Monoclonal Antibodies against Pig Ovarian Follicular Granulosa Cells Induce Apoptotic Cell Death in Cultured Granulosa Cells

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ABSTRACT. Two monoclonal antibodies capable of inducing granulosa cell apoptosis were produced against granulosa cells prepared from antral follicles of pig ovaries. The healthy follicles, 4–5 mm in diameter, were dissected from the ovaries of gilts, and then granulosa cells were isolated. BALB/c female mice were immunized with the isolated granulosa cells. Antibodies against the granulosa cells were detected by immunofluorescent staining using frozen ovarian sections. The isolated spleen cells prepared from immunized mice producing antibodies against the granulosa cells were fused with Sp2/O-Ag14 mouse myeloma cells by standard hybridization techniques. Two hybridoma clones, PFG-1 and PFG-2, which produced specific IgM antibodies against granulosa cells were selected. Western blotting analysis revealed that PFG-1 and PFG-2 antibodies specifically recognized cell-membrane proteins with molecular weights of 55 and 70 kD and isoelectric points of 5.9 and 5.4, respectively. The monoclonal antibodies immunohistochemically reacted with granulosa cells of healthy follicles. When the isolated granulosa cells prepared from healthy follicles were cultured in medium containing 0.1 or 10 μg/m*l* PFG-1 or PFG-2 antibodies, respectively, the cells underwent apoptosis as determined by nuclear morphology, DNA electrophoresis and flow cytometric analysis. In conclusion, these two monoclonal antibodies against granulosa cells have cell-killing activity in cultured granulosa cells.—KEY WORDS: apoptosis, follicular granulosa cell, monoclonal antibody, swine ovary.

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Apoptotic cell death of granulosa cells of rabbit Graafian follicles with atresia was first observed in 1885 by Flemming [12] who called it chromatolysis. Recent studies of pig follicular atresia have shown that the degeneration of the atretic follicles can be explained in part by apoptosis of granulosa cells [14, 15, 20, 25–27, 34–40, 44]. When the cells undergo apoptosis, their chromatin DNA is degraded at internucleosomal sites by endonucleases, giving rise to the characteristic 'ladder' pattern of oligonucleosomal-sized bands corresponding to multiples of about 180-bp [1, 4, 5, 10, 11, 23, 44]. From the results of our previous studies [25–27], granulosa cells scattered on the inner surface of the follicular wall appear to be the first to undergo apoptosis, and detachment and degeneration of the granulosa cell layer and fragmentation of basal lamina occur in the advanced stages of follicular atresia. However, no apoptosis occurs in cumulus cells during porcine follicular atresia before phagocytic cell invasion into the follicular antrum. In pig ovaries, the neutral Ca^{2+}/Mg^{2+}-dependent endonuclease, and not the neutral Ca^{2+}-dependent endonuclease, neutral Mg^{2+}-dependent endonuclease or acidic cation-independent endonuclease, is involved in granulosa cell apoptosis of the atretic antral follicles [26]. Moreover, no endonuclease activity was detected in cumulus cells prepared from the same atretic follicles. These findings suggest a lack of apoptotic cell death in cumulus cells in pig atretic follicles [25–27].

Apoptosis is the result of many stimuli which act through many signal transduction pathways culminating in the activation of endonucleases [9–11]. Apoptotic stimuli and intracellular signal transduction pathways involved in granulosa cell apoptosis remain to be determined. Therefore, understanding of the events that trigger activation of the endonucleases is essential to elucidate the pathways upstream of apoptosis. The aim of the present study was to produce monoclonal antibodies which recognize cell-surface antigens of pig granulosa cells and induce apoptosis to the granulosa cells. Such antibodies are considered to be a good probe for studying the intracellular pathway of granulosa cell apoptosis in pig atretic follicles.

MATERIALS AND METHODS

Preparation of follicular granulosa cells: Granulosa cells from healthy antral follicles of pig ovaries were prepared as described previously [25–27]. Briefly, the ovaries obtained from gilts at a slaughterhouse were used. Individual preovulatory antral follicles, 4–5 mm in diameter, were dissected in Medium 199 (Gibco BRL, Grand Island, NY, U.S.A.) with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Gibco) and 0.1% polyvinylalcohol (Wako Pure Chemical, Osaka, Japan). Under a surgical dissecting microscope (SZ11, Olympus, Tokyo, Japan), follicles were classified as morphologically healthy or atretic [7, 25]. The healthy follicles were punctured over a 1.5-m*l* microcentrifuge tube to collect follicular fluid. Fluid from each follicle was separated by

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centrifugation, and then estradiol-17β and progesterone levels were measured by radioimmunoassay (RIA) as described below to confirm the classification of the follicles [2, 6, 25–28]. Then, the granulosa cell layers were removed from the follicles in 25 mM HEPES-buffered Medium 199 containing 80 mg/m*l* kanamycin sulfate (Sigma Chemical, St. Louis, MO, U.S.A.) (HEPES-199). After washing with HEPES-199, the cell layers were incubated in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (Gibco) containing 10 mM ethylenediaminetetraacetic acid disodium salt (EDTA; Gibco) and 6.8 mM ethyleneglycol-bis-tetraacetic acid (EGTA; Sigma) for 15 min at room temperature (RT, 22–25°C), and then granulosa cells were isolated by pipetting. The isolated cells were washed twice in HEPES-199 containing 10% fetal calf serum (FCS; Gibco) (HEPES-199-FCS) by centrifugation. Cell number was counted using a hemocytometer plate, and cell viability was determined by trypan blue exclusion method. The isolated cells with viability of more than 95%, were used as antigens for immunization and as target cells in cell-killing activity assay as described below.

