Cell Death Processes of Postovulatory Cumulus Granulosa Cells in the Mouse Oviduct Ampulla after Mating

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ABSTRACT. In order to clarify the elimination process of the granulosa cells and excess spermatozoa in oviduct after ovulation, female reproductive organs of ICR mouse were observed at 3, 5, 8 and 12 hours after mating by immunohistochemistry and electron microscopy. All oviduct ampullas at five hours after two-hour mating contained several oocytes with numerous granulosa cells, and between 5 and 12 hours after mating, the majority of granulosa cells disappeared from the ampullas. At five hours after mating, approximately 5% of granulosa cells were TUNEL-positive and, at eight hours, 30% were TUNEL-positive. Ultrastructurally, there were nuclear changes mainly in compaction and margination of heterochromatin. These were designated as signs of classical apoptosis. In addition, some granulosa cells, approximately 2% at five hours and 13% at eight hours after mating, showed mitochondrial swelling and disruption of cellular, nuclear and organelle membranes. These were designated as typical signs of necrotic cell death. Therefore, postovulatory granulosa cells are eliminated from oviduct ampullas through two different cell death processes, and F4/80-positive macrophages appear after 5-8 hours after mating and remove mainly apoptotic cells.

Key words : oviduct ampulla — postovulatory granulosa cells — apoptosis — necrosis — mouse

It is well known that, in the development of eggs and follicular epithelial cells in the ovaries, the innermost three or four layers of granulosa cells become adherent to an ovum to form the corona radiata, and, at the moment of ovulation, the ovum is discharged into the peritoneal cavity together with cumulus granulosa cells of follicular epithelial cells.[1] After being swept into the oviduct, the ovum with corona stays in the oviduct ampulla to encounter spermatozoa ejaculated from a mated male. Thus, the oviduct ampulla after mating contains three different kinds of cell elements derived extrinsically ; ova and cumulus granulosa cells from ovary, and spermatozoa ejaculated from male.

At implantation of blastocysts, cumulus granulosa cells, which surround the oocytes in the oviduct, disappear. The fate of follicular epithelial cells

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in follicle atresia in the ovaries has been the subject of several investigations,[2-7] but very little information is available regarding the fate of postovulatory cumulus granulosa cells in the oviduct. To clarify the elimation process of the granulosa cells and excess spermatozoa, we observed oviduct ampullas after mating immunohistochemically and electron microscopically.

MATERIALS AND METHODS

A total of 26 ICR mice, 8-12 weeks old, which were purchased from Japan Laboratory (Tokyo, Japan), were used in this study. Twenty-one female mice were mated with males at 6 : 00 am for 2 hrs, and a successful mating was determined by the observation of a vaginal plug. Female mice were sacrificed at 3, 5, 8 and 12 hrs after mating. For a pilot study on changes of whole reproductive organs after mating, in addition to the two-hour-mated females, females were mated overnight, from 8 : 00 pm to 8 : 00 am, and three successfully mated females were sacrificed at 10 : 00 am, and, for normal controls, two females in estrus were also sacrificed. After the mice were deeply anesthetized with ether, they were perfused with 0.15M NaCl through the left ventricle of the heart, and then with 4% paraformaldehyde buffered with 0.1M sodium cacodylate (pH 7.4) for 30 min. For histological observation on whole reproductive organs, the ovaries, oviducts, uterus and vagina were removed altogether, and, after being pinned flat against a plastic plate, were placed in 4% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.4) overnight at 10°C. All the reproductive organs were then embedded in paraffin, and 5 μm sections were cut and stained with hematoxylin and eosin. Low magnification photographs of the whole reproductive organs were taken by a Minolta DiMAGE Scan Multi PRO.

Light microscopy

The oviducts of two-hour-mated females were removed and studied by conventional Mayer's hematoxylin and eosin staining, by the TUNEL method or by the F4/80 immunostaining procedure.

Cell death labeling procedure

The TUNEL method was preformed using the Apoptosis in situ Detection Kit, (Wako Pure Chemical Industries, Ltd. Tokyo, Japan) according to the manufacturer's instruction. Briefly, 4 μm sections were deparaffinized and treated with 0.01 mg/ml proteinase K for 10 min at 37°C. The 3'-hydroxyl ends of DNA were labeled with biotin-dUTP 1 μl/50 μl by terminal deoxynucleotidyl transferase, TdT. The sections were incubated in substrate solution for 10 min at 37°C and were put in 3% H_2O_2 solution to inactivate endogenic peroxidase (POD) at room temperature for 5 min. The labeled DNA was detected by incubation with POD-conjugated antibody for 10 min at 37°C, followed by incubation with diaminobenzidine (DAB). As positive controls, sections were exposed to DNase I for 15 min at 37°C before incubation with biotin-dUTP. For negative controls, sections were incubated with TdT substrate solution without biotin-dUTP after protease incubation.

