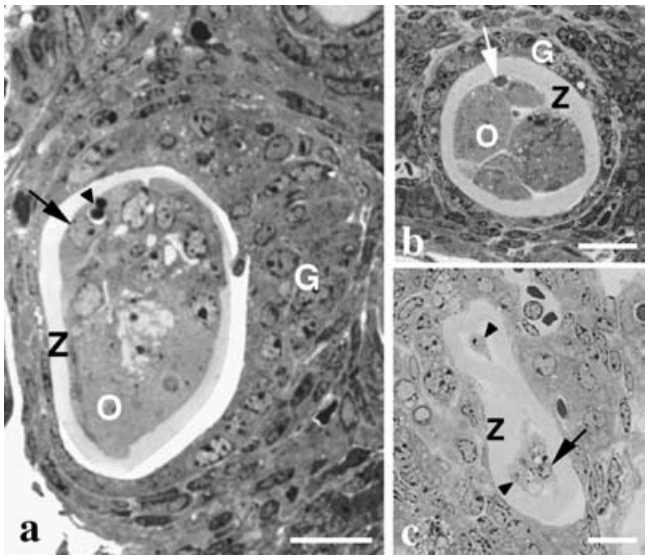

Figure 1 Atretic primary follicles at an advanced stage. (*a*) Atretic follicle containing a number of invasive cells in the zona pellucida. Arrowhead indicates an apoptotic cell. An invasive cell is phagocytosing the apoptotic cell (arrow). (*b*) Fragmentation of an oocyte. An invasive cell (arrow) is located behind the oocyte. (*c*) Folding formation of the zona pellucida at a more advanced stage. A fragment of oocyte (arrowheads) and the invasive cells (arrow) are observed. O, oocyte; G, granulosa cell; Z, zona pellucida. Scale bars represent 25 μm.

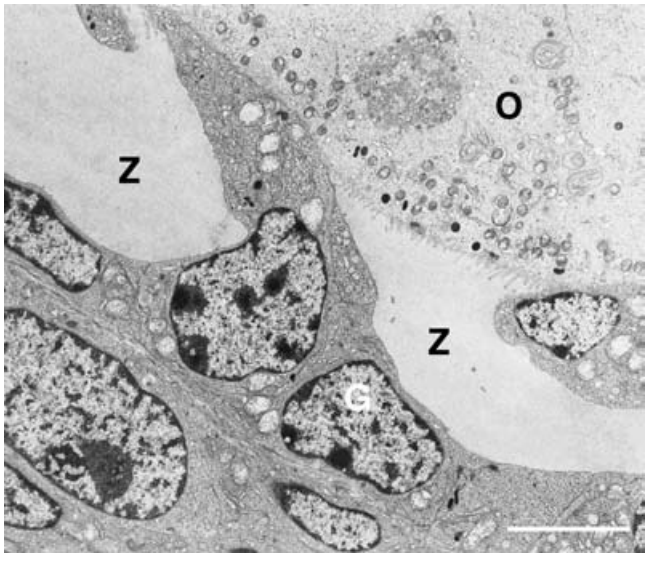

Figure 2 Electron micrograph showing an 'invading' cell. A cell adjacent to granulosa cells (G) is passing through the zona pellucida (Z) and directly in contact with the oocyte (O). Scale bar represents 5 μm.

Transmission electron microscopy findings

The results of electron microscopic analysis indicated that the ultrastructural characteristics of the invasive cells and granulosa cells were quite similar (Figs. 2, 3).

