Immunochemical characteristics of a novel cell death receptor and a decoy receptor on granulosa cells of porcine ovarian follicles

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Abstract

Previously, we prepared an IgM monoclonal antibody (PFG-1) which specifically recognized a cell-membrane glycoprotein (PFG-1 antigen; 55 kD, pI 5.9), immunohistochemically reacted with granulosa cells of healthy follicles but not of atretic follicles, and induced granulosa cell apoptosis. In the present study, an IgM monoclonal antibody (PFG-3) capable of inducing granulosa cell apoptosis and an IgG monoclonal antibody (PFG-4) not capable of inducing apoptosis were produced against granulosa cells prepared from healthy antral follicles of porcine ovaries. Two-dimensional Western blotting analysis revealed that PFG-3 specifically recognized two cell-membrane proteins (named PFG-3-1 and PFG-3-2/PFG-1 antigens; 42 kD, pI 5.2 and 55 kD, pI 5.9, respectively) of healthy granulosa cells, and that PFG-4 recognized the same two cell-membrane proteins. In atretic granulosa cells, PFG-3-2/PFG-1 antigen disappeared. Immunochemical reactions of these antibodies were only detected in follicular granulosa cells but not any other ovarian tissues or organs. PFG-3 and PFG-4 immunohistochemically reacted with granulosa cells of healthy and atretic follicles. When the isolated granulosa cells prepared from healthy follicles were cultured in medium containing PFG-3, the cells underwent apoptosis, and co-incubation with PFG-4 inhibited PFG-3-inducible apoptosis. These observations suggested that PFG-3-2/PFG-1 antigen is a novel cell death receptor which is different from the apoptosis-mediating receptors (Fas/Apo-1/CD95 or TNF receptor), and that PFG-3-1 antigen may act as a decoy receptor and inhibit apoptotic signal transmission.

Abbreviations: BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DIG-ddUTP, digoxigenin-11-2',3'-deoxyuridine-5'-triphosphate; EDTA, ethylenediaminetetraacetic acid disodium salt; EGTA, ethyleneglycol-bis-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HRP, horseradish peroxidase; IL, interleukin; IMDM, Iscove's modified Dulbecco's medium; NGF, nerve growth factor; PBS, phosphate buffered saline; RIA, radioimmunoassay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TDT, terminal deoxynucleotidyl transferase; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end-labeling.

Introduction

In mammalian ovaries, more than 99.9% of the follicles undergo the degenerative change known as at-

resia at varying stages of follicle development (Hirshfield, 1989, 1991). A number of studies of follicular atresia have revealed the morphological and biochemical characteristics of atretic follicles (Tilly and Hsue,

1992). Recent findings have suggested that apoptosis, originally described by Kerr et al. (1972), is the mechanism underlying ovarian follicular atresia. Apoptotic cell death of granulosa cells of rabbit Graafian follicles with atresia was first observed by Flemming (1885), who called it 'chromatolysis'. Unfortunately, the physiological roles of chromatolysis in granulosa cell of atretic follicles are not well understood. The degeneration of atretic follicles in mammalian ovaries can be explained, at least in part, by apoptotic cell death of granulosa and theca interna cells (Tilly et al., 1991; Hughes and Gorospe, 1991). However, the degenerative changes in cumulus cells during follicular atresia have not been investigated in detail (Tilly, 1993). Recently, we confirmed that apoptosis occurs in granulosa cells but not cumulus cells in the atretic Graafian follicles from porcine ovaries (Manabe et al., 1996a, 1997a). Briefly, *in situ* analysis of DNA fragmentation was performed on histological sections of follicles using the TUNEL method, and then conventional electron microscopic analysis was also performed. In healthy follicles, no apoptotic cells were observed among granulosa or cumulus cells, internal or external theca cells, or oocytes. In the early stage of atresia, apoptosis demonstrated histochemically by 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. Typical apoptotic features, i.e. condensed nuclei, were seen in the scattered granulosa cells, but cumulus cells with normal ultrastructure were also seen in the same follicles (Sugimoto et al., 1998). In the late to final stages of atresia, granulosa cells scattered on the inner surface of the follicular wall began to undergo apoptosis, but no TUNEL-positive cells were detected among the cumulus cells. Moreover, the neutral $\text{Ca}^{2+}/\text{Mg}^{2+}$ -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 (Manabe et al., 1996b). No endonuclease activity was detected in cumulus cells prepared from the same atretic follicles. These histological, cytological and biochemical findings confirmed that there were no apoptotic changes in the cumulus cells of the atretic follicles in immature or mature porcine ovaries (Manabe et al., 1996c, 1997b; Imai et al., 1999).

Previously, we prepared an IgM monoclonal antibody (named PFG-1) capable of inducing granulosa cell apoptosis (Myoumoto et al., 1997; Manabe et

al., 1997c, 1998a, b). PFG-1 was produced against granulosa cells prepared from healthy antral follicles of porcine ovaries, and specifically recognized a cell-membrane glycoprotein (PFG-1 antigen) with a molecular weight of 55 kD and isoelectric point of 5.9. PFG-1 immunohistochemically reacted with granulosa cells of healthy follicles but not of atretic follicles. When the isolated granulosa cells prepared from healthy follicles were cultured in medium containing PFG-1, the cells underwent apoptosis as determined by nuclear morphology, DNA electrophoresis and flow cytometric analysis. Immunochemical and biochemical characteristics of the PFG-1 antigen were different from those of known apoptosis-mediating receptors, Fas (Apo-1/CD95) or TNF receptors (TNFR), suggesting that the PFG-1 antigen is a novel cell death receptor located on the granulosa cells of healthy antral follicles in porcine ovaries. However, mechanisms of regulation of this novel cell death receptor are not well understood (Manabe et al., 1998a). In the present study, we produced two unique monoclonal antibodies to reveal the molecular mechanisms regulating follicular granulosa cell apoptosis in porcine ovaries. An IgM monoclonal antibody (PFG-3), which is capable of inducing granulosa cell apoptosis, and an IgG monoclonal antibody (PFG-4), which is not capable of inducing granulosa cell apoptosis and inhibits the PFG-1- and PFG-3-induced apoptosis, have been produced against granulosa cells prepared from healthy antral follicles. We used these antibodies as sensitive probes to elucidate the mechanisms responsible for granulosa cell apoptosis.