RIA of steroid hormones in the follicular fluid: Estradiol-17β and progesterone levels in follicular fluid diluted 100-fold with Medium 199 were quantified using [^{125}I]-RIA kits (Bio-Mérieux, Marcy-l'Etolle, France) as described previously [25–27]. In pigs, the progesterone/estradiol-17β ratio of follicular fluid in each follicle provides a good index of follicular atresia [2, 6, 25–28]. When the progesterone/estradiol-17β ratio of follicular fluid was less than 15, the follicle was classified as healthy according to our previous findings [25–28]. In the present study, the isolated granulosa cells (more than 95% viability estimated by trypan blue exclusion) prepared from healthy follicles categorized by progesterone/estradiol-17β ratio were used for immunization, cell-killing assay, observation of nuclear morphology, and DNA electrophoresis.

Preparation of monoclonal antibodies: Female BALB/c mice aged 8 weeks were purchased from Clea Japan (Tokyo, Japan), and housed in a controlled environment (lights on between 7:00 and 19:00; temperature 22 ± 2°C; humidity 70 ± 5%). All animals received humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" (Kyoto University Animal Care Committee according to NIH #86–23; revised 1985). Mice were immunized intraperitoneally with the isolated granulosa cells (10^6 cells/mouse, once a week) prepared from healthy follicles. Immunization was repeated four to six times. Antibody production was assessed by immunofluorescent staining as described below. Five days after the last immunization, fewer cells (10^4 cells/mouse) were injected intraperitoneally as a boost injection. Three days after boosting, the spleen cells from immunized mice, which produced anti-granulosa cell antibodies, were fused with Sp2/O-Ag14 mouse myeloma cells by standard hybridization techniques using the polyethylene glycol 1500 (Boehringer Mannheim, Indianapolis, IN, U.S.A.) [31, 43]. After washing with Iscove's modified Dulbecco's medium (IMDM; Gibco), the fused cells were suspended in IMDM containing 10% FCS and 1 unit/m*l* of interleukin 6 (Sigma) (IMDM-FCS-IL), and plated in 96-well cell culture plates (Falcon 3872, Becton Dickinson, Lincoln Park, NJ, U.S.A.). After 24 hr incubation at 37°C in 5% CO_2, HAT medium (1×10^{-4} M hypoxanthine, 0.8×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine; Boehringer Mannheim) was added to each well. Every 3 days, half of the culture medium was removed and replaced with IMDM-FCS-IL containing HAT medium (IMDM-FCS-IL-HAT). After three changes of IMDM-FCS-IL-HAT, the hybridoma cells were incubated in IMDM-FCS-IL containing HT medium (1×10^{-4} M hypoxanthine and 1.6×10^{-5} M thymidine; Boehringer Mannheim) (IMDM-FCS-IL-HT). Thereafter, half of the culture supernatant was replaced with fresh IMDM-FCS-IL-HT every 3 days. As only IgM antibodies against Fas-antigen had cell-killing activity [43], IgM antibody-producing hybridomas were selected during cell culture in IMDM-FCS-IL-HT. Antibody class was determined by an enzyme-linked immunosorbent assay (ELISA) as described below. Then, the hybridoma cells producing IgM antibodies against the granulosa cells were screened by immunofluorescent staining. The hybridoma cells, which produced antibodies with granulosa cell-killing activity assessed as described below, were selected. Finally, two hybridoma cells, named PFG-1 and PFG-2, were cloned two times by limiting dilution.

Female BALB/c mice aged 4 weeks were received intraperitoneally with 0.5 m*l*/mouse of pristane (2, 6, 10, 14-tetramethylpentadecane; Aldrich Chemical, Milwaukee, WI, U.S.A.). One month after pristane injection, PFG-1 or PFG-2 hybridoma cells (1×10^7 cells/mouse) were injected intraperitoneally. Within two weeks after injection, ascites were obtained from the mice and dialyzed against phosphate buffered saline (PBS), and then IgM-rich fractions were precipitated with 50% saturated ammonium sulfate (Wako). These crude antibodies were applied onto a hydroxylapatite column (Asahi Optical Inc., Tokyo, Japan). The IgM fractions were eluted with a 10–400 mM gradient of sodium phosphate, pH 7.4, by 10A-HPLC system (Simadzu, Kyoto, Japan). Eluted antibodies concentrated in ultrafiltration cells with XM50 ultrafiltration membrane (Amicon, Beverly, MA, U.S.A.) were heat-inactivated for 45 min at 56°C and sterilized by filtration through 0.22-μm Millipore filters (Marlborough, MA, U.S.A.). The optical densities at 280 nm of the antibody solutions were measured with a spectrophotometer (UV-1200, Simadzu) to determine protein concentration [8].