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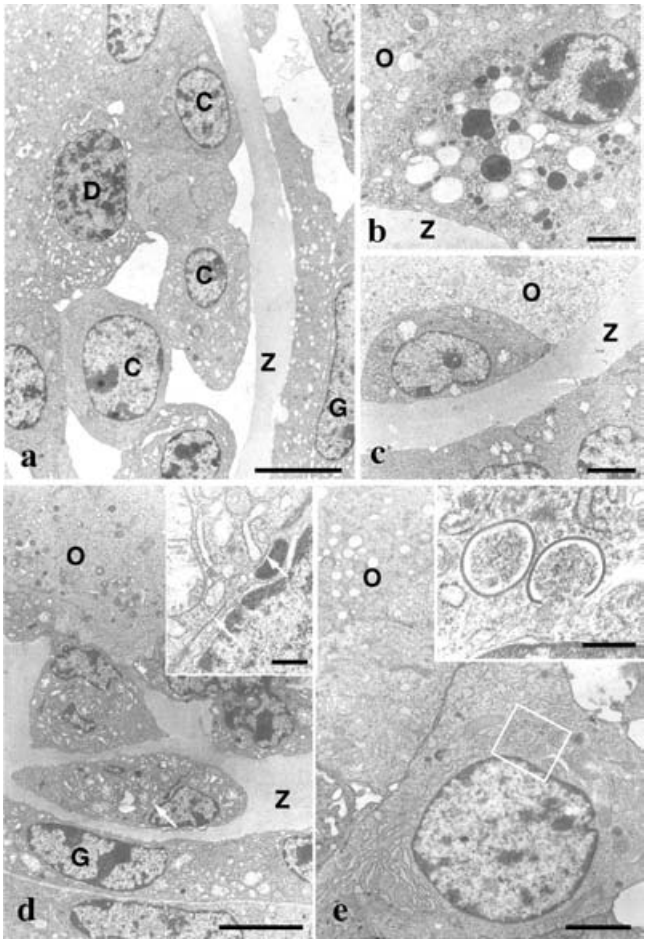

Figure 3 Electron micrographs showing the invasive cells in atretic primary follicles. (*a*) Invasive cells having a dark (D) or clear (C) nucleus. Scale bar represents 5 μm. (*b*) An invasive cell possessing lysosome-like dense bodies and vacuoles in the cytoplasm. Scale bar represents 1 μm. (*c*) An invasive cell that lacks a lysosome-like dense body. Scale bar represents 3 μm. (*d*) Gap junctions observed between 'invading' cells. Arrows indicate gap junctions between the cells. Scale bar represents 5 μm. Inset shows a higher-magnification view of the gap junction area. Scale bar represents 1 μm. (*e*) Annular gap junctions formation (in boxed area) in the invasive cell. Scale bar represents 2 μm. Inset shows a higher-magnification view of the boxed area. Scale bar represents 0.3 μm. O, oocyte; G, granulosa cell; Z, zona pellucida.

Some invasive cells had nuclei with relatively large amount of euchromatin, giving the nucleus a clear appearance (clear cells), whereas others had dark nuclei, i.e. the nuclei contained a relatively large amount of heterochromatin (dark cells) (Fig. 3a). The dark cells were frequently observed in the region directly in contact with the oocyte. The size of the cells, including the diameter and shape of the nucleus, did not differ significantly between the two groups. Some invasive cells had lysosome-like dense bodies in their cytoplasm (Fig. 3b), but others did not contain

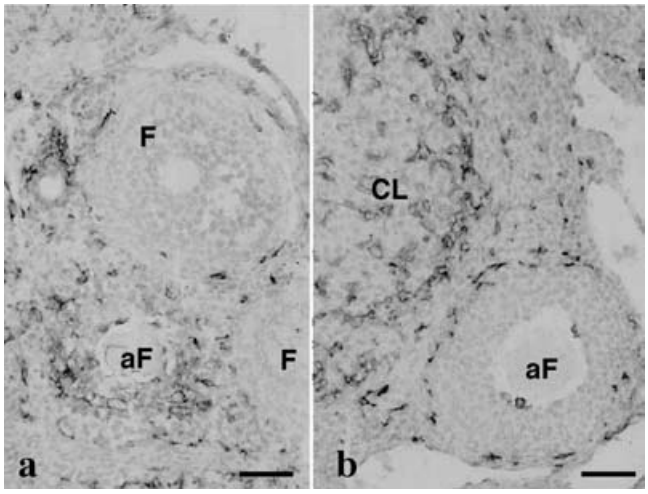

Figure 4 Immunoperoxidase localization of the antigen reactive with F4/80 in mouse ovary frozen sections. (*a*) Staining patterns of a healthy follicle (F) and an atretic follicle (aF). (*b*) Staining patterns of corpus luteum (CL) or an atretic follicle. No significant immunoreactivity was detected in healthy follicles. Scale bars represent 50 μm.

such features (Fig. 3c). These dense cytoplasmic bodies were observed more frequently in cells that had already invaded than in cells just beginning to pass through the zona pellucida ('invading' cells). No cytoplasmic processes, microvilli or cellular junctions were present in the invasive cells.