Immunohistochemistry

F4/80 positive cells were visualized using the avidin-biotin peroxidase complex method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, California USA). Sections were dewaxed, and, after incubation in normal rabbit serum for 30 min at 37°C, the sections were exposed to a 1 : 300 dilution of the macrophage-specific rat anti-mouse F4/80 monoclonal antibody (Cosmo Bio Co. Ltd. Tokyo, Japan) for 2 hrs at 37°C. Then they were incubated with a secondary antibody solution (Biotin-conjugated mouse anti-rat IgG monoclonal antibody), and the avidin-biotin-peroxidase complex (ABC) commercially obtained was used as advised. The reactions were visualized by incubation with a POD substrate solution containing 0.005% hydrogen peroxide, 0.2%DAB in 50mM Tris-HCl buffer (pH 7.6) for 3 min. Sections were then counterstained in Mayer's hematoxylin. As negative controls, sections were incubated with phosphate-buffered saline without primary antibody.

Electron microscopy

After perfusion fixation, oviducts were removed, and the ampullas were cut into small blocks and immersed in 4% paraformaldehyde with 5% glutaraldehyde in 0.1M cacodylate buffer, Karnovsky's fluid, pH 7.4 for 3 hrs at 4°C. Then they were postfixed in cold 1% osmium tetroxide in the same buffer for 2 hrs, and were embedded in Epon 812. Ultra-thin sections, 80 nm thick, were cut with a diamond knife on a Leica-Ultracut S ultramicrotome and were mounted on formvar film-coated one-pore copper mesh. The sections were then impregnated with 2% uranyl acetate and lead citrate and examined in a JEM-2000EX-II electron microscope at 80kV.

These experiments were approved by the Animal Research Committee of Kawasaki Medical School (No. 03-076) and conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School.

RESULTS

Reproductive organs of normal estrus and overnight-mated females

All the female reproductive organs, including the ovaries, oviducts, uterus and vagina, could be observed on a large section of flatly pinned specimens (Fig 1a and b). The ovaries of overnight-mated females contained numerous small primary and secondary follicles, and several small corpora lutea were freshly formed. The oviducts were coiled and located between the ovaries and the edge of uterine horns. Infundibulum and thin-walled ampullas were continuous with the narrow looped tube of the isthmus. The oviduct ampullas appeared as dilating sacs, having thin and smooth-surfaced mucous membranes, and the epithelium was of the simple cuboidal variety, and was not highly ciliated. The ampullas of the normal estrous females contained neither leukocytes nor mononuclear cells. The lumina of the overnight-mating females contained several oocytes ovulated with numerous granulosa cells (Fig 1c). The mucous membrane of the isthmus formed numerous folds, and columnar epithelium was highly ciliated. A few spermatozoa could be observed in the lumen of the isthmus of the overnight-mated females (Fig 1d). The "Y" shaped uterus consisted of two long horns and short corpus. Compared with the normal uterine horns (Fig 1a), the walls of those of the overnight-mated females became markedly thin, and the lumia were extremely dilated and segmented (Fig 1b). Each horn segment was full of spermatozoa ejaculated from mated males (Fig 1b, e, f). The endometrium, which was lined with a simple columnar epithelium, contained uterine glands, and a few spermatozoa could also be found in the uterine crypts. The majority of spermatozoa were aggregated at the central portion of the uterine lumen, but a few spermatozoa left spermatozoon aggregations, and their head were attached to the epithelial surface of the endometrium. The heads of the spermatozoa appeared to be phagocytosed by epithelial cells of the corpus uteri (Fig 1g). The corpus uteri projected into the vagina to form the cervix. The vagina was lined with stratified squamous epithelium, and the vaginas of the overnight-mated females contained a copulation plug (Fig 1b). The upper part of the vagina, vaginal-cervical region, also contained numerous spermatozoa, but no spermatozoa could be seen within the plug. The vaginal lumen between the copulation plug and the opening contained both numerous leukocytes and a few spermatozoa. The majority of the leukocytes were polymorphonuclear cells, mainly neutrophils, with the remaining ones being mononuclear cells. These cells were confined to the region near the vaginal opening, and they could not be observed in the uterine and oviduct lumina. However, the oviduct ampullas contained a few large mononuclear cells among granulosa cells around oocytes.

Granulosa cells surrounding oocytes in the oviduct ampullas after two-hour mating