Immunofluorescent staining: Pig ovaries were cut into small pieces, put on filter paper, mounted in OCT compound (Miles, Elkhart, IN, U.S.A.), and then frozen in liquid nitrogen. Serial sections (5 μm thick) were cut on a cryostat, mounted on glass slides precoated with 3-amino-propyltriethoxy-silane (Aldrich), and fixed with precooled acetone for 5 min at −80°C. After washing with PBS, the sections were preincubated with 1% normal goat serum diluted with PBS containing 1% bovine serum albumin

(BSA; Sigma) (PBS-BSA) for 2 hr at RT. The slides were washed with PBS containing 0.05% Tween 20 (Sigma) (PBS-Tw), and then the sections were incubated with mouse serum (1/10 dilution with PBS-BSA), hybridoma culture supernatant (1/100 dilution with PBS-BSA) or purified monoclonal antibody (1/400 dilution with PBS-BSA) for 18 hr at 4°C. As a negative control, adjoining sections were incubated with diluted normal mouse serum (1/10 dilution with PBS-BSA) or mouse IgM (1 μg/m*l* in PBS-BSA; Sigma). After washing with PBS-Tw, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM antibody (1/400 dilution with PBS-BSA; American Qualex, La Mirada, CA, U.S.A.) for 2 hr at RT. After washing with PBS-Tw, the sections were examined with a fluorescence microscope (BX50, Olympus) or a confocal laser scanning microscope (LSM 410, Carl Zeiss, Oberkochen, Germany).

ELISA for determination of antibody class: Falcon 3915-ELISA plates (Becton Dickinson) were filled with 50 μ*l*/well of goat anti-mouse IgM antibody (1/800 dilution with PBS; American Qualex), incubated overnight at 4°C, blocked with PBS-BSA containing 3% skimmed milk for 1 hr at RT, and then rinsed with PBS-Tw. After 1 hr incubation with 50 μ*l*/well of each hybridoma culture supernatant at RT, the plates were washed with PBS-Tw, and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgM antibody (1/400 dilution with PBS-BSA; American Qualex) for 1 hr at RT. After washing with PBS, the HRP-reaction was visualized by addition of 0.4 mg/m*l* *o*-phenylenediamine dihydrochloride (Sigma) in 0.1 M phosphate-citrate buffer, pH. 5.6, containing 0.02% H_2O_2. The color development was stopped with 4 N H_2SO_4 solution. Optical density measurement was made at 492 nm using a microplate reader (M450, Bio-Rad, Hercules, CA, U.S.A.) [43].

Cell-killing activity assay: The trypan blue exclusion method was used to determine the cell-killing activity [43]. Briefly, the isolated granulosa cells (10^6 cells/m*l*) in 96-well culture plates were cultured in HEPES-199-FCS containing 10% each hybridoma culture supernatant or monoclonal antibody (0.01–100 μg/m*l* of PFG-1 or PFG-2) for 1 to 6 hr at 37°C. As a negative control, the granulosa cells were cultured in HEPES-199-FCS without any additive. The cells were resuspended by pipetting and stained by trypan blue, and then viable cells were quantified by microscopy (BX50, Olympus).

Nuclear morphology: To observe the morphology of the nuclei, the isolated granulosa cells (10^6 cells/m*l*) on 13-mm plastic coverslips (Nunc 174650, Japan Intermed, Tokyo, Japan) placed in the wells of 24-well culture plates (Falcon 3047, Becton Dickinson) were cultured in HEPES-199-FCS containing monoclonal antibody (0.01 to 100 μg/m*l* PFG-1 or PFG-2 antibody) for 1 to 6 hr at 37°C. As a negative control, the cells were cultured in HEPES-199-FCS without any additive. The cells were fixed with cold acetone for 5 min, washed with PBS, and stained with Hoechst 33258 (4 μg/m*l* in distilled water; Molecular Probes, Eugene, OR, U.S.A.) for 5 min. After washing with PBS, the cells were examined with a fluorescence microscope.

DNA electrophoresis: The isolated granulosa cells were cultured in HEPES-199-FCS containing monoclonal antibody (0.1 or 10 μg/m*l* PFG-1 or PFG-2, respectively) for 3 hr at 37°C, resuspended by pipetting, and washed with modified HEPES-199-FCS. DNA fractions were separated from the granulosa cells by centrifugation at 9,000 g for 20 min at 4°C [25]. DNA contents were determined by 4',6-diamidino-2-phenylindole dihydrochloride-fluorescence assay using calf thymus DNA (Sigma) as a standard [22], and then DNA samples were electrophoresed in 2% agarose gels with 40 mM Tris-acetate (pH 8.1) containing 2 mM EDTA, 18 mM NaCl, and 10 μg/m*l* ethidium bromide at 60 V for 90 min. Gels were photographed on an ultraviolet transilluminator [25].

Flow cytometric analysis: Degraded DNA in granulosa cells was determined by flow cytometry [14]. Briefly, the isolated granulosa cells (10^6 cells/m*l*) were cultured in HEPES-199-FCS containing monoclonal antibody (0.1 or 10 μg/m*l* PFG-1 or PFG-2, respectively) for 3 hr at 37°C. They were resuspended by pipetting, washed with modified HEPES-199-FCS, and fixed in 80% ethanol at 4°C for 60 min. After washing with PBS, the cells were stained with 50 μg/m*l* propidium iodide (Sigma) in PBS containing 0.1% Triton X-100 (Sigma), 0.1 mM EDTA, and 2.5 U/m*l* RNase (Worthington Biochemical, Freehold, NJ, U.S.A.), and filtered through 35 μm nylon mesh (Wako). Determination of the percentage of cells with degraded DNA and of cell cycle distributions for single cells were carried out using a flow cytometer (Epics-MCL, Coulter Co., Hialeah, FL, U.S.A.). Cell cycle analysis was carried out with the Multicycle program (Phoenix Flow System, Phoenix, AZ, U.S.A.) using an IBM computer.