Materials and methods

Preparation of follicular granulosa cells

Granulosa cells from healthy antral follicles of porcine ovaries were prepared as described previously (Myoumoto et al., 1997). Briefly, 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 HEPES (Gibco) and 0.1% polyvinylalcohol (Wako Pure Chemical, Osaka, Japan) from the ovaries obtained from mature pigs at a slaughterhouse. Under a surgical dissecting microscope (SZ11; Olympus, Tokyo, Japan), follicles were classified as morphologically healthy or atretic (Dailey et al., 1975). The healthy follicles were punctured over 1.5-ml microcentrifuge tubes to collect follicular fluid. Fluid

from each follicle was separated by centrifugation, and then estradiol-17 β and progesterone levels were measured by [125 I]-RIA as described below to confirm the classification of the follicles (Manabe et al., 1996a). Then, the granulosa cell layers were removed from the follicles in 25 mM HEPES-buffered Medium 199 containing 80 mg ml $^{-1}$ 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 EDTA (Gibco) and 6.8 mM 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% FCS (Gibco) (HEPES-199-FCS) by centrifugation. Cell number was counted using a hemocytometer plate, and cell viability was determined by the 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-17 β and progesterone levels in follicular fluid diluted 100-fold with Medium 199 were quantified using [125 I]-RIA kits (Bio-Mérieux, Marcy-l'Etoile, France) as described previously (Manabe et al., 1996a). In pigs, the progesterone/estradiol-17 β ratio of follicular fluid in each follicle provides a good index of follicular atresia (Babalola and Shapiro, 1988; Coley et al., 1994; Guthrie et al., 1995). When the progesterone/estradiol-17 β ratio of follicular fluid was less than 15, the follicle was classified as healthy according to our previous findings (Manabe et al., 1996a).

Preparation of monoclonal antibodies

Eight-week-old female BALB/c mice purchased from Clea Japan (Tokyo, Japan) were used. 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). They were housed in a controlled environment (lights on between 7:00 and 19:00; temperature 22 $\pm$ 2 °C; humidity 70 $\pm$ 5%) and were immunized intraperitoneally with isolated healthy granulosa cells (10 6 cells/mouse, once a week). Immunization was repeated four to six times. Antibody production was assessed by conventional immunofluorescence staining as described below. Five days after

the last immunization, fewer cells (10 4 cells/mouse) were injected intraperitoneally as a booster 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 polyethylene glycol 1500 (Boehringer Mannheim, Indianapolis, IN, U.S.A.) (Oi and Herzenberg, 1980). After washing with IMDM (Gibco), the fused cells were suspended in IMDM containing 10% FCS and 1 unit ml $^{-1}$ of interleukin 6 (Sigma) (IMDM-FCS-IL6), and plated in 96-well cell culture plates (Falcon 3872; Becton Dickinson, Lincoln Park, NJ, U.S.A.). After 24 h incubation at 37 °C in 5% CO $_2$, HAT medium (1 $\times$ 10 $^{-4}$ M hypoxanthine, 0.8 $\times$ 10 $^{-7}$ M aminopterin and 1.6 $\times$ 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-IL6 containing HAT medium (IMDM-FCS-IL6-HAT). After three changes of IMDM-FCS-IL6-HAT, the hybridoma cells were incubated in IMDM-FCS-IL6 containing HT medium (1 $\times$ 10 $^{-4}$ M hypoxanthine and 1.6 $\times$ 10 $^{-5}$ M thymidine; Boehringer Mannheim) (IMDM-FCS-IL6-HT). Thereafter, half of the culture supernatant was replaced with fresh IMDM-FCS-IL6-HT every 3 days. The hybridoma cells producing antibodies against the granulosa cell surface were screened by conventional immunofluorescence staining. Then, antibody class was determined by ELISA as described previously (Myoumoto et al., 1997). Two hybridoma cell lines, which produced IgM and IgG antibody against the granulosa cell surface, were selected, cloned twice by limiting dilution, and named PFG-3 and PFG-4, respectively.

Four-week-old female BALB/c mice received intraperitoneal injection of 0.5 ml/mouse of pristane (2, 6, 10, 14-tetramethylpentadecane; Aldrich Chemicals, Milwaukee, WI, U.S.A.). One month after pristane injection, PFG-3 and PFG-4 hybridoma cells (1 $\times$ 10 7 cells/mouse) were injected intraperitoneally. Within two weeks after injection, ascites were obtained from the mice and dialyzed against PBS (pH 7.4), and then immunoglobulin-rich fractions were precipitated with 50% saturated ammonium sulfate (Wako). These crude antibodies were applied to a hydroxyapatite column (Asahi Optical Inc., Tokyo, Japan). The IgM and IgG fractions were eluted with a 10–400 mM gradient of sodium phosphate, pH 7.4, by preparative HPLC (Simadzu, Kyoto, Japan). Eluted antibodies concentrated in ultrafiltration cells with XM50 ultra-

filtration membrane (Amicon, Beverly, MA, U.S.A.) were heat-inactivated for 45 min at 56 °C and sterilized by filtration through 0.22- μ m porefilters (Millipore, Marlborough, MA, U.S.A.). The optical densities at 280 nm of the antibody solutions were measured with a spectrophotometer (Ultrospec 3000; Pharmacia Biotech, Uppsala, Sweden) to determine protein concentration.

Immunofluorescence staining

Porcine ovaries obtained at a slaughterhouse were cut into small pieces, put on filter paper, mounted in OCT compound (Miles Lab., Elkhart, IN, U.S.A.), and then rapidly frozen in liquid nitrogen. Serial sections (5 μ m thick) were cut on a cryostat (Jung CM1500; Leica, Heidelberg, Germany), 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% BSA (Sigma) (PBS-BSA) for 2 h 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 h at 4 °C. As negative controls, adjoining sections were incubated with diluted normal mouse serum (1/10 dilution with PBS-BSA) or mouse IgM or IgG (1 μ g ml⁻¹ in PBS-BSA; Sigma). After washing with PBS-Tw, the sections were incubated with FITC-conjugated goat anti-mouse IgM or IgG antibody (1/400 dilution with PBS-BSA; American Qualex, La Mirada, CA, U.S.A.) for 2 h at RT. After washing with PBS-Tw, the sections were examined with a fluorescence microscope (BX50-fluoro system, Olympus) or a confocal laser scanning microscope (LSM 410; Carl Zeiss, Oberkochen, Germany).

