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Interactions of Apoptosis and Extracellular Matrices in Granulosa Cells of Atretic Follicles in Porcine Ovaries

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Abstract: *The process of apoptosis of follicular granulosa cells in porcine ovaries during follicular atresia was investigated by in situ DNA 3'end-labeling at the level of individual cells. Histochemical changes in the follicular basement membrane (BM) were visualized by immunofluorescent staining of BM extracellular matrix (ECM) components, i.e. type IV collagen, laminin and fibronectin. At the first stage, granulosa cells located on the inner surface of the granulosa layer appeared to undergo apoptosis, followed by neighboring granulosa cells. No apoptotic granulosa cells making tight contact with intact BM were observed. Detachment and degeneration of the granulosa cell layer and fragmentation of BM occurred in follicles at the advanced stage of atresia. Finally, intermittent structures of BM and subsequent invasion of macrophages and fibroblasts were observed. Therefore we concluded that granulosa cell apoptosis is an initial symptom of follicular atresia in the porcine ovaries, and the degradation of BM follows granulosa cell apoptosis in the pig. Our results suggest that ECM components of follicular BM act as survival factors on follicular granulosa cells in porcine ovaries.*
Key words: *Apoptosis, Extracellular Matrix, Granulosa cell, Atretic follicle, Porcine ovary.*

More than 99% of ovarian follicles undergo atresia at various stages of development in mammalian ovaries [1, 2]. Follicular atresia is a key reproductive phenomenon which decided the follicles that will not ovulate in the ovary. Despite its critical role during the recruitment of follicles for ovulation, the mechanisms of the cellular and molecular events are not well understood yet. Despite species-specific differences, it is generally accepted that the earlier stages of ovarian follicular atresia are correlated with disorganization and degeneration of follicular granulosa cells [2, 3]. Early studies [4–7] showed that both enhanced DNA degradation and ladder formation, hallmarks of apoptotic cell death, occur in granulosa cells of atretic follicles, and that apoptosis, originally reported by Kerr *et al.* [8], plays a key role in mammalian follicular atresia. Our previous detailed studies of the mechanism of follicular atresia in porcine ovaries demonstrated that degeneration of the atretic follicles can be explained in part by apoptosis of granulosa cells [9–21], and that the alteration of glycoconjugates of cell-surface and intercellular components in granulosa cells during follicular atresia are involved in some processes of ovarian follicular atresia and granulosa cell apoptosis [22–25].

The extracellular matrix (ECM) components play important roles in the maintenance of homeostasis of organs and cells [26]. The relationship between ECM and cells that adhere to it can play important regulatory roles in many basic cellular processes by influencing enzyme activity [27, 28] and phospholipid metabolism [29], and by modifying transcriptional and transitional activities of the cell [30]. These regulatory influences can control key events in the life of a cell, such as cell shape, deformation and its motility [31], cell proliferation, growth and differentiation [31, 32], and cell survival and death [33–35]. Disruption of existing cellular interactions with the ECM, due to experimental, pathological or normal physiological changes were also shown in early studies to be closely linked to changes in the functional capacity of cells. Early investigations [26–35] showed that cell-substratum contact is closely linked to cell growth and cell death, and that the relationship be-

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tween substratum contact and cell growth and differentiation is positive for nonpathological cells. The basement membrane (BM), which mainly consists of type IV collagen, laminin and fibronectin and makes direct contact with epithelial cells, has various prominent roles such as polarization of cells, induction of cell differentiation, support of cell movement, and maintenance of metabolism and structure [36–38]. When ovarian follicles are allowed to undergo atresia, apoptosis occurs in follicular epithelial cells, i.e. granulosa cells [9–25], and the follicular BM becomes fragmented [39]. Alteration of the ovarian follicular BM is considered to be a critical change during follicular atresia. However, the details of the changes in the BM during atresia in porcine ovarian follicles have not yet been established clearly.

In the present study, to elucidate the relationship between the degradation of follicular BM and granulosa cell apoptosis during atresia in the pig ovarian follicles, we applied a histochemical technique for the *in situ* visualization of apoptosis at the single-cell level, and immunofluorescence-confocal microscopy techniques for estimation of ECM components in follicular BM.