Western blotting analysis: For sodium dodecyl sulfate (SDS)-polyacryl-amide-gel electrophoresis (PAGE), cell membrane samples of the isolated granulosa cells prepared according to the method of Thom *et al*. [41] were electrophoresed through to 4% polyacrylamide slab gels [43]. Then, the cell membrane samples were separated by SDS-PAGE. After electrophoresis, separated protein bands in a gel were stained with Coomassie brilliant blue solution (Wako). For two-dimensional PAGE (2D-PAGE), the granulosa cell samples were solubilized in 2% Triton X-100 containing 9.5 M urea, 5% 2-mercaptoethanol, and 2% carrier ampholyte (pH 3.5–10; Pharmacia Biotech, Uppsala, Sweden). 2D-PAGE was performed according to the method of O'Farrell [29]. Briefly, the cell membrane lysates were separated by isoelectric focusing on 4% polyacrylamide cylindrical gels, separated by SDS-PAGE using 7.5% polyacrylamide slab gels, and then separated protein spots in the gels were detected by silver staining (Wako). After electrophoresis, the proteins were transferred onto a nitrocellulose membranes (Wako), which then were preincubated with 3% skimmed milk in PBS for 1 hr at 37°C. After washing with PBS-Tw, the membranes were incubated with monoclonal antibodies at 10 μg/m*l*, and

immunological reaction products were visualized with an ABC staining kit (Vector Lab., Burlingame, CA, U.S.A.) according to the manufacturer's instructions.

RESULTS

Hybridoma selection: Two hybridoma cells, named PFG-1 and PFG-2, producing IgM antibodies to pig granulosa cell surface components with associated granulosa cell-killing activity were selected and cloned. The characteristics of the monoclonal antibodies produced by these two hybridoma cell clones were as follows.

Characterization of granulosa cell antigens by Western blotting: The antigens on healthy granulosa cells were characterized by Western blotting. Plasma membrane fraction of the healthy granulosa cells was subjected to PAGE in the presence of SDS. After electrophoresis, the proteins were transferred on nitrocellulose sheets and the granulosa cell antigens were analyzed. A specific band with a molecular weight of 55 kD was observed on the nitrocellulose filters treated with PFG-1 antibody (Fig. 1, lane 1). When the nitrocellulose filters were treated with PFG-2 antibody, a specific band with a molecular weight of 70 kD was observed (Fig. 1, lane 2). Then, 2D-PAGE analysis was performed. Figure 2A shows the 2D-PAGE plate, and protein spots in the slab-gel were detected by silver staining. Figures 2B and C show the results of Western blotting analysis on nitrocellulose membranes. The two specific spots of PFG-1 and PFG-2 antibodies were also observed when SDS-PAGE was carried out in the presence of 2-mercaptoethanol. Isoelectric points of these cell-surface proteins with molecular weights of 55 and 70 kD were 5.9 and 5.4, respectively (Figs. 2B and C, respectively).

Immunohistological characterization of monoclonal antibodies: Indirect immunofluorescence analysis on cryostat ovarian sections was used to determine the target specificity of the monoclonal antibodies. PFG-1 antibody was strongly reactive with granulosa cells of healthy follicles (Fig. 3A). PFG-2 antibody showed weak fluorescent staining on granulosa cells (Fig. 3B). These antibodies did not label theca interna or externa cells, basement membrane, or ovarian stroma cells (Fig. 3A and B). Moreover, these antibodies showed neither specific binding to pig stomach, small intestine, large intestine, liver, pancreas, lung, kidney, testis, adrenal grand, heart, spleen, nor brain (data not shown).

Granulosa cell apoptosis mediated by the monoclonal antibodies: In the screening procedure, granulosa cell-killing activities of the monoclonal antibodies were assessed by cell-killing activity assay as described above (data not shown). Granulosa cell apoptosis mediated by the selected antibodies, PFG-1 and PFG-2, was determined by assessment of nuclear morphology, DNA electrophoretic analysis, and cell cycle analysis by flow cytometry. The isolated granulosa cells were co-cultured with concentrations varying from 0.01 to 100 μg/m*l* of PFG-1 or PFG-2, for 1 to 6 hr at 37°C, and then the morphology of the nuclei was observed by staining with Hoechst 33258 under a fluorescent microscope. Figure 4A showed the isolated granulosa cells were cultured without any additive for 3 hr at 37°C. Weak round fluorescence of healthy granulosa cell nuclei was observed. When the isolated granulosa cells were co-cultured with at least 0.1 μg/m*l* PFG-1 for 3 hr at 37°C, many small condensed fluorescent signals representing apoptotic bodies (morphological hallmark of apoptotic cell death) were observed (Fig. 4B). Such apoptotic bodies were also observed in isolated cells co-cultured with at least 10 μg/m*l* PFG-2 for 3 hr at 37°C (Fig. 4C).

After co-incubation with PFG-1 and PFG-2, DNA samples of these isolated granulosa cells were electrophoresed in 2% agarose gels, and the DNA displayed a ladder pattern (biochemical hallmark of apoptosis) (Fig. 5, lanes 2 and 3, respectively). However, DNA samples of the isolated cells co-cultured without any additive displayed no such pattern on electrophoresis (Fig. 5, lane 1).