At three hours after mating, the oviduct ampullas of one mated female out of three contained oocytes together with corona radiata cells, and some of the granulosa cells' nuclei already appeared to be pyknotic. Oocytes could not be found in the oviduct ampullas of the remaining two females. At five hours after mating, every oviduct ampulla contained several oocytes with granulosa cells ovulated from the ovary (Fig 2a), and the ampullas appeared markedly dilated. Each oocyte was large and round, approximately 40 μm in diameter, and, between the oocytes and granulosa cells, there existed a wide perivitelline space and a zona pellucida. The perivitelline spaces were irregularly dilated, and the heads of spermatozoa could be seen in the space (Fig 2b). The zona pellucida appeared faintly eosinophilic, ranging from 1 μm to 3 μm in width, and, outside of the zona pellucida, granulosa cells were diffusely located (Fig 2b). The granulosa cells were round, about 5-8 μm in diameter, and had spherical nuclei. The inner layer of these cells made contact with the zona pellucida. Approximately 5% of the granulosa cells showed changes in nuclear morphology at five-hours after mating. The nucleus became pyknotic, and the chromatin became greatly condensed. Some of the granulosa cells contained a few small nuclear fragments. TUNEL stained approximately 5% of granulosa cell nuclei, 44 cells out of 852 from five sections (Fig 2c, d). In addition, approximately 2% of the granulosa cells, 18 cells out of 870 from five sections, had a foamy appearance. The cells tended to swell and lyse, and the cell's contents spilled into the extracellular space. There appeared a few large mononuclear cells, 10-12 μm in diameter, among the granulosa cells, and they included several dying cells and cell fragments (Fig 2e). These large mononuclear cells were F4/80-positive, and their inclusions were often TUNEL-positive (Fig 2f).

At eight hours after mating, the ampullas also contained several oocytes, but the numbers of granulosa cells markedly decreased. The corona radiata cells became detached from the zona pellucida, and the majority of them had an irregularly shaped nucleus. About 30% of the nuclei of all the granulosa cells, 113 cells out of 377 from eight sections, were TUNEL-positive. A few F4/80 positive cells were also observed in eight hours sections. Foamy cell debris, in approximately 13% of the cells, 48 cells out of 356 from eight sections, could also be observed among granulosa cells.

At 12 hours after mating, the oviduct ampullas contained a few ova, although many of ova had moved to the isthmus. Granulosa cells around individual ovum markedly decreased in number and some of the ova had no accompanying granulosa cells at all. About 40% of the nuclei of the remaining granulosa cells, 72 out of 181 from five sections, were TUNEL-positive, and no F4/80-positive cells were observed in ampullas. Foamy cell debris, approximately 10% of the cells, 20 cells out of 183 from five sections, could also be observed.

Ultrastructure of granulosa cells undergoing cell death and macrophages in the ampullas after two-hour mating

Between 3 and 12 hours after two-hour mating, the granulosa cells surrounding oocytes showed a dramatic decrease in number. Until 12 hours after mating, the granulosa cells exhibited various morphological signs of cell death through two different cell death processes, and they finally disappeared from the oviduct ampullas. Between three and five hours after mating, the majority of granulosa cells remained intact. The intact granulosa cells had a round cell profile, 5-8 μm in diameter, with a round or oval nucleus. As shown in Figure 3a, the nuclei sometimes had an irregular outline, and the heterochromatin was in the form of small and irregular masses, which were scattered throughout the nucleus. One to three small nucleoli were frequently seen. The cytoplasm contained a few lipid droplets, smooth and rough endoplasmic reticulum, numerous ribosomes and mitochondria. The cell surface was characterized by a few short microvillous projections (Fig 3a). In the ampullas at five hours after mating, macrophages appeared among the granulosa cells. The cells were larger than intact granulosa cells, approximately 8-10 μm in diameter. Impressive ultrastructural features of the macrophages included a large number of fingerlike projections on the cell surface and pinocytotic vesicles at the cell periphery (Fig 3b). The majority of vesicles were round and clear, 0.25-1 μm in diameter. The nuclei had irregular profiles and the cytoplasm also contained a few strands of rough endoplasmic reticulum and small lysosomal granules, 0.25 μm in diameter (Fig 3c).