A previous report from our research group clearly demonstrated that the invading cells were connected with neighbouring granulosa cells by gap junctions, a cellular structure that is typical of cells of epithelium origin (Inoue *et al*., 2000). In the present study, gap junctions were frequently observed not only between invading and granulosa cells but also between invading cells (Fig. 3d). Occasionally, annular gap junctions were noted in the cytoplasm of the invasive cells (Fig. 3e). Thus, no strong morphological evidence was obtained of features common to both invasive cells and macrophages.

Immunohistochemical analysis using macrophage-specific antibodies

We characterized the invasive cells in atretic follicles more precisely by means of immunohistochemical studies using two antibodies that have been reported to be specific to mouse macrophages – F4/80 and Mac-1 – to evaluate whether the cells expressed these classical marker molecules for macrophages. As expected, F4/80-positive cells were detected in the ovarian medulla, corpus luteum and atretic follicles (Fig. 4). However, no F4/80-positive cells were detected in normal follicles (Fig. 4a). A similar staining tendency was confirmed using the Mac-1 antibody (data not

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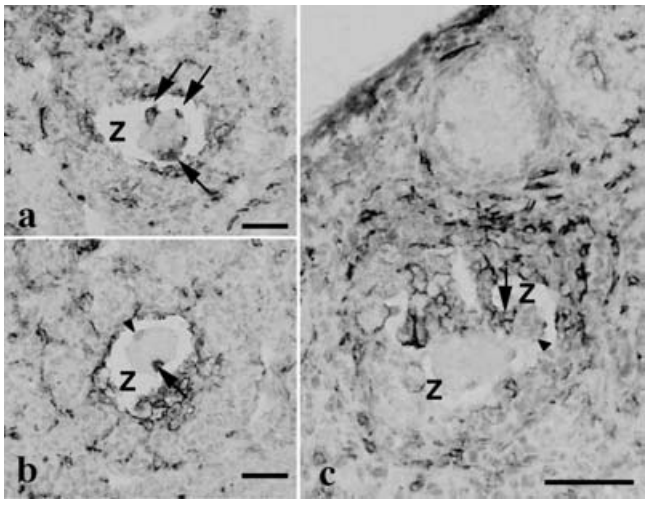

Figure 5 Immunoperoxidase localization of the antigen reactive with F4/80 of the invasive cells in advanced atretic follicles. (*a*) A follicle showing that most of the invasive cells are immunopositive. (*b*) A follicle showing that a small number of the invasive cells are immunopositive. (*c*) A follicle showing a mixed localization of immunopositive and immunonegative invasive cells. Arrows indicate immunopositive cells, and arrowheads represent immunonegative cells. Z, zona pellucida. Scale bars represent 50 μm.

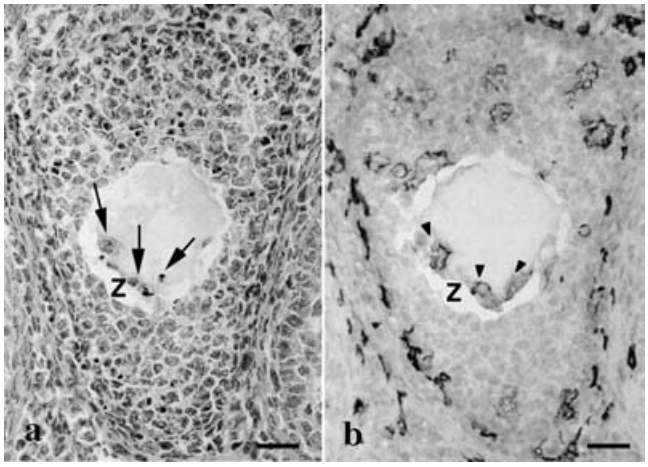

Figure 6 Correlation between apoptotic cells and F4/80 immunopositive cells within the zona pellucida of advanced atretic primary follicles. (*a*) A H-E staining pattern of atretic follicle, and (*b*) immunoperoxidase localization of the antigen reactive with F4/80 in the serial cross-section. Immunopositive cells (arrowheads) are located in parallel with the position of apoptotic cells (arrows) in the zona pellucida. Z, zona pellucida. Scale bars represent 50 μm.

shown). In the granulosa cell layer of the atretic primary follicle, the positive cells were located throughout the layer in some cases (Fig. 4a); occasionally, only a few positive cells were seen (Fig. 4b). No correlation between the distribution of positive cells in the follicle and the sexual maturity of the animal was observed.