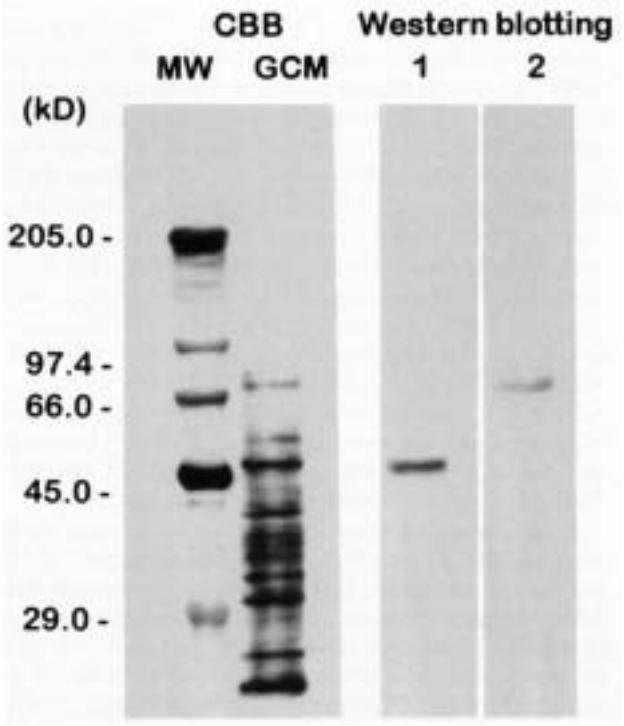


Fig. 1. Representative results of Western blotting analysis of granulosa cell-membrane antigens recognized by PFG-1 and PFG-2. Granulosa cell membrane (GCM) fractions were prepared from healthy granulosa cells, and separated by SDS-PAGE using 4% polyacrylamide slab gels. Separated protein bands in the gels were stained with Coomassie brilliant blue (CBB) solution (CBB-GCM). After electrophoresis, the proteins were transferred onto nitrocellulose sheets, and the granulosa cell antigens were visualized by immunostaining. Specific bands with molecular weights of 55 and 70 kD were identified on the nitrocellulose filters treated with PFG-1 (lane 1) and PFG-2 (lane 2) antibodies, respectively. Molecular weight markers (MW) stained with CBB are shown in the left lane (CBB-MW).

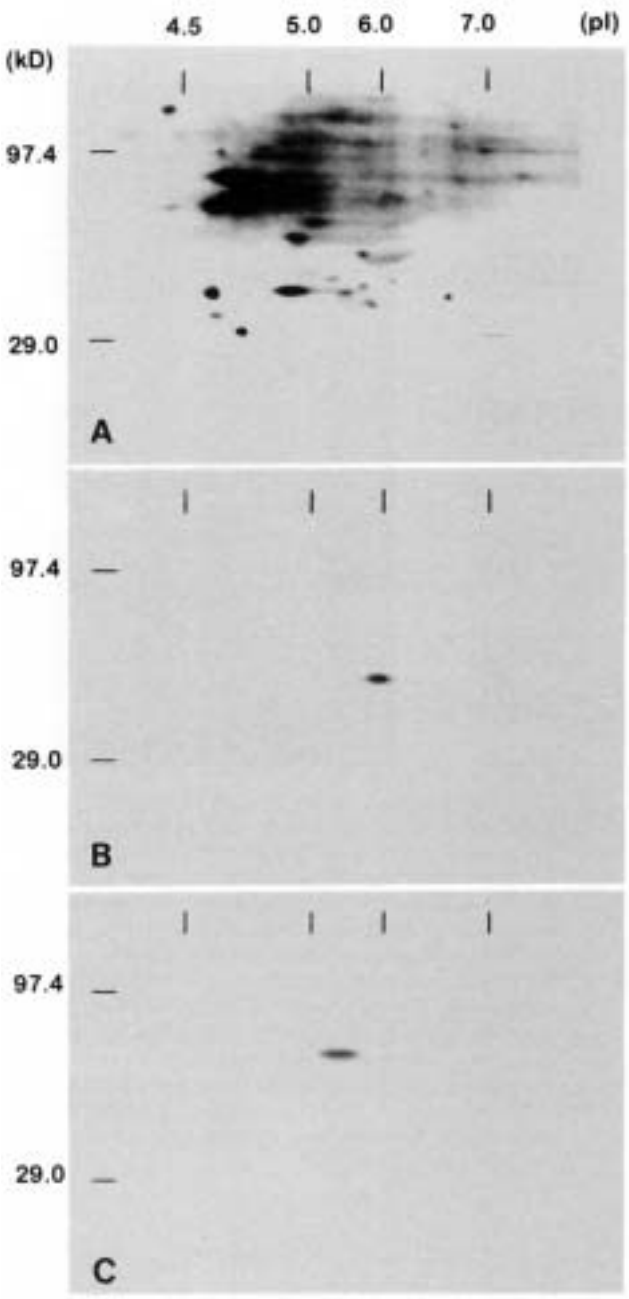


Fig. 2. Representative results of two-dimensional Western blotting analysis of granulosa cell-membrane antigens recognized by PFG-1 and PFG-2. Granulosa cell membrane fractions were prepared from the healthy granulosa cells, and separated by two-dimensional PAGE (2D-PAGE). Separated protein spots in gels were detected by silver staining (A). After electrophoresis, the proteins were transferred onto a nitrocellulose sheets and the granulosa cell antigens were visualized by PFG-1 (B) and PFG-2 (C). One specific spot (molecular weight of 55 kD and isoelectric point of 5.9) reacted with PFG-1 antibody. PFG-2 antibody also reacted with one s.pot (molecular weight of 70 kD and isoelectric point of 5.4).