Materials and Methods

Tissue preparation and conventional histopathology

Ovaries obtained from a local slaughterhouse were fixed with 10% phosphate buffered formalin (pH 7.2), for conventional histopathological evaluation. For immunohistochemical and *in situ* detection of DNA fragmentation, the ovaries were cut into small pieces, put on filter paper, mounted in OCT compound (Ames Co., Elkhart, IN, USA), and then rapidly frozen in dry ice-cooled isopentane. The frozen tissue samples were kept at –80°C until use. Formalin-fixed tissues were dehydrated through a graded ethanol series and embedded in Histosec (Merck Co., Darmstadt, Germany). Sections 3 μm thick were prepared on a microtome, mounted on glass slides precoated with 3-aminopropyltrimethoxysilane (Aldrich Chemical, Milwaukee, WI, USA), deparaffinized in xylene, and rehydrated through a graded ethanol series. For conventional histopathological evaluation, the sections were stained with hematoxylin-eosin according to the standard method. ECM structure was assessed on sections stained with Sirius red solution, saturated picric acid in distilled water containing 0.1% (w/v) Sirius red F3B (BDH Chemicals Ltd., Poole, UK) [40, 41]. All slides were mounted with Entelan (Merck), and then light microscopic examination was performed.

In situ detection of fragmented DNA

Apoptotic cells were displayed histochemically by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) method using a commercial kit (FITC-ApopTag; Oncor Inc., Gaithersburg, MD, USA) [9]. Briefly, serial frozen sections (5 and 20 μm thick for conventional and confocal microscopy, respectively) were cut on a cryostat, mounted on 3-aminopropyltrimethoxy silane-precoated slides and then fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4°C for 10 min. Fixed sections were incubated with proteinase K (20 μg/ml; Wako Pure Chemical Co., Osaka, Japan) for 10 min at room temperature (RT; 22–25°C) and washed in phosphate buffered saline (PBS; pH 7.2). They were incubated with terminal deoxynucleotidyl transferase (TDT) solution containing digoxigenin-11-dUTP (DIG-UTP) and dATP for 1 h at 37°C, and then immersed in stop solution (2 × salt sodium citrate buffer) for 30 min at 37°C. After washing, the sections were incubated with fluorescein isothiocyanate (FITC)-labeled anti-DIG antibody solution for 30 min at RT. The slides were washed with PBS, mounted with glycerol, and then examined with a confocal laser scanning microscope (LSM-GB200, Olympus, Tokyo, Japan) and/or a fluorescence microscope (BHS-RFC, Olympus, Tokyo, Japan). The following positive and negative controls were included in each experimental run; as negative controls, the sections were incubated with omission of either TDT or DIG-UTP, while as a positive control tissue sections were treated with DNase I (1 μg/ml; Wako) for 10 min at RT before exposure to TDT, and sections prepared from young adult rat testis were used as physiological positive controls [42].

Immunohistochemistry for ECM components

For immunofluorescence staining for ECMs, adjoining frozen sections (5 and 20 μm thick for conventional and confocal microscopy, respectively) were cut on a cryostat, mounted on 3-aminopropyltrimethoxysilane-precoated slides and then fixed with precooled acetone for 10 min at –80°C. After washing with PBS, the sections were preincubated with diluted normal goat serum (1/100 dilution with PBS containing 4% bovine serum albumin; PBS-BSA) for 20 min at RT, and then rinsed with PBS including 0.05% Tween 20 (PBS-Tw). The slides were incubated with each rabbit anti-ECM antibody (anti-type IV collagen, anti-laminin and anti-fibronectin antibodies; Chemicon International, Temecula, CA, USA) diluted (1/100) with PBS-BSA or with diluted normal rabbit serum (1/100 dilution with PBS-

BSA) for 18 h at 4°C. After washing with PBS-Tw, the sections were incubated with FITC-conjugated goat anti-rabbit IgG antibody (1/400 dilution; Sigma Chemical, St. Louis, MO, USA) for 2 h at RT. After 3 washes with PBS-Tw, the slides were mounted with glycerol and then examined with a confocal laser scanning microscope and/or a fluorescence microscope. As negative controls, sections incubated without anti-ECM antibody were prepared in each experimental run [41].