Conventional and two-dimensional Western blotting analyses

For conventional Western blotting analysis, homogenized samples of ovarian tissues (granulosa cells and luteal bodies), oviduct, uterus, testis, liver, kidney, adrenal gland, pancreas, stomach, small intestine, large intestine, spleen, thymus, brain, heart, lung or skeletal muscle were electrophoresed through 4% polyacrylamide slab gels as described previously (Myoumoto et al., 1997). After SDS-PAGE, separated protein bands

in the gels were stained with a Coomassie brilliant blue staining kit (Wako) according to the manufacturer's instructions.

As previously reported (Myoumoto et al., 1997), cell membrane samples of the isolated granulosa cells were separated by two-dimensional PAGE (2D-PAGE) which was performed according to the method of O'Farrell (1975). Briefly, the cell membrane samples prepared from healthy and atretic follicles were solubilized in 2% Triton X-100 (Sigma) containing 9.5 M urea, 5% 2-mercaptoethanol and 2% carrier ampholyte (pH 3.5–10; Pharmacia Biotech). The lysates were separated by isoelectric focusing on cylindrical 4% polyacrylamide gels, separated by SDS-PAGE using 7.5% polyacrylamide slab gels, and then separated protein spots in the gels were stained with a Coomassie brilliant blue staining kit (Wako) according to the manufacturer's instructions. After 2D-electrophoresis, the proteins were transferred onto nitrocellulose membranes (Wako), which then were preincubated with 3% skimmed milk in PBS for 1 h at 37 °C. After washing with PBS-Tw, the membranes were incubated with monoclonal antibodies at 10 μ g ml⁻¹, and immunological reaction products were visualized with an ABC staining kit (Vector Laboratories, Burlingame, CA, U.S.A.) according to the manufacturer's instructions.

Cell-killing activity assay

The trypan blue exclusion method was used to determine cell-killing activity (Myoumoto et al., 1997). Briefly, the isolated healthy granulosa cells (10⁶ cells ml⁻¹) in 96-well culture plates were cultured in HEPES-199-FCS containing 10% each hybridoma culture supernatant or monoclonal antibody (0.001 to 100 μ g ml⁻¹ of PFG-1, PGF-3 and/or PFG-4) for 1 to 6 h at 37 °C. As a negative control, the granulosa cells were cultured in HEPES-199-FCS without any additives. The cells were resuspended by pipetting and stained with trypan blue, then viable cells were quantified by microscopy (BX50, Olympus).

TUNEL and nuclear staining

Apoptotic cells were demonstrated histochemically by the TUNEL method as described previously (Manabe et al., 1997b), and the nuclei of cultured cells were stained with Hoechst 33258 (Molecular Probes, Eugene, OR, U.S.A.) to observe their morphology. Briefly, the isolated healthy granulosa cells (10⁶ cells ml⁻¹) 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 (0.001 to 100 $\mu\text{g ml}^{-1}$ of PFG-3 and/or PFG-4) for 1 to 18 h at 37 °C. As a negative control, the cells were cultured in HEPES-199-FCS without any additives.

For TUNEL staining, the cells attached to the plastic coverslips were fixed in 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) in 0.1 M phosphate buffer (pH 7.4) for 10 min at 4 °C, then incubated with proteinase K (10 $\mu\text{g ml}^{-1}$) for 10 min at 20 °C, and washed in distilled water. The cells were incubated with TDT (1 U μl^{-1} ; Boehringer Mannheim solution containing 45 μM ddATP and 5 μM DIG-ddUTP (Boehringer Mannheim) for 1 h at 37 °C, and then immersed in double-strength salt sodium citrate buffer. After washing with PBS, the cells were incubated with FITC-labeled anti-digoxigenin antibody solution (Boehringer Mannheim) at 1:100 dilution with PBS containing 2% BSA for 1 h at RT. After washing with PBS, the cells were examined with a fluorescence microscope or a confocal laser scanning microscope. The following positive and negative controls were included in each experimental run: as negative controls, the cell samples were incubated with omission of either TDT or DIG-ddUTP; as a positive control, the cell samples were treated with DNase I (1 $\mu\text{g ml}^{-1}$; Boehringer Mannheim) for 5 min at RT before exposure to TDT, and paraffin sections prepared from young adult rat testis were used as physiological positive controls (Manabe et al., 1996a).

To observe the morphology of the nuclei, the cells were fixed with cold acetone for 5 min, washed with PBS, and stained with Hoechst 33258 (4 $\mu\text{g ml}^{-1}$ in distilled water) for 5 min. After washing with PBS, the cells were examined with a fluorescence microscope.

DNA electrophoresis

To assess the DNA fragmentation in cultured granulosa cells, DNA samples prepared from cultured cells were electrophoresed (Manabe et al., 1996a). Briefly, after incubation with monoclonal antibody as described above, cultured granulosa cells were resuspended by pipetting, and washed with modified HEPES-199-FCS. DNA fractions were separated from the cells by centrifugation at 9,000 g for 20 min at 4 °C. DNA contents were determined by DAPI-fluorescence assay using calf thymus DNA (Sigma) as a standard (Kapusinski and Skoczylas, 1977), 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 $\mu\text{g ml}^{-1}$ ethidium bromide at 60 V for 90 min. Gels were photographed on an ultraviolet transilluminator.

Results

Hybridoma selection

Two hybridoma cell lines, named PFG-3 and PFG-4, producing IgM and IgG antibodies to porcine granulosa cell-surface components, were selected and cloned. The characteristics of the monoclonal antibodies produced by these hybridoma cell clones were as follows.