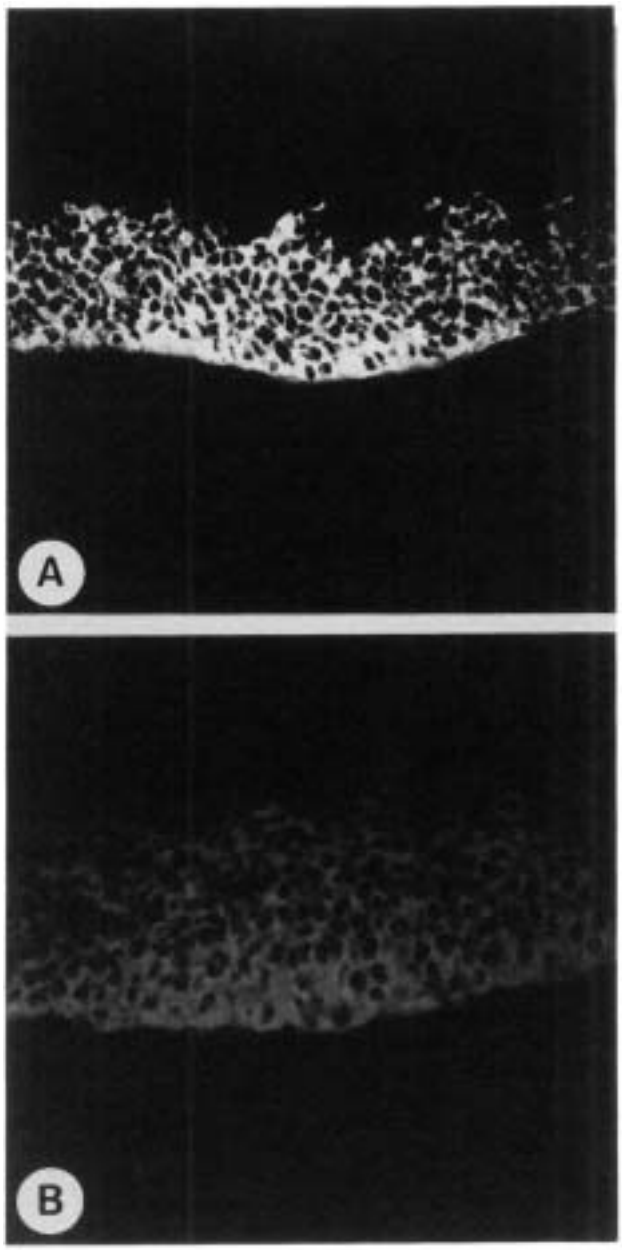


Fig. 3. Composite confocal images of ovarian follicles from pigs. Frozen sections were stained with PFG-1 (A) and PFG-2 (B) antibodies and with FITC-conjugated goat anti-mouse IgM to show the distribution of granulosa cell membrane antigens. The follicles were optically sectioned at 0.5 μm and five serial images were generated using the confocal microscope. Granulosa cells of healthy follicles were strongly stained with PFG-1 antibody, but they were weakly stained with PFG-2 antibody. No fluorescent staining of theca interna or externa cells, basement membrane, or ovarian stroma cells was observed.

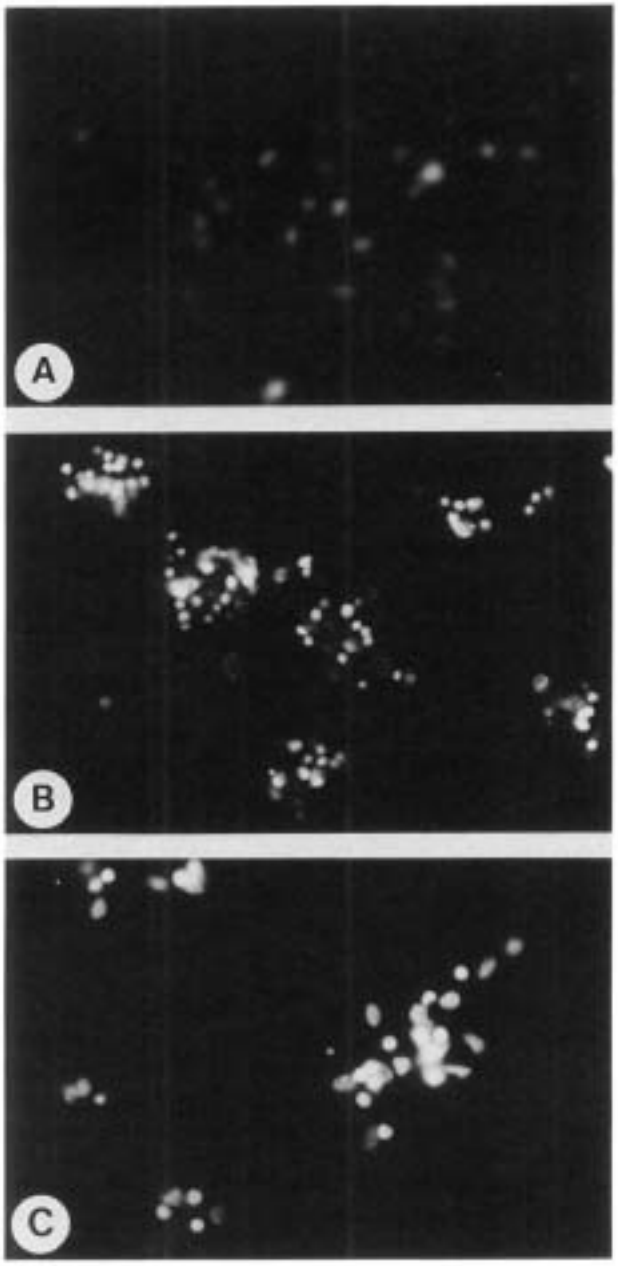


Fig. 4. Fluorescence photomicrographs of the isolated granulosa cells stained with Hoechst 33258 for observation of nuclear morphology. The granulosa cells were cultured with 0.1 μg/ml PFG-1 (B) or 10 μg/ml PFG-2 (C) for 3 hr at 37°C. In controls, the cells were cultured without any additive (A). Weak round fluorescent signals of healthy granulosa cell nuclei were observed in controls. In PFG-1-treated cells, many small condensed bodies (apoptotic bodies) were observed. Such apoptotic bodies were also observed in PFG-2-treated cells.