Results

Follicular apoptotic (TUNEL-positive) cells and BM components (type IV collagen, laminin and fibronectin) were visualized histochemically. Three-dimensional images, (composite confocal images) were generated using a confocal laser scanning microscope. Briefly, ovarian tissue sections stained histochemically were optically sectioned at 0.5 μm intervals using a confocal laser scanning microscope, and twenty serial optical sections in each tissue section were saved in the computer system of the confocal laser scanning microscope. Then, composite confocal images from the 20 serial images saved in the computer system were generated. Finally, overlapping images of TUNEL and ECM-stained serial tissue sections were generated using the NIH-image program on a Macintosh computer.

In healthy antral follicles, oocytes were surrounded by cumulus cells directly adjacent to granulosa cell layers (oocyte-cumulus cell complex), and granulosa cells and thecal cells were well preserved. No cells with TUNEL-positive nuclei (apoptotic nuclei) were observed among oocytes, granulosa cells, cumulus cells, or thecal cells (Fig. 1A-center). Strong positive staining for BM components (laminin, type IV collagen and fibronectin), especially for laminin (Fig. 1A-left), was observed along the BM. The BM of healthy follicles had no shrinkage, cleavage or laceration, and was considered to be intact. As positive reactions for laminin were seen in the BM of capillary blood vessels, the fluorescence intensity indicates the density of capillary blood vessels in the theca layer. Strong staining for laminin was demonstrated in the theca layers of the healthy follicles.

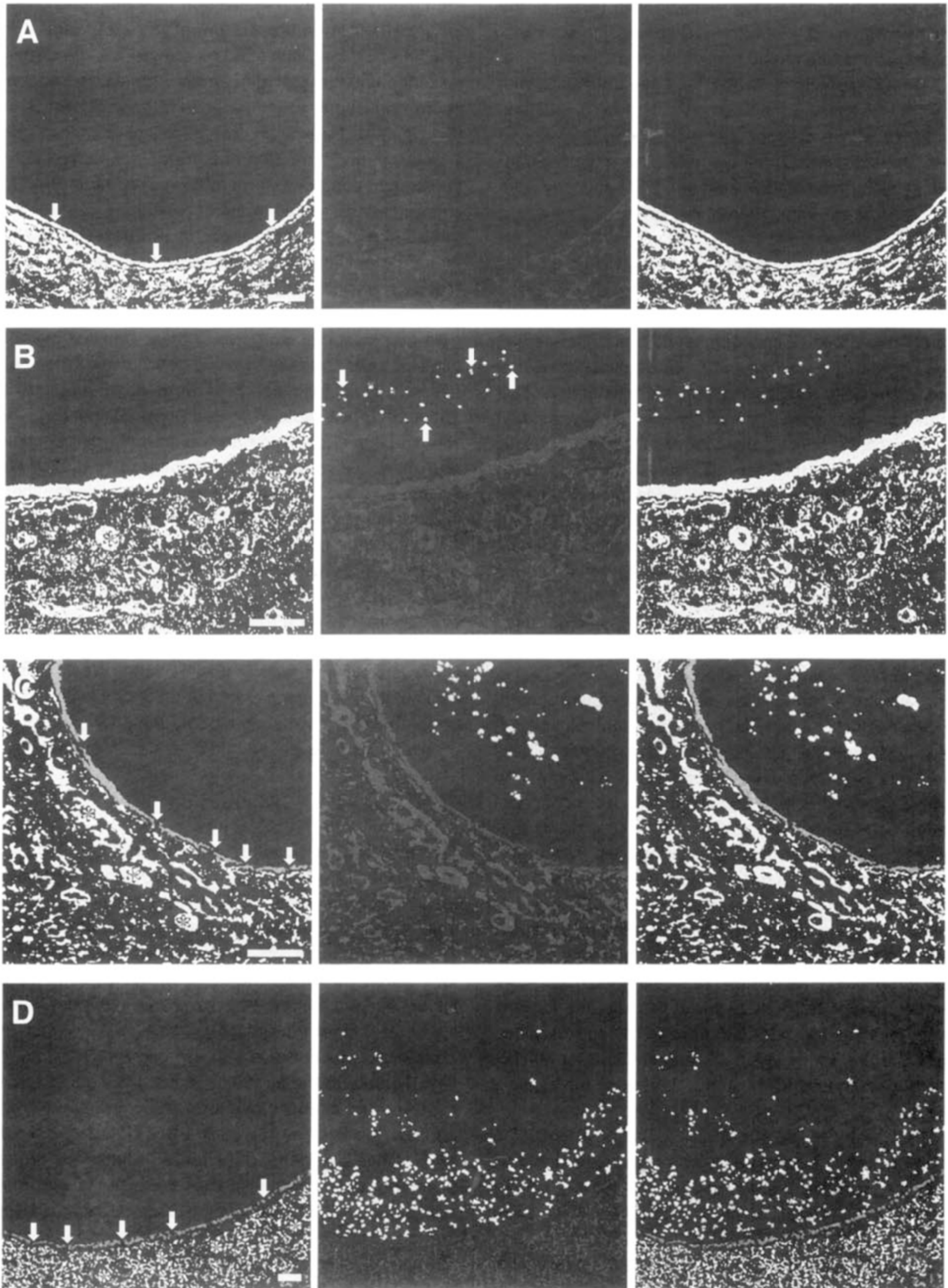
In the early stage of ovarian follicular atresia, the oocyte-cumulus cell complex was preserved and was negative for TUNEL staining (Fig. 1B-center). Scattered single TUNEL-positive granulosa cells were observed on the inner side of the granulosa cell layers. No obvious changes were noted in the BM as assessed by immunofluorescence staining when compared with the healthy follicles (Fig. 1B-left). Neighboring granulosa cells continued to undergo apoptosis (Fig. 1C-center). Along with follicular atresia, the BM showed discontinuity, that is a diffuse and fragmented appearance, and began to exhibit less intense fluorescence (Fig. 1C-left). Healthy (TUNEL-negative) granulosa cells, located on the outer side of the granulosa layer, maintained tight contact with morphologically intact BM. No differences were seen in the fluorescence intensities of the ECM proteins in the theca layers between healthy and early atretic follicles, indicating no difference in the blood supply for the follicles between them.