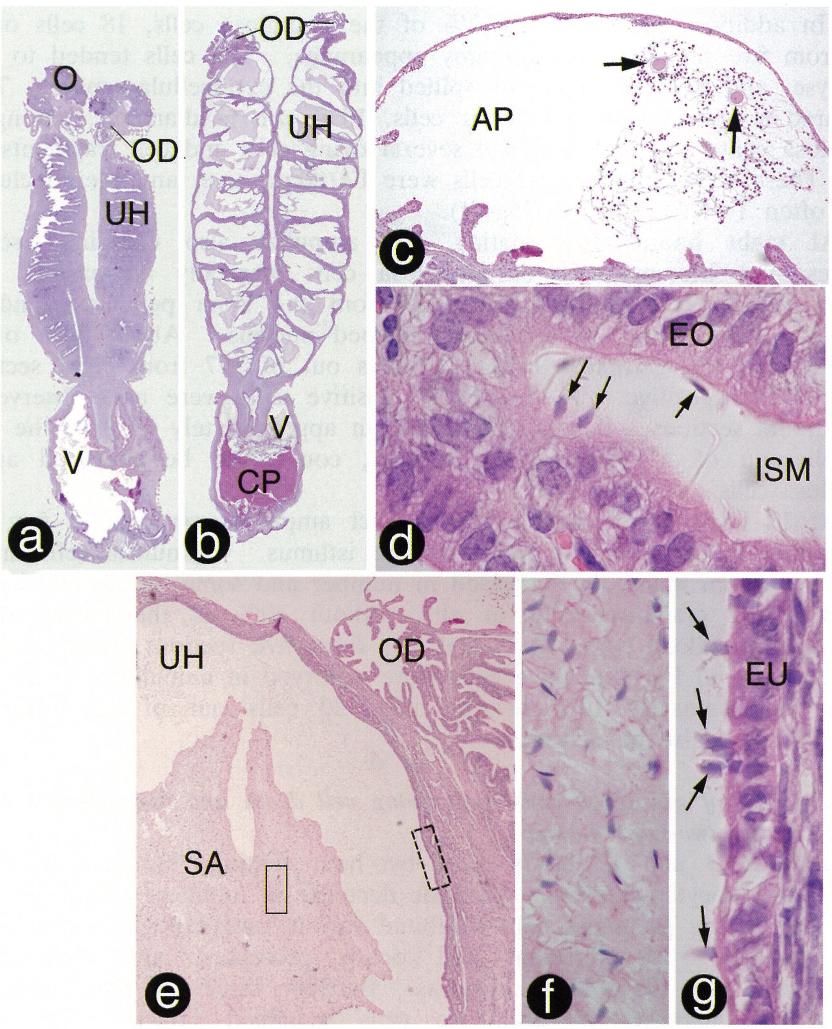

Fig 1. Light micrographs of reproductive organs of normal estrus and overnight-mated females. H-E staining.

a. A low-power micrograph of all the reproductive organs of a normal estrus female. O : ovary, OD : oviduct, UH : uterine horn, V : vagina. ×2.3.

b. All the reproductive organs of an overnight-mated female. The uterine horn (UH) appears segmented and extremely dilated, and the lumina of extended segments contain aggregations of sperm. A large copulation plug (CP) exists in the vaginal lumen (V). OD : oviduct. ×2.3.

c. Ampulla of an oviduct of an overnight-mated female. A dilated lumen of an ampulla (AP) contains two ova (arrows) with corona radiata cells. ×54.

d. A high-power micrograph of the isthmus of an oviduct of an overnight-mated female. Within the lumen of the isthmus (ISM), a few spermatozoa (arrows) can be found, but no other cell elements could be identified. The heads of spermatozoa often attached to the epithelial surface of the oviduct (EO). ×640.

e. Uterine horn of an overnight-mated female. Each segment of the uterine horn (UH) contains a sperm aggregation (SA). OD : oviduct. ×24.

f. A high-power micrograph of the sperm aggregation in the solid-line rectangle in figure (e).

No cellular elements other than sperms can be observed in the sperm aggregation. ×600.

g. A high-power micrograph of the dotted-line rectangle in figure (e).
The heads of spermatozoa (arrows) are attached to the surface of the endometrium of uterine horn (EU). ×750.

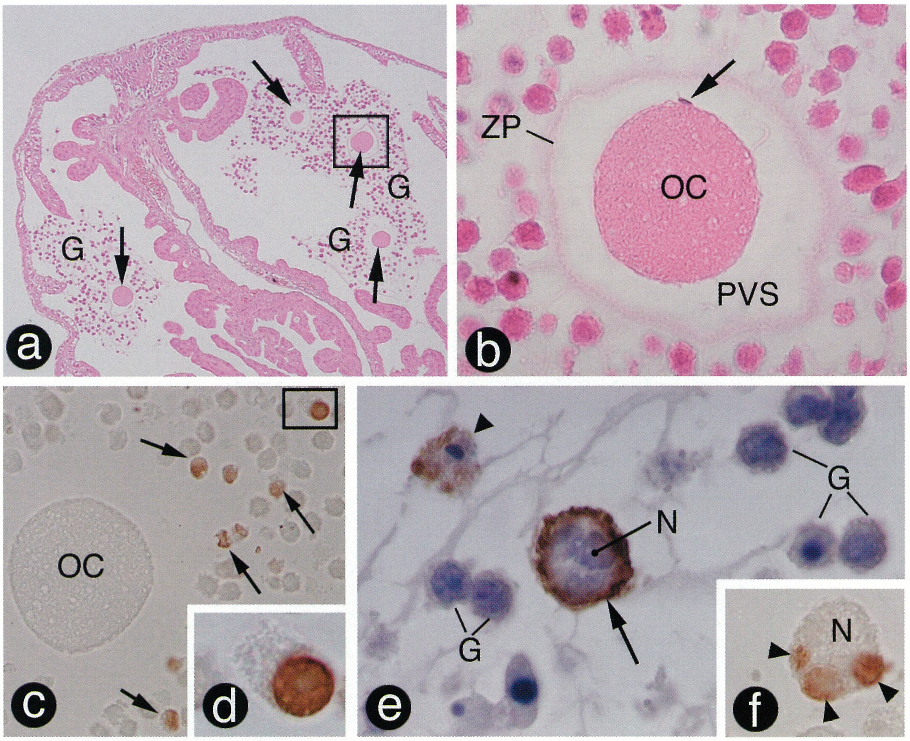

Fig 2. Oviduct ampullas at five hours after two-hour mating.