In the invasive cells, immunoreactivity against reportedly macrophage-specific antibodies was clearly identified. Immunoreactivity was noted in most of the invasive cells in some atretic follicles (Fig. 5a), but in other cases not all the invasive cells were immunopositive (Fig. 5b, c). The percentage of immunoreactive cells was greater in follicles with relatively large numbers of cells. Although only a few invasive cells were seen at the end stage of follicular atresia, these cells were usually immunopositive.

Attempts were then made to clarify the morphological characteristics of the immunopositive cells using H-E-stained serial cross-sections. In most atretic follicles, immunopositive invasive cells and apoptotic cells had a clear tendency to be located close together (Fig. 6).

Discussion

The basement membrane isolates ovarian follicles from the theca cell layer and the ovarian medulla. Therefore, a follicle can be considered, structurally, to be a closed epithelium, because granulosa cells, a major component of follicles, originate from the genital ridge, which originally proliferates from the ectoderm (Langman, 1975). Numerous reports have been published concerning the elimination of apoptotic granulosa cells from atretic follicles during the process of follicular atresia. The results of some immunohistochemical studies using reportedly macrophage-specific (or related) antibodies have suggested that macrophages are involved in the phagocytosis of apoptotic granulosa cells and the subsequent elimination of atretic follicles (Bukovsky *et al*., 1993, 1995; Kasuya, 1995, 1997). However, many more papers, including our previous studies, have concluded, based mainly on morphological evidence, that granulosa cells adjacent to apoptotic granulosa cells are capable of phagocytosis, and that granulosa cells, not macrophages, play the major role in eliminating dead cells from atretic follicles (Hay *et al*., 1976; Byskov, 1979; Peluso *et al*., 1980; Spanel-Borowski, 1981; Kuryszko & Adamski, 1987; Logothetopoulos *et al*., 1995; Takeo & Hokano, 1995; Inoue *et al*., 2000; Araki, 2003). Previously, we demonstrated that granulosa-like cells are also present in the region of the zona pellucida that contacts the oocyte, where they are presumably involved in phagocytosing atrophic oocytes (Inoue *et al*., 2000). We speculated that these cells were granulosa cells, and not macrophages, because the invading cells were connected with neighbouring granulosa cells by gap junctions.

The present study has demonstrated, for the first time, the immunoreactivity of the invasive cells against

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F4/80 and Mac-1 (antibodies widely used for the identification of macrophages) during follicular atresia. These results suggest the possibility that the invasive cells are immunohistochemically macrophages. However, we do not have definitive evidence to conclude that the immunopositive cells are macrophages, since the electron microscopic observations of the cells did not support speculation as to a macrophagic origin for the cells (Figs. 2, 3).

Previously, Gaytan *et al*. (1998) reported the existence of macrophages in atretic rat ovarian follicles. Macrophages identified in rat ovarian follicles exhibited reactivity to ED1, a mouse monoclonal antibody to rat macrophages (Dijkstra *et al*., 1985), as well as morphological characteristics that are typically observed in macrophages (e.g. irregular nuclei with a distinct chromatin pattern, or a number of vacuoles, or phagocytosed cell debris in the cytoplasm). Recent studies by Kasuya (2002) demonstrated relatively large round cells (20–30 μm diameter) located in atretic follicles at the early stage of atresia in guinea pigs. The cytoplasm of the cells had lysosome-like granules containing dead granulosa cell debris at different stages of degradation. In addition, these cells were acid-phosphatase-positive and reacted strongly to MR-1, a monoclonal antibody that reacts with macrophages and monocytes in guinea pig (Kraal *et al*., 1988). These results suggested that the large cells observed in atretic follicles were macrophages (Kasuya, 2002). It should, however, be noted that the conclusion in both rat or guinea pig that the phagocytic cells in atretic follicles are macrophages was based on cellular evidence demonstrating (1) a positive reaction to reportedly macrophage-specific antibody(ies); (2) cellular characteristics, such as nuclear or cytoplasmic patterns, typically observed in macrophages; and (3) the frequent observation of lysosomal granules in phagocytic cells.