Conventional Western blotting analysis

After SDS-PAGE, the proteins were transferred onto nitrocellulose sheets, and then the specific antigens against PFG-3 and PFG-4 were analyzed (Figure 1). In the homogenized samples of granulosa cells prepared from healthy follicles, two specific bands with molecular weights of 42 and 55 kD were observed on the nitrocellulose filters treated with PFG-3 and PFG-4. A dense band of 42 kD was seen in the samples from healthy follicles (lanes 3 and 5). In the granulosa cell samples prepared from atretic follicles, a specific band with a molecular weight of 42 kD was observed on the nitrocellulose filters treated with PFG-3 and PFG-4, but the band of 55 kD disappeared (lanes 4 and 6). In addition, when the nitrocellulose filters were treated with PFG-1, which is an IgM monoclonal antibody capable of inducing granulosa cell apoptosis (Manabe et al., 1997b; Myoumoto et al., 1997), a specific band with a molecular weight of 55 kD was detected only in the granulosa cell samples prepared from healthy but not atretic follicles (data not shown). When the homogenized samples of granulosa cells prepared from healthy follicles were immuno-absorbed with PFG-1 monoclonal antibody before SDS-PAGE, the band of 55 kD disappeared (data not shown). No positively stained bands were detected in the homogenized samples of the luteal body, oviduct, uterus, testis, liver, kidney, adrenal gland, pancreas, stomach, small intestine, large intestine, spleen, thymus, brain, heart, lung, adrenal gland, thyroid gland or skeletal muscle (data not shown).

Figure 1. Representative results of Western blotting analysis of granulosa cell-membrane antigens recognized by PFG-3 and PFG-4. Granulosa cell membrane fractions were prepared from healthy and atretic 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 (lanes 1 and 2, respectively). After SDS-PAGE, the proteins were transferred onto nitrocellulose sheets, and the granulosa cell antigens were visualized by immunostaining. In the samples prepared from healthy granulosa cells, two specific bands with molecular weights of 42 and 55 kD were identified on the nitrocellulose filters treated with PFG-3 and PFG-4 (lane 3 and 5, respectively). A dense band of 42 kD was seen in the samples prepared from atretic follicles, the specific band of 55 kD disappeared (lane 4 and 6; PFG-3 and PFG-4, respectively). Molecular weight markers (MW) stained with CBB are shown in the left lane.

Characterization of antigens by 2D-Western blotting

The antigens on the granulosa cells prepared from healthy and atretic follicles were characterized by 2D-Western blotting. Plasma membrane fractions of the granulosa cells prepared from healthy and atretic follicles were subjected to 2D-PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose sheets and the granulosa cell antigens were analyzed. Representative 2D-PAGE results of healthy and atretic follicle samples are shown in Figure 2. Protein spots in the slab-gel were detected by conventional Coomassie brilliant blue staining (Figures 2A and B). Figures 2C–H showed the results of 2D-Western blotting analysis on nitrocellulose membranes. In healthy

follicle samples, the two specific spots (42 kD, pI 5.2 and 55 kD, pI 5.9; named PFG-3-1 and PFG-3-2/PFG-1 antigens, respectively) of PFG-3 were demonstrated (Figure 2C), and the same two spots of PFG-4 were detected (Figure 2E). A large and dense spot of PFG-3-1 antigen was seen in the samples of healthy follicles (Figures 2C and E), and moderate expression of PFG-3-1 antigen was demonstrated in the samples of atretic follicles (Figures 2D and F). However, in atretic follicle samples, PFG-3-2/PFG-1 antigen disappeared (Figures 2D and F). In addition, 2D-Western blotting against PFG-1 showed a specific spot (55 kD, pI 5.9; PFG-3-2/PFG-1 antigen) in healthy follicle samples (Figure 2G), but this spot disappeared in atretic follicle samples (Figure 2H). As same as conventional West-

Figure 2. Representative results of two-dimensional (2D) Western blotting analysis of granulosa cell-membrane antigens recognized by PFG-3 (C and D) and PFG-4 (E and F) and additionally by PFG-1 (G and H). Granulosa cell membrane fractions were prepared from healthy (A, C, E and G) and atretic (B, D, F and H) granulosa cells, and separated by 2D-PAGE. Separated protein spots in gels of HGC and AGC samples were detected by Coomassie brilliant blue (A and B, respectively). After electrophoresis, the proteins were transferred onto nitrocellulose sheets and the granulosa cell antigens were visualized by PFG-3, PFG-4 and PFG-1. In healthy follicle samples, two specific spots (42 kD, pI 5.2 and 55 kD, pI 5.9; arrow and arrowhead, respectively) of PFG-3 (C) were observed, and the same two spots of PFG-4 were detected (E). A large and dense spot of 42 kD, pI 5.2 (arrow) was seen in the samples of healthy follicles (C and E), and moderate expression of the same spot was demonstrated in the samples of atretic follicles (D and F). The specific spot of 55 kD, pI 5.9 disappeared (D and F). In addition, one specific spot (55 kD, pI 5.9; arrowhead in G) immunoreactive with PFG-1 antibody was detected in healthy follicle samples, but the same spot disappeared in atretic follicle samples (H).

Figure 3. Composite confocal images of porcine ovarian follicles. Frozen sections were stained with PFG-3 (A and B) and PFG-4 (C and D) and with FITC-conjugated goat anti-mouse IgM and IgG, respectively, to show the distribution of granulosa cell membrane antigens. The follicles were optically sectioned at $0.5\ \mu\text{m}$ and five serial images were generated using the confocal microscope. Granulosa cells of healthy and atretic follicles were stained with PFG-3 (A and B, respectively). PFG-4 showed moderate fluorescent staining on granulosa cells of healthy and atretic follicles (C and D, respectively). No fluorescent staining of theca interna or externa cells was observed in either healthy or atretic follicles ($\times 200$).

ern blotting analysis, when the homogenized samples of granulosa cells prepared from healthy follicles were immuno-absorbed with PFG-1 before 2D-PAGE, PFG-3-2/PFG-1 antigen disappeared (data not shown).