In the follicles at the advanced stage of atresia, oocyte-cumulus cell complex and/or oocytes without cumulus cells and many TUNEL-positive granulosa cells suspended in follicular fluid were observed, but no apoptotic thecal cells were seen (Fig. 1D-center). BM with shrinkage, cleavage and laceration was observed, and then discontinuous BM appeared (Fig. 1D-left). Weak staining for ECM proteins was seen in the theca layers of the advanced atretic follicles.

At the final stage of follicular atresia, phagocytotic macrophages invaded into the antra of the follicles from clefts in the BM, and numbers of apoptotic granulosa cells decreased as a result of phagocytosis by the infiltrating macrophages (Fig. 1E-center). The discontinuous BM was collapsed radially (Fig. 1E-left). Weak staining for laminin was demonstrated in the theca layers of the atretic follicles, indicating blood supply for follicles decreased at the advanced and final stages of atresia.

Discussion

The ECM components in BM control key events in basic cellular functions, i.e. cell morphology, cell polarization, cell movement, cell proliferation, growth and differentiation, cell survival, and programmed cell death [31–35]. Moments after a suspended cell makes contact with ECM components, there is a tremendous increase in protein and RNA synthesis in the cell, showing that signals generated by cell-ECM contact are rapidly transduced to changes in gene expression [26, 31]. Therefore, the formation of cell-ECM contacts can have a rapid anabolic effect on cells, and can provide an important regulatory step in basic cellular processes [26]. Cells are generally separated into two groups according to the nature of their interactions with ECM components. Epithelial/endothelial cells form cell layers that are organized into structured tissues, while mesenchymal cells tend to be isolated and migratory. Epithelial cells, such as follicular granulosa cells in the ovaries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associate with well-structured connective tissue components, especially with BM. The BM is comprised of specialized ECM structures and is highly enriched with glycoproteins, laminin, fibronectin and type IV collagen. Other structural molecules involved in cell-ECM adhesion such as vitronectin, tenascin and heparan sulfate proteoglycan are also present [26]. The BM typically forms a continuous layer that provides an attachment substratum at the basal surface of epithelial cells. BM proteins when used as a substrate for cell culture *in vitro*, can promote cell attachment, spreading and polarization, and stimulate cell proliferation and differentiation [31–35]. In atretic follicles of porcine ovaries, characteristic changes in sugar chains of glycoproteins and/or proteoglycan in granulosa cells and follicular BM were demonstrated by lectin histochemical and lectin blotting techniques [23, 24]. Up-regulation of α2,6-sialyltransferase mRNA was detected in apoptotic granulosa cells, and this increased the amounts of glycoconjugates containing α2,6-linked sialic acid residue proteoglycans [25]. Such glycobiological changes may play key roles in detachment of apoptotic granulosa cells and phagocytosis by neighboring granulosa cells and invading macrophage in atretic follicles [43]. The present findings show that before the BM breakdown stage apoptotic granulosa cells were mainly located on the inner surface of the granulosa cell layer, but no phagocytotic macrophages were detected, and that after the BM breakdown stage most granulosa cells were positive for TUNEL staining and many invading macrophages were seen in the follicular antrum space. These findings support those of previous electron microscopic studies [43].

