MOLECULAR REGULATORY MECHANISMS OF GRANULOSA CELL APOPTOSIS DURING FOLLICULAR ATRESIA IN PORCINE OVARIES

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Introduction

In mammalian ovaries, more than 99% of the follicles undergo the degenerative change known as atresia at varying stages of follicle development. A number of studies of follicular atresia have revealed the morphological and biochemical characteristics of atretic follicles. Recent findings have suggested that the degeneration of atretic follicles in mammalian ovaries can be explained, at least in part, by apoptotic death of granulosa and theca interna cells. We confirmed that apoptosis occurs in granulosa cells but not cumulus cells in the atretic Graafian follicles from porcine ovaries[1]. Apoptosis is the result of many stimuli that act through many signal transduction pathways culminating in the activation of endonucleases. 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. In the present study, we produced monoclonal antibodies that recognize cell-surface antigens of granulosa cells and induce apoptosis of cultured granulosa cells. Such antibodies, especially PFG-1 that induces apoptosis of cultured granulosa cells, are good probes for studying the intracellular pathways of granulosa cell apoptosis in porcine atretic follicles.

Materials and Methods

The isolated granulosa cells prepared from healthy antral follicles of pig ovaries were used as antigens for immunization and as target cells in cell-killing activity assay, for observation of nuclear morphology and DNA electrophoresis. According to the standard procedure, female BALB/c mice

were immunized with the isolated granulosa cells, and then the spleen cells from immunized mice, which produced anti-granulosa cell antibodies, were fused with Sp2/O-Ag14 mouse myeloma cells. As only IgM antibodies against Fas-antigen had cell-killing activity[2], IgM antibody-producing hybridomas were selected. The hybridoma cells producing IgM antibodies against the granulosa cells were screened by ELISA and immunofluorescent staining techniques. Then, the hybridoma cells that produced antibodies with granulosa cell-killing activity were selected. Finally, one hybridoma clone, named PFG-5, was selected. Indirect immunofluorescence analysis of cryostat sections of ovaries was used to determine the target specificity of the PFG-5 antibody. The antigens on healthy granulosa cells were characterized by two-dimensional (2D)-Western blotting. To confirm granulosa cell apoptosis mediated by PFG-5 antibody, the isolated granulosa cells prepared from healthy follicles were co-cultured with PFG-5 antibody at concentrations varying from 0.0001 to 1,000 μg/ml, for 1 to 48 hr at 37 °C, and then the morphology of the nuclei was observed by staining with Hoechst 33258 under a fluorescence microscope, apoptosis was determined by DNA electrophoretic analysis and cell cycle analysis using a fluorescence activated cell sorter (FACS).

Results and Discussion

As shown in Fig. 1, PFG-5 antibody, an IgM antibody against the granulosa cells, was strongly reactive with granulosa cells. The antibody did not label theca interna or externa cells, basement membrane, or ovarian stroma cells. Moreover, the antibody showed no specific binding to pig stomach, small intestine, large intestine, liver, pancreas, lung, kidney, testis, adrenal grand, heart, spleen or brain, or ovaries of rodents or ruminants.