a. A low-power micrograph of an oviduct ampulla.
The lumina of ampullas contain four oocytes (arrows) with granulosa cells (G) ovulated from the ovary. H-E staining. ×70.

b. An oocyte and surrounding granulosa cells of the rectangle in figure (a).
Between the oocyte (OC) and the granulosa cells, a wide perivitelline space (PVS) and a zona pellucida (ZP) exist, and the head of a spermatozoa (arrow) can be seen on the oocyte. H-E staining. ×560.

c. An oocyte and granulosa cells stained by TUNEL.
TUNEL positive nuclei (arrows) can be seen in corana radiata around an oocyte (OC). ×420.

d. A high-power micrograph of the rectangle in figure (c).
A granulosa cell nucleus is TUNEL-positive. ×1,000.

e. Immunohistochemical F4/80 stained corona radiata.
The arrow indicates an F4/80-positive mononuclear cell, which appears among granulosa cells (G). A peripheral part of F4/80-positive mononuclear cell is indicated by an arrowhead. The section is counterstained with hematoxylin. N : nucleus of the mononuclear cell. ×980.

f. The TUNEL stained mononuclear cell in the adjacent section of figure (d).
The mononuclear cell cytoplasm contains three TUNEL-positive inclusions (arrowheads). N : nucleus of the mononuclear cell. ×980.

The first cell death process of granulose cells was early characterized by nuclear changes including compaction and margination of nuclear heterochromatin and condensation of the cytoplasm (Fig 4a, b). The

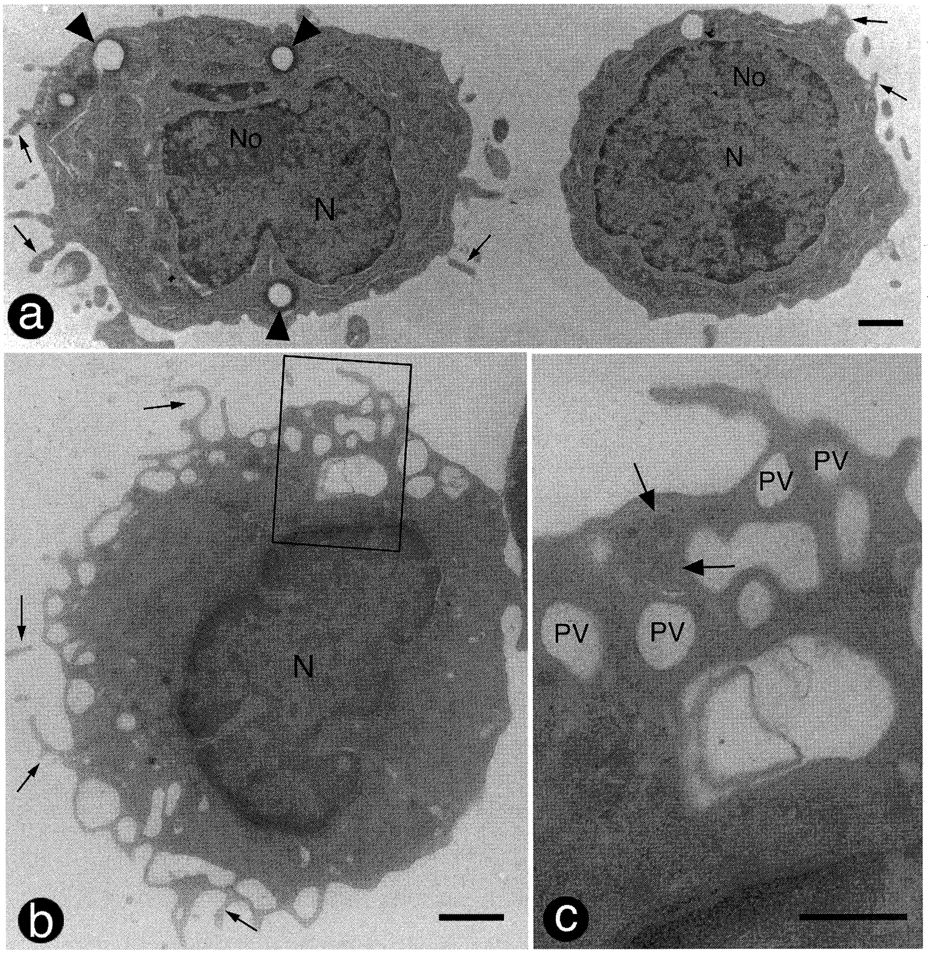

Fig 3. Electron micrographs of granulosa cells and a macrophage of the corona radiata in an oviduct ampulla at five hours after two-hour mating.