Immunohistochemical characterization of monoclonal antibodies

Immunofluorescence analysis on serial cryostat sections of porcine ovaries was used to determine the target specificity of the monoclonal antibodies. PFG-3 was reactive with granulosa cells of healthy and atretic follicles (Figures 3A and B, respectively). Strong immunostaining was seen in the section of healthy follicles. PFG-4 showed moderate fluorescent staining on granulosa cells of healthy and atretic follicles (Figures 3C and D, respectively). These antibodies did not label theca interna or externa cells, basement membrane, or ovarian stroma cells in either healthy or atretic follicles. Moreover, these antibodies showed neither specific binding to the luteal body, oviduct, uterus, testis, liver, kidney, adrenal gland, pancreas, stomach, small intestine, large intestine, spleen, thymus, brain, heart, lung or skeletal muscle (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, the trypan blue exclusion method, as described above. After selection of hybridoma clones, *in vitro* granulosa cell apoptosis mediated by the selected antibodies was confirmed by assessment of TUNEL staining, nuclear morphology and DNA electrophoretic analysis. The isolated granulosa cells prepared from healthy follicles were co-cultured with concentrations varying from 0.001 to $100\ \mu\text{g ml}^{-1}$ of PFG-3 and/or PFG-4, for 6 h at $37\ ^\circ\text{C}$. After incubation, apoptotic cells were determined by TUNEL staining (Figure 4). No TUNEL-positive granulosa cells were observed in the isolated granulosa cells cultured without any additive (Figure 4A). When the isolated granulosa cells were co-cultured with at least $0.01\ \mu\text{g ml}^{-1}$ PFG-3, many TUNEL-positive round nuclei and small condensed nuclear fractions (apoptotic bodies; a morphological hallmark of apoptotic cell death) were observed (Figure 4B). However, when co-cultured with $100\ \mu\text{g ml}^{-1}$ PFG-4, no positive cells were observed (Figure 4C). When the cells

Figure 4. Fluorescence photomicrographs of the cultured granulosa cells stained by the TUNEL method to assess apoptosis. The granulosa cells prepared from healthy follicles were cultured without any additive (A; as control), or with $0.01 \mu\text{g ml}^{-1}$ PFG-3 (B) or $100 \mu\text{g ml}^{-1}$ PFG-4 (C) for 6 h at 37°C . No TUNEL-positive cells were observed in the control. In PFG-3-treated cells, many TUNEL-positive round nuclei and small condensed nuclear fractions (apoptotic bodies) were observed. No positive cells were seen among those cultured with PFG-4. When the cells were co-cultured with both 0.01 and $100 \mu\text{g ml}^{-1}$ PFG-3 and PFG-4 respectively, no positive cells were seen (PFG-3/PFG-4; D). In addition, when the cells were co-incubated with 0.01 and $100 \mu\text{g ml}^{-1}$ PFG-1 and PFG-4 respectively, no TUNEL-positive cells were observed (PFG-1/PFG-4; E) ($\times 400$).

were co-cultured with 0.01 and $100 \mu\text{g ml}^{-1}$ of PFG-3 and PFG-4 (PFG-3/PFG-4) respectively, no TUNEL-positive cells were observed (Figure 4D). Thus, apoptotic cell death induced by PFG-3 was inhibited by PFG-4. In addition, when the cells were co-incubated with 0.01 and $100 \mu\text{g ml}^{-1}$ of PFG-1 and PFG-4 (PFG-1/PFG-4) respectively, no TUNEL-positive cells were observed (Figure 4E).

After co-incubation with PFG-3 and/or PFG-4, DNA samples of these isolated granulosa cells were electrophoresed in 2% agarose gels (Figure 5). DNA samples of the isolated cells co-cultured without any additive displayed no ladder pattern (biochemical hallmark of apoptosis) on electrophoresis (lane 1). PFG-3

co-incubated DNA samples displayed a ladder pattern (lanes 2), but no such ladder pattern was seen in the DNA samples prepared from PFG-4, PFG-3/PFG-4 or PFG-1/PFG-4 co-incubated cells (lanes 3, 4 and 5, respectively).

Discussion

In the present study, we selected two hybridoma clones, named PFG-3 and PFG-4, producing monoclonal IgM and IgG antibodies, respectively, against cell-membrane proteins of granulosa cells. These antibodies showed specific binding to granulosa cells

Figure 5. Electrophoretic analysis of DNA fragments in DNA samples prepared from cultured granulosa cells. The isolated granulosa cells were cultured with $0.01 \mu\text{g ml}^{-1}$ PFG-3 or $100 \mu\text{g ml}^{-1}$ PFG-4 for 6 h at 37°C . Equal amounts of DNA samples ($10 \mu\text{g/lane}$) prepared from cultured granulosa cells were electrophoresed in 2% agarose gels, and then DNA was visualized by ethidium bromide staining under ultraviolet illumination. DNA samples from control cells displayed no DNA ladder formation on electrophoresis (lane 1). The DNA from PFG-3-treated granulosa cells displayed a ladder pattern (lane 2), but no such ladder pattern was seen in the DNA samples prepared from cells treated with PFG-4 (lane 3). When the cells were co-cultured with 0.01 and $100 \mu\text{g ml}^{-1}$ PFG-3 and PFG-4 (PFG-3/PFG-4; lane 4), respectively, or with 0.01 and $100 \mu\text{g ml}^{-1}$ PFG-1 and PFG-4 (PFG-1/PFG-4; lane 5) respectively, no ladder pattern was seen. Molecular weight markers (MW) are indicated on the left side of the figure.