The most fundamental and important issue in immunohistochemical studies is the true specificity of the antibody used. The present study clearly demonstrates the lack of immunopositive staining in healthy follicles using the F4/80 antibody (Fig. 4). Although not shown, a similar staining pattern was also obtained with the Mac-1 antibody. The lack of reactivity in healthy follicles seems to imply that there are no problems with the specificity of these antibodies when used against ovarian follicle antigens. It is indeed striking that immunopositive cells did not appear in follicles until they progressed toward atresia (Figs. 5, 6). In addition, it should be noted that most of the invasive cells present at the end stage of follicular atresia were immunopositive (Fig. 5). On the other hand, the majority of the invasive cells were morphologically identified as having an epithelial origin, resembling granulosa cells, because the cells frequently had gap junctions (Figs. 2, 3). These conflicting results led us to consider that the 'macrophage-specific' molecule(s) recognized by the F4/80 and Mac-1 antibodies is actually an antigen expressed by the invasive cells, even though they lack the morphological characteristics of mouse macrophage.

Apoptosis is an important pattern-forming tool in embryonic development; it is used to eliminate unwanted cells and to sculpt various tissues of the developing embryo (Jacobson *et al*., 1997). Although massive numbers of cells die during development, they are rapidly eliminated by phagocytic cells. Macrophages are generally considered to be important for the elimination of dead cells. However, a recent study with macrophage-null mice clearly demonstrated that mesenchymal cells in the mouse embryo were competent to act as stand-in phagocytes during sculpting of the mouse footplate, even though macrophages had appeared to be responsible for almost all the clearance of the footplate apoptotic debris (Wood *et al*., 2000). This clear example of cell redundancy may provide an explanation for the discrepancy between the immunoreactivity of invasive cells against macrophage-specific antibodies and their morphological characteristics. Because cell-specific molecules are generally related to that cell's function (for example, CD4 on helper T cells), it would not be surprising if the invasive cells can express a macrophage-like molecule recognized by reportedly macrophage-specific antibodies. Ovarian follicles are structurally isolated and functionally quite unique, as exemplified by the atresia observed throughout life; thus they may have developed a specific mechanism (tentatively termed 'natural cell redundancy') for the elimination of dead cells in the absence of macrophages.

At present, correlations between the immunoreactivity and phagocytic ability of the invasive cells are inconclusive. If we are correct in speculating that the immunopositive cells have phagocytic ability, the cells should be located behind apoptotic cells within the zona pellucida of degenerated oocytes. The results shown in Fig. 6 may confirm our speculation. However, further careful studies are needed to clarify the issue, including immunoelectron microscopic examinations.

In summary, we have characterized, for the first time, the invasive cells in the atretic follicles in mice. We found from electron microscopic and immunohistochemical studies that (1) the invasive cells were typically observed within atretic primary follicles in the advanced stage; (2) lysosome-like granules were frequently identified in the cytoplasm of the cells after passing through the zona pellucida, indicating that the cells may be phagocytic; and (3) most of the cells were immunopositive for reportedly macrophage-specific

antibodies. However, the cells morphologically resembled granulosa cells, not macrophages. In addition, gap junctions were identified in the cells, suggesting that the invasive cells originated from granulosa cells. At present, although we do not have direct evidence to indicate whether the invasive cells are essential for the elimination of degenerated oocytes, the results of this study strongly suggest that they have an important role in the elimination of oocytes during the process of follicular atresia.

Acknowledgements

We are indebted to Mr Hitoshi Suzuki (Yamagata University) and the staff at the Laboratory Animal Center, Yamagata University for their technical support. This work was supported by Grants-in-Aid for Scientific Research, nos. 14571537 and 16591635, from the Ministry of Education, Science, Technology, Sports and Culture, Japan.

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https://doi.org/10.1017/S0967199404002825 Published online by Cambridge University Press