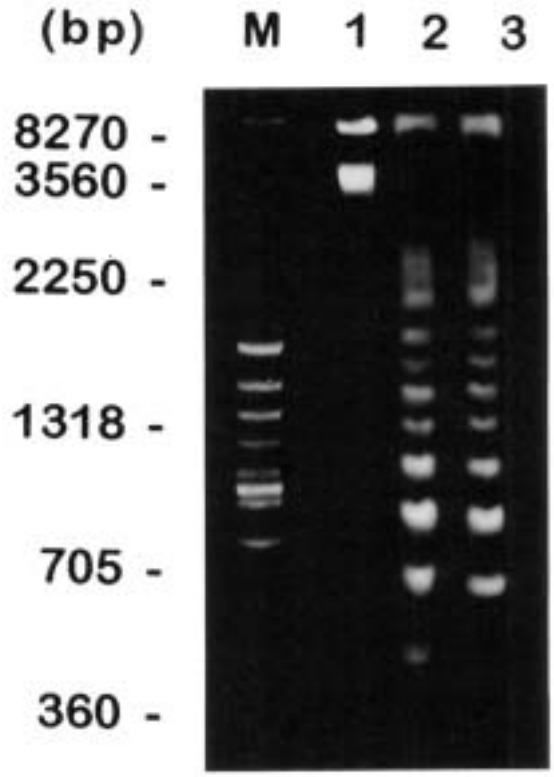
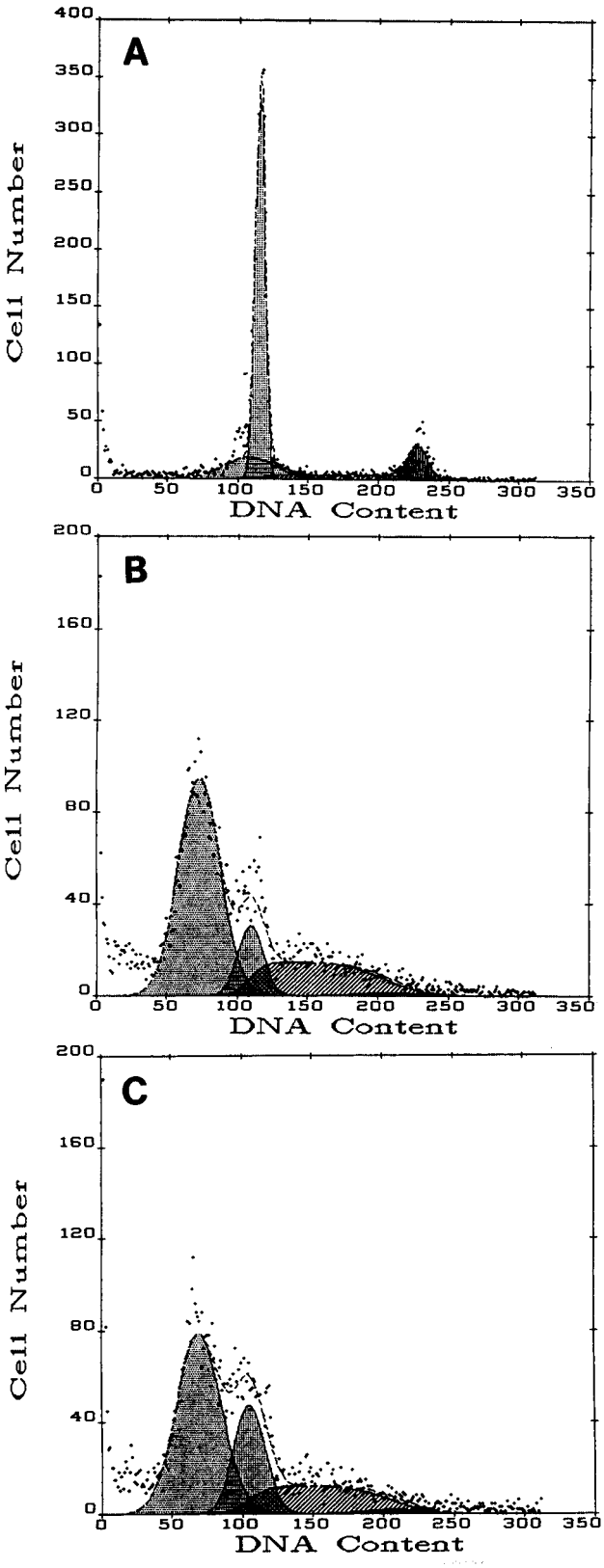


Fig. 5. Electrophoretic analysis of DNA fragments in DNA samples prepared from isolated granulosa cells. The isolated granulosa cells were cultured with 0.1 or 10 μg/ml PFG-1 or PFG-2, respectively, for 3 hr at 37°C. Equal amounts of DNA samples (10 μg/lane) prepared from the granulosa cells were electrophoresed in 2% agarose gels, and then DNA was visualized by ethidium bromide staining under ultraviolet illumination. The DNA from PFG-1- and PFG-2-treated granulosa cells displayed a ladder pattern, a hallmark of apoptosis, on electrophoresis (lane 2 and 3, respectively). DNA samples from control cells displayed no such DNA ladder formation (lane 1). Molecular weight markers (lane M) are indicated on the left side of the Figure.