a. Two intact granulosa cells.
Granulosa cells have a round or ovoid cell profile, and the surface has a few short microvillous projections (arrows). The cytoplasms contain a few lipid droplets (arrowheads), strands of smooth and rough endoplasmic reticulum, numerous ribosomes and mitochondria. N : nucleus, No : nucleolus. Bar : 1 μm

b. A macrophage in the corona radiata.
The prominent features of the macrophage are a large number of fingerlike projections (arrows) at cell surface and numerous pinocytotic vesicles at the cell periphery. N : nucleus. Bar : 1 μm

c. High-power micrograph of the rectangle in figure (b).
The cytoplasm contains numerous clear pinocytotic vesicles (PV) and a few small lysosomal granules (arrows). Bar : 500 nm

endoplasmic reticulum began to expand to form numerous small sized vacuoles, approximately 0.3 μm in diameter. Mitochondria became somewhat swollen, but the cristae remained intact. Finally, fragmentations of the nucleus and budding of the whole cell were followed by production of membrane-bounded bodies (Fig 4c). The first cell death process could be designated as typical apoptosis, which appeared as TUNEL-positive cells

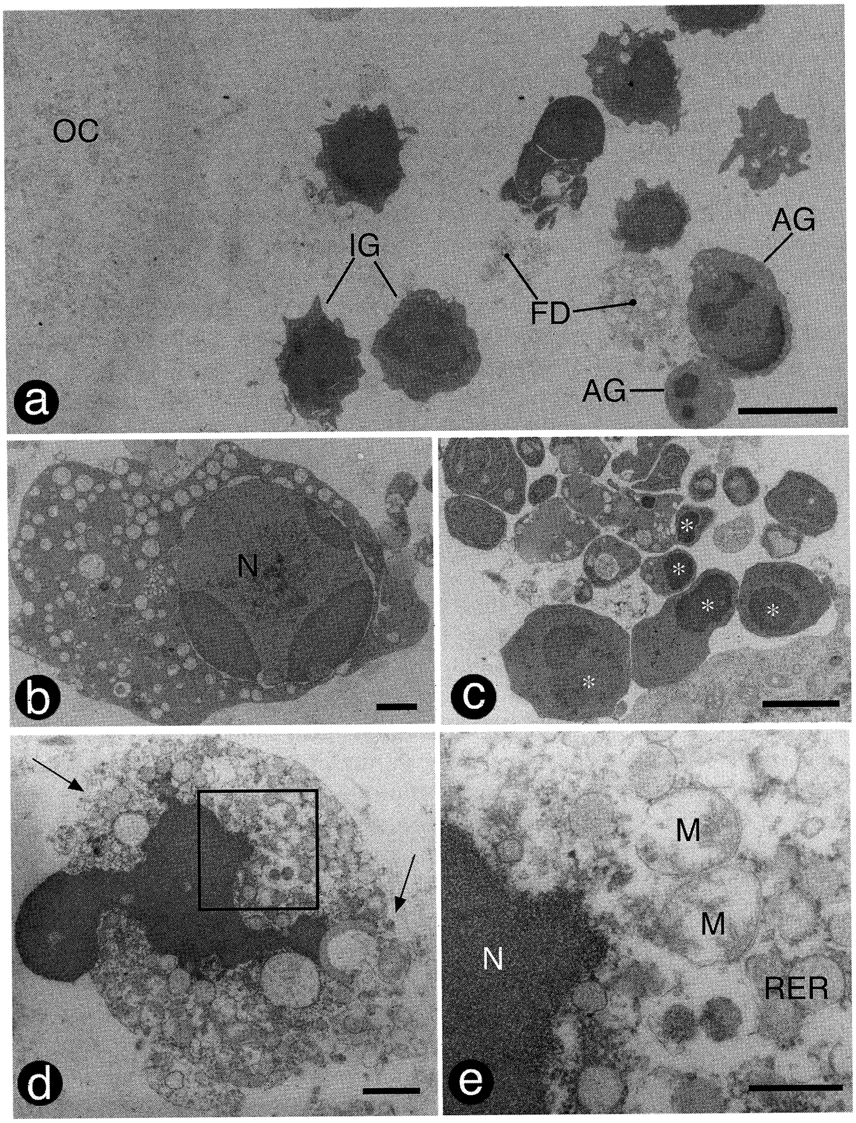

Fig 4. Ultrastructure of dying granulosa cells in oviduct ampulla after 2-hour mating.