of ovarian follicles, but no specific binding to the luteal body, oviduct, uterus, testis, liver, kidney, adrenal gland, pancreas, stomach, small intestine, large intestine, spleen, thymus, brain, heart, lung, adrenal gland, thyroid gland or skeletal muscle. 2D-Western blotting analysis revealed that both PFG-3 and PFG-4 antibodies specifically recognized two cell membrane proteins. One of cell membrane protein with a molecular weight of 42 kD and isoelectric point of 5.2 (named PFG-3-1 antigen) was detected in the granulosa cells of both healthy and atretic

follicles, but other cell membrane protein with a molecular weight of 55 kD and isoelectric point of 5.9 (named PFG-3-2/PFG-1 antigen) disappeared in atretic follicles. Abundant PFG-3-1 antigen expression was noted in the granulosa cells of healthy follicles. Immunohistochemical staining confirmed that PFG-3-1 and PFG-3-2/PFG-1 antigens were present only in the cell membrane of follicular granulosa cells. Moreover, PFG-3 but not PFG-4 induced apoptotic cell death in cultured granulosa cells prepared from healthy antral follicles. Previously, we generated a unique monoclonal IgM antibody, named PFG-1, and 2D-Western blotting analysis showed that this antibody recognized a cell membrane protein (55 kD, pI 5.9, named PFG-1 antigen) of porcine granulosa cells (Manabe et al., 1997c; Myoumoto et al., 1997). PFG-1 antigen is a novel cell death receptor which was present only in the cell membrane of granulosa cells of healthy antral follicles. Similarly to one of PFG-3-recognized antigen (55 kD, pI 5.9), PFG-1 antigen was immunochemically and immunohistochemically detected only in the granulosa cells of healthy but not atretic follicles (Myoumoto et al., 1997). Additional experiments in the present study showed that PFG-1 could not recognize PFG-3-1 antigen. Moreover, when the cell membrane samples of granulosa cells prepared from healthy follicles were immuno-absorbed with PFG-1 before 2D-PAGE, PFG-3-2/PFG-1 antigen disappeared. These findings indicated that PFG-1 antigen is the same membrane protein as one of PFG-3-recognized antigen (55 kD, pI 5.9), and thus we named PFG-3-2/PFG-1 antigen, a novel cell death receptor which is specifically expressed in granulosa cells of healthy porcine follicles.

Fas, a member of the TNFR superfamily, is a transmembrane protein that mediates apoptosis in a variety of lymphoid and tumor cells through Fas and Fas-ligand binding (Yonehara et al., 1989; Itoh et al., 1991). Fas mRNA is expressed in the thymus, liver, heart, lung and ovary (Ogasawara et al., 1993), but the physiological and pathological roles of the Fas and Fas-ligand system are not well understood in the ovary. In rodent ovaries, Fas is expressed in follicular granulosa cells and mediates granulosa cell apoptosis in ovarian follicle atresia (Hakuno et al., 1996; Sakamaki et al., 1997). Immunohistochemical staining of rat ovaries revealed intense positive immunostaining for Fas and Fas-ligand in granulosa cells of atretic small and medium antral follicles, and intense Fas-ligand staining was evident in the theca interna cells of healthy small antral follicles. These observations

indicated that Fas-ligand may be the signal which induces granulosa cell apoptosis during atresia in rats (Kim et al., 1998). In mouse luteal bodies, which contain luteal, stromal and endothelial cells, fibroblasts and surface epithelial cells, Fas is expressed abundantly in surface epithelial cells and mediates apoptosis of the surface epithelial cells (Quirk et al., 1997). Recent *in vitro* studies demonstrated that other receptors (angiotensin II type 2 receptor, gonadotropin-releasing hormone receptor etc.) mediate follicular atresia (Murdoch, 1995; Yamada et al., 1996), but the *in vivo* physiological roles of these receptors in selection of atretic follicles have not been determined. TNF also can induce apoptosis in a variety of tumor cells, and the TNFR, a transmembrane protein, can also mediate apoptosis (Stauber et al., 1988). The molecular weights of Fas and TNFR are 45–46 (Ogasawara et al., 1993; Hakuno et al., 1996; Yoo et al., 1996) and 65 kD (Stauber et al., 1988), respectively. As described above, the molecular weights of the granulosa cell-surface antigens recognized by PFG-3 and PFG-4 were 42 and 55 kD. Fas was immunohistochemically detected in the granulosa cells and luteal cells of both healthy and atretic follicles in rodent ovaries, but TNFR was not detected in ovarian follicles or luteal bodies (Sakamaki et al., 1997; Manabe et al., 1998b). However, PFG-3-2/PFG-1 antigen visualized histochemically by both PFG-1 and PFG-3 was only detected in the granulosa cells of healthy but not atretic follicles. Based on their biochemical and histochemical characteristics, PFG-3-2/PFG-1 antigen is different from the known apoptosis-mediating receptors Fas and TNFR, and is considered to be a novel cell death receptor expressed specifically on the granulosa cells of healthy follicles.

The physiological properties of PFG-3-1 antigen, however, are not well understood. In this study, we showed that PFG-1 and PFG-3 but not PFG-4 can induce the apoptotic cell death of porcine granulosa cells in primary culture. It is interesting that the cultured granulosa cells were not killed by PFG-1 or PFG-3 antibodies in the presence PFG-4. As described above, PFG-3-2/PFG-1 antigen is considered to be a cell death receptor, and its expression disappeared in atretic granulosa cells. Abundant expression of PFG-3-1 antigen was noted in healthy granulosa cells, and moderate to trace expression was demonstrated in atretic cells. Both PFG-3 and PFG-4 bind to PFG-3-1 and PFG-3-2/PFG-1 antigens, and PFG-3-2/PFG-1 antigen expressed only in healthy follicles. Thus, it is unlikely that PFG-3-1 antigen is a survival