The isolated granulosa cells were co-cultured with 0.1 or 10 μg/ml PFG-1 or PFG-2, respectively, for 3 hr at 37°C, and then the percentages of cells with degraded DNA were determined by flow cytometric analysis. No degraded DNA was observed in cells co-cultured without any additive (Fig. 6A). However, high percentages of degraded DNA, representing apoptotic cell death, were demonstrated in cells co-cultured with PFG-1 and PFG-2 (Figs. 6B and C, respectively).

DISCUSSION

In the present study, two hybridoma clones, PFG-1 and PFG-2, were selected. These clones produced monoclonal IgM antibodies against cell-membrane proteins of granulosa cells. Western blotting analysis revealed that the PFG-1 antibody specifically recognized a cell membrane protein with a molecular weight of 55 kD and isoelectric point of



5.9, and that the PFG-2 antibody identified a membranous protein with a molecular weight of 70 kD and isoelectric point of 5.4. Moreover, these antibodies induced apoptotic cell death in cultured granulosa cells prepared from healthy antral follicles of pig ovaries.

In mammalian ovaries, more than 99.9% of the follicles undergo the degenerative change known as atresia at varying stages of follicular development [19, 42]. Many studies of follicular atresia have been performed, and they have revealed the morphological and biochemical characteristics of the atretic follicles which include detachment and degeneration of the granulosa cell layer [17], fragmentation of the basal lamina of the follicles [3], reduced DNA synthesis [13, 18], decreased estrogen production [2, 6, 15, 28], etc. Recent findings have suggested that apoptosis, originally described in 1972 by Kerr *et al.* [24], is the mechanism underlying ovarian follicular atresia [14, 15, 20, 25-27, 34–40, 44]. Our previous studies of the process of follicular atresia in pig ovaries [25–27] revealed that granulosa cells scattered on the inner surface of the follicular wall undergo apoptosis in the earliest stage, and detachment and degeneration of the granulosa cell layer and fragmentation of the basal lamina occur in the advanced stages of atresia. No apoptotic cell death, however, was demonstrated in cumulus cells during atresia before phagocytic cell invasion into the follicular antrum [16, 17]. In the granulosa cells but not cumulus cells prepared from atretic follicles, elevated activity of neutral Ca^{2+}/Mg^{2+}-dependent endonuclease was detected [17, 18]. These previous findings confirmed that granulosa cells undergo apoptosis and that no apoptotic cell death occurs in cumulus cells in pig atretic follicles. However, has not been determined which trigger molecules induce granulosa cell apoptosis, or how intercellular apoptotic signals are transmitted in the granulosa cells [39, 40]. A specific monoclonal antibody, which recognizes a cell-surface trigger molecule and induces apoptosis, is essential to define the molecular mechanism of apoptotic signal transmission

Fig. 6. Representative DNA histogram of flow cytometric analysis for determination of degraded DNA in cultured granulosa cells. DNA content is indicated on the X-axis, the number of cells is shown on the Y-axis. The isolated granulosa cells were cultured with 0.1 or 10 μg/m*l* PFG-1 or PFG-2, respectively, for 3 hr at 37°C. After staining with propidium iodide, determination of the percentage of cells with degraded DNA and of cell cycle distributions for single cells was carried out using a flow cytometer. Cell samples were analyzed for 10,000 events. In control cultures (A), most cells were in G_0/G_1 (1N: DNA content is approximately 130 region) and G_2 (2N: DNA content is approximately 230 region) phase. When the cells were cultured with PFG-1 and PFG-2 (B and C, respectively), cell populations in the apoptotic phase (DNA content is approximately 80 region) indicating high percentages of degraded DNA were observed.

pathways in the granulosa cells. In the present study, we generated two specific monoclonal antibodies that reacted against cell-surface proteins and induced apoptosis in cultured granulosa cells from healthy follicles.

Fas/APO-1/CD95 protein (Fas antigen) can mediate granulosa cell apoptosis in ovarian follicle atresia in rats [16, 32], but this protein has not yet been identified in pigs. In rodents and human, Fas antigen is a transmembranous glycoprotein that belongs to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family and which mediates apoptosis in a variety of lymphoid and tumor cells [21, 30, 43]. TNF induces apoptosis in a variety of tumor cells, and the TNF-receptor, a transmembranous protein, can also mediate apoptosis [33]. The molecular weights of Fas antigen and TNF-receptor are 45 [16] and 65 kD [33], respectively. As described above, the molecular weights of the granulosa cell-surface antigens recognized by PFG-1 and PFG-2 were 55 and 70 kD, respectively. Based on their molecular weights, these cell-surface antigens, cell death receptors, are considered to be different from the apoptosis-mediating receptors, Fas antigen and TNF-receptor. Moreover, Fas antigen was immunohistochemically detected in the granulosa cells of both healthy and atretic follicles in mouse ovaries [16, 32]. However, these antigens visualized histochemically by both PFG-1 and PFG-2 were only detected in the granulosa cells of healthy follicles in pig ovaries. These observations indicate that these antigens were not Fas antigen. We considered these antigens to belong to the TNF/NGF receptor family. Biochemical details of these cell death receptors of the porcine granulosa cells should be elucidated in future studies. In our laboratory, PFG-1 and PFG-2 antibodies have been used to screen for cell death receptors on the granulosa cell membrane. These antibodies will be useful probes to investigate the cell death receptors on the granulosa cell membrane and their natural ligands, and to define the intercellular pathway of apoptotic signal transmission in granulosa cells of pig ovaries.

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