a. A low-power micrograph of the corona radiata.
Not only intact granulosa cells (IG) but also many dying cells can be recognized in the corona radiata around an oocyte (OC). Two different types of cell death can be identified; one characterized by apoptotic granulosa cells (AG), and, the other by foamy debris from necrotic granulosa cells (FD). Five hours after two-hour mating. Bar : 5 μm

b. An apoptotic granulosa cell.
Compaction and margination of heterochromatin can be seen in the nucleus (N). The endoplasmic reticulum begins to expand to form numerous small sized vacuoles, and mitochondria become swollen. Eight hours after mating. Bar : 1 μm

c. Apoptotic bodies.
Nuclear fragments can be found in large apoptotic bodies (*). Eight hours after mating. Bar : 1 μm

d. A necrotic granulosa cell.
The cell is swollen and lysed, giving it a foamy appearance. Both the nuclear

envelope and the cell membrane have disappeared, and the nuclear contents spills into extracellular space. Arrows indicate plasma membrane lysis. Eight hours after mating. Bar : 1 μm

e. High-power micrograph of the rectangle in figure (d).
Mitochondria (M) have become extremely swollen and only a few cisternae can be recognized. Rough endoplasmic reticulum (RER) is also distended, resulting in a round profile. N : degrading nucleus. Bar : 500 nm

under light microscopy. Macrophages seemed to phagocytose and remove not only apoptotic cells but also apoptotic bodies in the ampullas of the oviducts.

On the other hand, the second process was characterized by quite different features than those of the first one (Fig 4a). Ultrastructurally, the second process was characterized by mitochondrial swelling and irregular clumping of nuclear chromatin (Fig 4d). Disruption of cell, nuclear and organelle membranes was followed by dissolution of the overall cell structure (Fig 4d, e). The second cell death process of granulose cells was designated as typical necrosis.

DISCUSSION

Our present study revealed that granulosa cells rapidly disappeared from the oviduct ampullas after mating by undergoing cell death through two different processes; apoptosis and necrosis, and that phagocytosis by macrophages appeared to be represented one of the cell disposal pathways in the oviduct ampullas. Previous histochemical and ultrastructure studies on the fate of follicular epithelial cells in the follicle atresia in ovaries have revealed that massive groups of granulosa cells simultaneously undergo apoptosis,[2-4] and this apoptosis has been considered to be essential for the elimination of atretic follicles from the ovary.[5] As shown in our results, oviduct ampulla after ovulation contains several oocytes surrounded by corona radiata, and granulosa cells disappear quickly from the ampulla during 12 hours after mating also by massive cell death. On the basis of our TUNEL reaction and electron microscopic observations, it was concluded that granulosa cells degenerated and died by the classical apoptotic process first described in 1972 by Kerr *et al*.[8] It has also been reported that the apoptotic process in oviduct ampullas starts as early as three or four hours after ovulation.[9] In the present study, however, in addition to this apoptosis, the corona radiata contained a considerable number of not only necrotic granulosa cells but also numerous foamy cell debris, which were diffusely scattered among intact and apoptotic granulosa cells. D'Herde *et al*[10] reported that three different types of cell death coexisted in the granulosa cells of the follicle atresia: apoptosis, autophagic cell death and primary cell necrosis, and that both apoptosis and autophagic cell death are the exclusive mode of active cell death in follicle atresia. Among postovulatory granulosa cells in the oviduct ampullas, unlike follicle atresia in ovary, a large proportion degenerated throuh apoptosis and necrosis, and the occurrence of autophagic cell death appeared inconspicuous in the ampullas.

According to earlier investigations of normal cell death in ontogeny, cell death had been considered to take two distinct forms, necrosis seen under acute pathological conditions, and apoptosis under normal physiological conditions.[8,11] In necrosis, marked swelling of mitochondria is finally followed by dissolution of the overall cell structure, whereas, in apoptosis, condensation of the cytoplasm and nuclear chromatin is finally accompanied by the formation of membrane-bounded bodies. As mentioned in our results, the corona radiata cells around oocytes underwent both apoptosis and necrosis. It is surprising that two opposite kinds of cell death simultaneously occur in the oviduct ampulla under a physiological condition accompanied by ovulation. During the past 15 years, the necrosis-verous-apoptosis dichotomy has been gradually replaced by various classifications of cell death mainly on destruction mechanisms,[12,13] and interplay leading to cell death between apoptosis and necrosis has been widely explored in normal cell death.[14] The significance of the coexistence of apoptosis and necrosis in oviduct ampullas after mating remains unclear, but postovulatory granulosa cells could become a useful experimental model for an investigation of the mechanisms which are responsible for cell death pathway selection.

Antigen F4/80 is a membrane glycoprotein marker expressed by monocytes and macrophages in many tissues of the mouse,[15] and mouse ovaries have been known to contain F4/80-positive cells.[16] In the atretic follicles of prereproductive young female mice, Inoue *et al*[5] found no evidence for the involvement of macrophages, and, due to both repeated apoptosis and phagocytosis by granulosa cells, atretic follicles disappeared from the ovary. However, as shown in our results, a few F4/80 positive phagocytes appeared among the postovulatory granulosa cells, and these contained several TUNEL-positive cell elements in their cytoplasm, although the number of macrophages in the oviduct did not seem enough for the elimination of dying granulosa cells in a short period. Therefore, postovulatory granulosa cells in the ampullas appeared to undergo not only apoptosis but also necrosis to be lysed and dissolved into the lumina.