receptor. Recently, TNF-related apoptosis-inducing ligand (TRAIL, also called Apo2 ligand), which is a novel CD95 ligand (Fas-ligand) homologous cytotoxic cytokine, was shown to belong to the TNF family, and to activate rapid apoptosis in various tumor cells (Macfarlane et al., 1997; Marsters et al., 1997; Emery et al., 1998; Rieger et al., 1998). TRAIL induces apoptosis upon binding to its cytoplasmic death domain-containing receptors, death receptor 4 and 5 (DR4 and DR5, respectively). Both DR4 and DR5 independently bind to their specific ligand TRAIL and engage the caspase cascade to induce apoptosis similarly to Fas and TNFR (Sheikh et al., 1998). Two additional TRAIL binding membrane proteins, named decoy receptor 1 and 2 (DcR1 and DcR2, respectively), were identified (Pan et al., 1997; Sheridan et al., 1997). These decoy receptors are glycopospholipid-anchored cell surface proteins, lack functional cytoplasmic death domains, and act as antagonist decoy receptors that inhibit TRAIL signaling. Moreover, apoptosis and decoy receptors are expressed on the same tumor cells. It is considered that overexpression of decoy receptor on the surface of tumor cells inhibits apoptotic cell death induced by TRAIL. Thus, a cell-surface mechanism exists for the regulation of cellular responsiveness to pro-apoptotic stimuli. The present results indicated that PFG-3-1 antigen is abundantly expressed on the surface of healthy granulosa cells and inhibits an apoptotic signal induced by ligand-like antibodies (PFG-1 and PFG-3), and that PFG-3-1 antigen is a modulator of PFG-3-2/PFG-1 antigen which transmits an apoptotic signal. Moreover, we confirmed that up-regulated expression of the α 2,6-sialyltransferase mRNA increases membranous glycoconjugates containing α 2,6-linked sialic acid residues in apoptotic granulosa cells of porcine atretic follicles (Kimura et al., 1998, 1999). As these properties of PFG-3-1 antigen are similar to the decoy receptors in TRAIL/DcR1 and DcR2 system, we presumed that PFG-3-1 antigen acts as a decoy receptor in granulosa cells of porcine follicles.

In the present study, we demonstrated that PFG-3-2/PFG-1 antigen mediates apoptosis. We hypothesized that PFG-3-2/PFG-1 antigen is a novel cell death receptor and a member of the TNFR superfamily, and that PFG-3-1 antigen is a decoy receptor involved in regulation of granulosa cell survival and death. As these two receptors may play an important role in control of ovarian functions, detailed biochemical studies should be performed. In our laboratory, PFG-1, PFG-3 and PFG-4 antibodies have been used to screen

for cell death receptors on the granulosa cell membrane and to identify the cell death ligands binding to these receptors. Thus, these antibodies will be useful probes to investigate the cell death receptors on the granulosa cell membrane and their natural ligands, to elucidate cell surface mechanisms for the regulation of apoptosis, and to define the intercellular pathway of apoptotic signal transduction in granulosa cells of porcine ovaries.

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References

- Babaloo GO and Shapiro BH (1988) Correlation of follicular steroid hormone profiles with ovarian cyclicity in sows. *J Reprod Fertil* 84: 79–87.
- Coley AJ, Howard HJ, Slinger WD and Ford JJ (1994) Steroidogenesis in the preovulatory porcine follicle. *Biol Reprod* 51: 655–661.
- Dailey RA, Clark JR, First NL, Chapman AB and Casida LE (1975) Loss of follicles during the follicular phase of the estrous cycle of swine as affected by genetic group and level of feed intake. *J Anim Sci* 41: 835–841.
- Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, Dul E, Appelbaum ER, Eichman C, Diprinzio R, Dodds RA, James IE, Rosenberg M, Lee JC and Young PR (1998) Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *J Biol Chem* 273: 14363–14367.
- Flemming W (1885) Über die Bildung von Richtungsfiguren in Säugethiereiern beim Untergang Graaf'scher Follikel. *Arch Anat EntGesch* 221–244.
- Guthrie HD, Cooper BS, Welch GR, Zakaria AD and Johnson LA (1995) Atresia in follicles grown after ovulation in the pig: Measurement of increased apoptosis in granulosa cells and reduced follicular fluid estradiol-17 β . *Biol Reprod* 52: 920–927.
- Hakuno N, Koji T, Yano T, Kobayashi N, Tsutsumi O, Taketani Y and Nakane PK (1996) Fas/APO-1/CD95 system as a mediator of granulosa cell apoptosis in ovarian follicle atresia. *Endocrinology*, 137: 1938–1948.
- Hirshfield AN (1989) Rescue of atretic follicles *in vitro* and *in vivo*. *Biol Reprod* 40: 181–190.
- Hirshfield AN (1991) Development of follicles in the mammalian ovary. *Int Rev Cytol* 124: 43–101.
- Hughes Jr FM and Gorospe WC (1991) Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. *Endocrinology* 129: 2799–2801.
- Imai Y, Manabe N, Uchio K, Kinoshita A, Kimura Y, Nakayama M, Nishihara S, Wada S, Sugimoto M and Miyamoto H (1999) Interactions of apoptosis and extracellular matrices in granulosa cells of atretic follicles in porcine ovaries. *J Mamm Ova Res* 16: 59–66.
- Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S-I, Sameshima M, Hase A, Seto Y and Nagata S (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 66: 233–243.
- Kapuscinski J and Skoczylas B (1977) Simple and rapid fluorimetric method for DNA microassay. *Anal Biochem* 83: 252–257.
- Kerr JFR, Wyllie AH and Currie AR (1972) Apoptosis: A basic biological phenomenon with wide ranging implication in tissue kinetics. *Br J Cancer* 26: 239–257.
- Kim JM, Boone DL, Auyeung A and Tsang BK (1998) Granulosa cell apoptosis induced at the penultimate stage of follicular development is associated with increased levels of Fas and Fas ligand in the rat ovary. *Biol Reprod* 58: 1170–1176.
- Kimura Y, Manabe N, Matsushita H, Tajima C, Myoumoto A and Miyamoto H (1998) Examination of granulosa cell glycoconjugates which change during follicular atresia in the pig ovaries. *J Reprod Dev* 44: 35–44.
- Kimura Y, Manabe N, Nishihara S, Matsushita H, Tajima C, Wada S and Miyamoto H (1999) Up-regulation of the α 2,6-sialyltransferase messenger ribonucleic acid increases glycoconjugates containing α 2,6-linked sialic acid residues in granulosa cells during follicular atresia of porcine ovaries. *Biol Reprod* 60: 1475–1482.
- Macfarlane M, Ahmad M, Srinivasula SM, Fernandes AT, Cohen GM, Alnemri ES (1997) Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *J Biol Chem* 272: 25417–25420.
- Manabe N, Imai Y, Ohno H, Takahagi Y, Sugimoto M and Miyamoto H (1996a) Apoptosis occurs in granulosa cells but not cumulus cells in the atretic antral follicles in the pig ovaries. *Experientia* 52: 647–651.
- Manabe N, Imai Y, Kimura Y, Myoumoto A, Sugimoto M, Miyamoto H, Okamura Y and Fukumoto M (1996b) Ca²⁺/Mg²⁺-dependent endonuclease but not Ca²⁺-dependent, Mg²⁺-dependent or cation-independent endonuclease is involved in granulosa cell apoptosis of pig atretic follicles. *J Reprod Dev* 42: 247–253.
- Manabe N, Imai Y, Kimura Y, Myoumoto A, Sugimoto M, Miyamoto H, Okamura Y and Fukumoto Y (1996c) Signal transmission of granulosa cell apoptosis in the atretic follicles in the pig ovaries. *J Reprod Dev* 42: 135–141.
- Manabe N, Kimura Y, Myoumoto A, Imai Y, Sakamaki K, Sugimoto M and Miyamoto H (1997a) Apoptosis occurs in granulosa cells but not cumulus cells in the atretic antral follicles in the porcine ovaries. *J Reprod Dev* 43: 179–180.
- Manabe N, Imai Y, Myoumoto A, Kimura Y, Sugimoto M, Okamura Y, Fukumoto M, Sakamaki K and Miyamoto H (1997b) Apoptosis occurs in granulosa cells but not cumulus cells in the atretic graafian follicles in multiparous pig ovaries. *Acta Histochem Cytochem* 30: 85–92.
- Manabe N, Myoumoto A, Kimura Y, Imai Y, Sugimoto M and Miyamoto H (1997c) A monoclonal antibody against pig ovarian follicular granulosa cells recognizes a novel cell death receptor and induces granulosa cell apoptosis. In: Kawashima S (ed.) *Advances in Comparative Endocrinology* (pp. 1793–1797).