Separation of epithelial cells from the BM deprives the detached cells of ECM-derived signals, and apoptosis occurs [44]. In the skin epithelial cells, apoptosis may

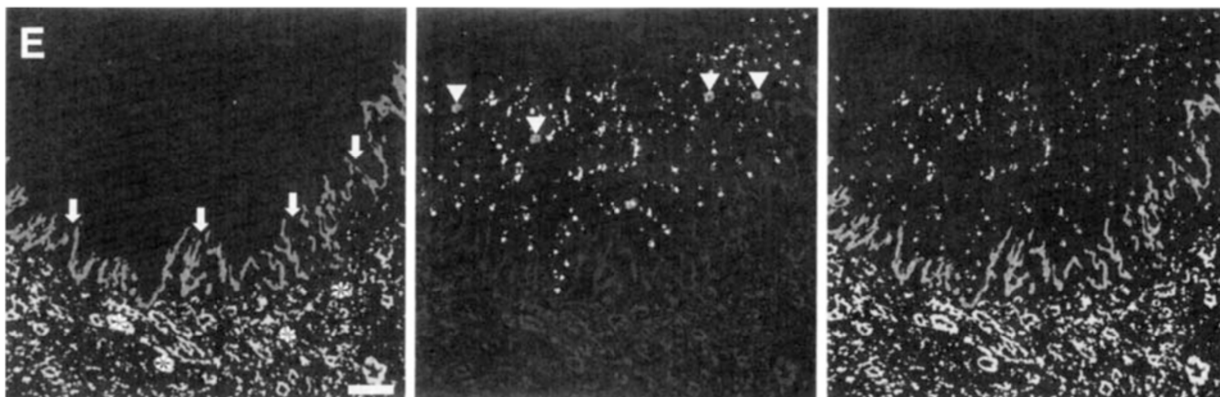

Fig. 1. Composite confocal images of antral follicles of porcine ovaries. Healthy follicle (A) and the follicles at the early (B and C) and late (D and E) stages of atresia were cut. Serial sections were histochemically stained for laminin, which is a major ECM component of the basement membrane (BM), and for TUNEL, which is an *in situ* detection method of apoptosis by the histochemical DNA 3′ end-labeling technique. The follicles were optically sectioned at 0.5 μm and twenty serial images were generated using the confocal microscope. Serial images of laminin (left) and TUNEL staining (center) were overlapped using a computer system (right). Bars indicate 200 μm. In healthy follicles, strong positive staining for laminin was observed along the intact BM (arrows) which had no shrinkage, cleavage or laceration (A-left). Strong staining for laminin indicated high density of capillary blood vessels (asterisks) in the theca layer. No TUNEL-positive oocytes, granulosa cells, cumulus cells or thecal cells were seen (A-center). At the early stage of atresia, no obvious changes in the laminin staining were noted (B-left). Oocyte-cumulus cell complex was preserved and was negative for TUNEL staining, but scattered single TUNEL-positive granulosa cells (arrows) were observed on the inner side of the granulosa cell layers (B-center). Neighboring granulosa cells continued to undergo apoptosis (C-center), and weak and discontinuous (arrows) staining of laminin was seen in the follicular BM (C-left). At the advanced stage of atresia, weak, diffuse and fragmented staining for laminin with shrinkage, cleavage and laceration was observed in the BM (arrows) (D-left). Oocyte-cumulus cell complex or oocytes without cumulus cells, or thecal cells were negative for TUNEL, and many TUNEL-positive granulosa cells suspended in follicular fluid were seen (D-center). At the final stage of atresia, phagocytotic macrophages (arrowheads) invaded into the antral space from cleavages of the BM (arrows). Weak and discontinuous staining for laminin was radially collapsed, and weak fluorescence was also demonstrated in the theca layers, indicating blood supply for follicles decreased at the advanced and final stages of atresia (E-left). TUNEL-positive granulosa cells decreased as a result of phagocytosis by the infiltrating macrophages (E-center).