The third extrinsic element in the oviduct ampulla after mating is spermatozoa ejaculated from a mating male. Aggregations of spermatozoa after mating were found not only in the vagina but also in the uterus, and, at five hours after mating, the heads of the spermatozoa could be identified in the perivetelline space of oocyte in oviduct ampullas. For the removal of excess spermatozoa from the female genital tract after coitus, evidence of involvement of the vaginal epithelium,[17] uterine endothelium,[18] oviduct epithelium,[19] and cumulus cells of the ovum[20] have been reported. As shown in our results, spermatozoa in the oviducts and uterus often lay in close association with the epithelial cells, and no macrophages showing a close relationship with spermatozoon aggregations were found in the uterine and oviduct cavities. After mating, a few macrophages showing active phagocytosis appeared only in the oviduct ampullas, and the number was so small that their functional roles for the elimination of extrinsic elements from the genital tract lumina seemed to be very limited.

In conclusion, postovulatory granulosa cells are eliminated from oviduct ampullas through two different cell death processes, necrosis and classical apoptosis. Macrophages appear in oviduct ampullas after ovulation to remove mainly apoptotic granulosa cells, but they are not found to be associated with removal of excess spermatozoa in oviduct.

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REFERENCES

1) Page EW, Villee CA, Villee DB : Human Reproduction. 3rd ed, Philadelphia, WB. Saunders. 1981, pp 32-38
2) Hughes, FM, Gorospe WC : Biochemical identification of apoptosis (programmed cell death) in granulosa cells : evidence for a potential mechanism underlying follicular atresia. Endocrinology **129** : 2415-2422, 1991
3) Kasuya K : Elimination of apoptotic granulosa cells by intact granulosa cells and macrophages in atretic mature follicles of the guinea pig ovary. Arch Histol Cytol **60**(2) : 257-264, 1995
4) Kasuya K : The process of apoptosis in follicular epithelial cells in the rabbit ovary, with special reference to involvement by macrophages. Arch Histol Cytol **58**(2) : 175-184, 1997
5) Inoue S, Watanabe H, Saito H, Hiroi M, Tonosaki A : Elimination of atretic follicles from the mouse ovary : A TEM and immunohistochemical study in mice. J Anat **196** : 103-110, 2000
6) Hay MR, Cran DG, Moor RM : Structural changes occurring during atresia in sheep ovarian follicles. Cell Tissue Res **169**(4) : 515-529, 1976
7) Logothetopoulos J, Dorrington J, Bailey D, Stratis M : Dynamics of follicular growth and atresia of large follicles during the ovarian cycle of the guinea pig : fate of the degenerating follicles, a quantitative study. Anat Rec **243**(1) : 37-48,1995
8) Kerr JFR, Wyllie AH, Currie AR : Apoptosis : a basic biological phenomenon with wide-varying implications in tissue kinetics. Brit J Cancer **26** : 239-257, 1972
9) Szoltys M, Tabarowski Z, Pawlik A : Apoptosis of postovulatory cumulus granulosa cells of the rat. Anat Embryol (Berl) **202**(6) : 523-529, 2000
10) D'Herde, B De Prest, Roels F : Subtypes of active cell death in the granulosa of ovarian atretic follicles in the quail. Reprod Nutr Dev **36** : 175-189, 1990
11) Walker NI, Harmon BV, Gobe GC, Kerr JFR : Patterns of cell death. Meth. Achiev. Exp. Pathol **13** : 18-54, 1988
12) Clarke PGH : Developmental cell death: morphological diversity and multiple mechanisms. Anat Embryol **181** : 195-213, 1990
13) Zakeri Z, Bursch W, Tenniswood M, Lockshin RA : Cell death : programmed, apoptosis, necrosis, or other ? Cell Death Differentiation **2** : 87-96, 1995
14) Lockshin RA, Zakeri Z : Caspase-independent cell deaths. Curr Opin Cell Biol **14**(6) : 727-733, 2002
15) Morris L, Graham CF, Gordon S : Macrophages in haemopoietic and other tissues of the developing mouse detected by the monoclonal antibody F4/80. Develop **112**(2) : 517-526, 1991
16) Austyn JM, Gordon S : F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur J Immunol **11** : 805-815, 1981
17) Phillips DM, Mahler S : Phagocytosis of spermatozoa by the rabbit vagina. Anat Rec **189**(1) : 61-72, 1977
18) Austin CR : Fate of spermatozoa in the female genital tract. J Reprod Fertil **1** : 151-156, 1960
19) Murakami M, Nishida T, Shiromoto M, Inokuchi T : Phagocytosis of spermatozoa and latex beads by epithelial cells of the ampulla vasis deferentis of the rabbit : a combined SEM and TEM study. Arch Histol Jpn **48**(3) : 269-277, 1985
20) Thompson RS, Smith DM, Zamboni L : Fertilization of mouse ova in vitro : an electron microscopic study. Fertil Steril **25**(3) : 222-249, 1974