- Monduzzi, Editore Spa, Bologna.
- Manabe N, Myoumoto A, Kimura Y, Imai Y, Sugimoto M, Sakamaki K, Okamura Y, Fukumoto M and Miyamoto H (1998a) Regulatory mechanisms of granulosa cell apoptosis in porcine ovarian follicle atresia. In: Miyamoto H and Manabe N (eds) *Reproductive Biology Update* (pp. 23–35). Nakanishi Pub Co, Kyoto.
- Manabe N, Kimura Y, Myoumoto A, Matsushita H, Tajima C, Sugimoto M and Miyamoto H (1998b) Role of granulosa cell apoptosis in ovarian follicle atresia. In: Yamada T and Hashimoyto Y (eds) *Apoptosis: Its Roles and Mechanism* (pp. 97–111). Jpn Acad Soc Book Cent, Tokyo.
- Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, Yuan J, Gurney A, Goddard AD, Godowski P and Ashkenazi A (1997) A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Cur Biol* 7: 1003–1006.
- Murdoch WJ (1995) Immunolocalization of a gonadotropin-releasing hormone receptor site in murine endometrium that mediates apoptosis. *Cell Tissue Res* 282: 527–529.
- Myoumoto A, Manabe N, Imai Y, Kimura Y, Sugimoto M, Okamura Y, Fukumoto M, Sakamaki K, Yonehara S, Niwano Y and Miyamoto H (1997) Monoclonal antibodies against pig ovarian follicular granulosa cells induce apoptotic cell death in cultured granulosa cells. *J Vet Med Sci* 59: 641–649.
- O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250: 4007–4021.
- Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T and Nagata S (1993) Lethal effect of the anti-Fas antibody in mice. *Nature* 364: 806–809.
- Oi VT and Herzenberg LA (1980) Immunoglobulin producing hybrid cell lines. In: Mishell BB and Shijji SM (eds) *Selected Methods in Cellular Immunology* (pp. 351–372). Freeman Pub, San Francisco.
- Pan G, Ni J, Wei YF, Yu GL, Gentz RA and Dixit VM (1997) An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* 277: 815–818.
- Quirk SM, Cowan RG, Huber SH (1997) Fas antigen-mediated apoptosis of ovarian surface epithelial cells. *Endocrinology* 138: 4558–4566.
- Rieger J, Naumann U, Glaser T, Ashkenazi A and Weller M (1998) APO2 ligand: A novel lethal weapon against malignant glioma? *FEBS Lett* 427: 124–128.
- Sakamaki K, Yoshida H, Nishimura Y, Nishikawa SI, Manabe N and Yonehara S (1997) Involvement of Fas antigen in ovarian follicular atresia and leuteolysis. *Mol Reprod Dev* 47: 11–18.
- Sheikh MS, Burns TF, Huang Y, Wu GS, Amundson S, Brooks KS, Fornace Jr AJ and El Deiry WS (1998) p53-dependent and -independent regulation of the death receptor Killer/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. *Cancer Res* 58: 1593–1598.
- Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, Goddard AD, Godowski P and Ashkenazi A (1997) Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* 277: 818–821.
- Stauber GB, Aiyer RA and Aggarwal BB (1988) Human tumor necrosis factor- α receptor: Purification by immunoaffinity chromatography and initial characterization. *J Biol Chem* 263: 190–198.
- Sugimoto M, Manabe N, Kimura Y, Myomoto A, Imai Y, Ohno H and Miyamoto H (1998) Ultrastructural changes in granulosa cells in porcine antral follicles undergoing atresia indicate apoptotic cell death. *J Reprod Dev* 44: 7–14.
- Tilly JL, Kowalski KI, Johnson AL and Hsueh AJW (1991) Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology* 129: 2799–2801.
- Tilly JL and Hsueh AJW (1992) Apoptosis as the basis of ovarian follicular atresia. In: Hillier SG (ed.) *Gonadal Development and Function* (pp. 157–165). Raven Press, New York.
- Yamada T, Horiuchi M and Dzau VJSO (1996) Angiotensin II type 2 receptor mediates programmed cell death. *Prod Natl Acad Sci USA* 93: 156–160.
- Yonehara S, Ishii A and Yonehara M (1989) A cell-killing monoclonal antibody (Anti-Fas) to a cell surface antigen co-down-regulated with the receptor of tumor necrosis factor. *J Exp Med* 169: 1747–1756.
- Yoo J, Stone RT and Beattie CW (1996) Cloning and characterization of the bovine Fas. *DNA Cell Biol* 15: 227–234.