be regulated by expression of bcl-2, an inhibitor of apoptosis, by cells in the basal layer of the skin epithelium but not in more superficial layers [45]. Apoptosis is a poorly understood cellular phenomenon characterized by endonuclease-mediated intranucleosomal cleavage of DNA [8]. In the mammalian ovaries, apoptosis in granulosa cells, follicular epithelial cells, may play a key role in selective follicular atresia [4–7]. We have been studying the mechanisms of selective atresia of porcine ovarian follicles [9–25, 43]. Briefly, porcine ovary samples were prepared for histochemical and ultrastructural analyses. *In situ* analysis of DNA fragmentation was performed on histological sections of follicles using the TUNEL method. No apoptotic cells were observed in healthy follicles. In atretic follicles, apoptotic TUNEL staining was seen in scattered granulosa cells located on the inner surface of the follicular wall, but not in cumulus cells, internal or external theca cells, or oocytes. Nuclear condensation, a typical feature of apoptosis was seen only in scattered granulosa cells. The neutral Ca^{2+}/Mg^{2+}-dependent endonuclease is involved in granulosa cell apoptosis. No endonuclease activity was detected in cumulus cells. An IgM monoclonal antibody (named PFG-1) capable of inducing granulosa cell apoptosis was then introduced against granulosa cells prepared from healthy antral follicles. Two-dimensional Western blotting analysis revealed that PFG-1 specifically recognized a cell-membrane protein (PFG-1 antigen, 55 kD, pI 5.9). PFG-1 immunohistochemically reacted with granulosa cells of healthy follicles but not those of atretic follicles. When the isolated granulosa cells prepared from healthy follicles were cultured in medium containing 0.1 μg/ml of PFG-1, the cells underwent apoptosis. The PFG-1 antigen, which is considered to be a novel cell death receptor, is different from the known apoptosis-mediating receptors Fas antigen or tumor necrosis factor receptor 1. Thus, we have demonstrated that the degeneration of atretic follicles in the ovaries can be explained, at least in part, by receptor-mediated apoptotic death of granulosa and theca interna cells, and that apoptosis occurs only in granulosa cells but not cumulus cells in the atretic Graafian follicles [9–25].

However, the interaction between granulosa cells and ECMs of follicular BM has not been studied. In the present study, we histochemically demonstrated the relationship between granulosa cell apoptosis and follicular BM degeneration during follicular atresia in the porcine ovaries. The healthy (TUNEL-negative) granulosa cells in the healthy follicles remained in contact with the BM and/or neighboring granulosa cells. In the atretic follicles, the granulosa cells which lost contact with BM or each other and did not have a shrunken nucleus were positive for TUNEL staining at the onset of apoptosis. Similar to skin epithelial cells *in vitro*, which showed high tyrosine kinase activity after losing contact with the BM and the onset of apoptosis [45], high tyrosine kinase activities were detected in apoptotic granulosa cells and were considered to be playing prominent roles in the regulation of granulosa cell apoptosis during ovarian follicular atresia of pigs [19, 21].

In the porcine ovaries, no apoptosis occurs in granulosa cells which maintain close contact with the morphologically intact BM, or in cumulus cells which maintain contact with the morphologically normal oocyte until the advanced stage of atresia. Such attachment to the BM and the oocyte is considered to reduce apoptosis in the granulosa cells of pig ovarian follicles. In contrast, our preliminary studies showed that the granulosa cells which were in contact with the BM began to undergo apoptosis at the earliest stage of follicular atresia in the cow (data not shown). In the ovaries of rodents, apoptotic granulosa cells are scattered randomly in the granulosa cell layer [46–48]. Thus, there are species-specific differences in the mechanism of granulosa cell apoptosis-ECM interaction and in the mechanism of induction of apoptosis in the granulosa cells. Further comparative studies are currently in progress to elucidate the species-specific mechanism of atresia and the process of follicular atresia including the death of the oocyte.

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