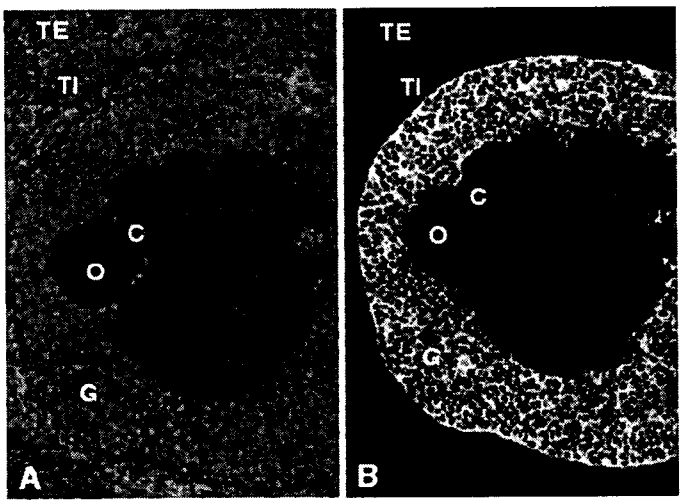

Fig. 1. Composite confocal images of healthy follicles. Frozen section of porcine ovary was stained with Hoechst 33258 (A) to visualize the cell nuclei. The same section was stained with PFG-5 antibody and with FITC-conjugated anti-mouse IgM, to visualize the distribution of granulosa cell membrane antigens (B). The

follicle was optically sectioned at 0.5 μm and five serial images were generated using the confocal microscope. PFG-5 antibody showed strong fluorescent staining on granulosa cells (G). No fluorescent staining of cumulus cells (C), oocyte (O), theca interna (TI) or externa (TE) layers was observed. x 200.

The antigens on healthy granulosa cells were characterized by 2D-Western blotting, and one specific spot (PGF-5 antigen; 55 kD, pI 5.9) was observed.

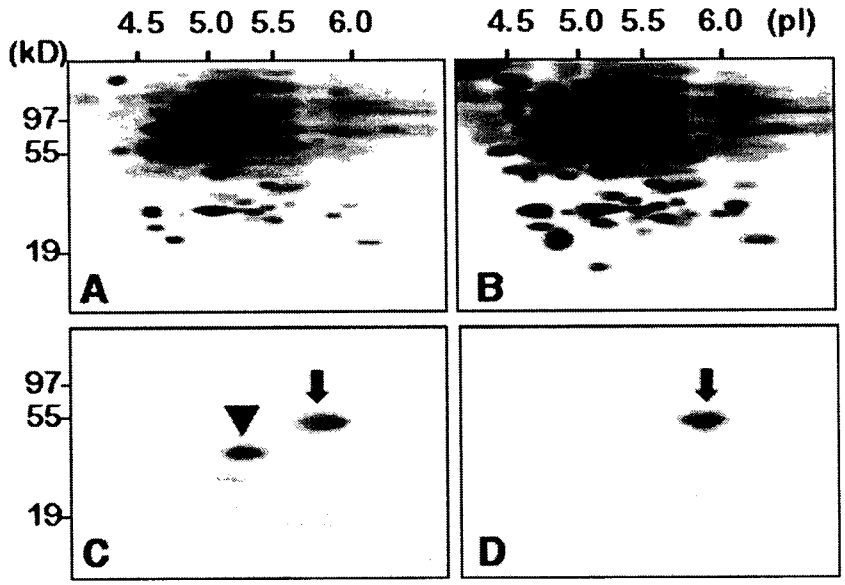

Fig. 2. Representative results of 2D-Western blotting analysis of granulosa cell-membrane antigens recognized by PFG-5 antibody (C and D). Granulosa cell membrane fractions prepared from healthy (A and C) and atretic (B and D) follicles were separated by 2D-PAGE. Separated protein spots in gels were detected by Coomassie brilliant blue staining (A and B). After electrophoresis, the protein spots were transferred onto nitrocellulose sheets and the antigens were visualized by PFG-5 antibody. Two specific spots (42 kD-pI 5.2 and 55 kD-pI 5.9: arrowhead and arrow, respectively) were observed in healthy follicles, (C), but the spot of 42 kD-pI 5.2 disappeared in atretic follicles (D).

Granulosa cell apoptosis mediated by PFG-5 was determined by assessment of nuclear morphology, DNA electrophoretic analysis and cell cycle analysis using a fluorescence activated cell sorter (FACS). The isolated granulosa cells were co-cultured with PFG-5 at concentrations varying from 0.001 to 100 μg/ml, for 1 to 48 hr at 37 °C, and then the morphology of the nuclei was observed by staining with Hoechst 33258 under a fluorescence microscope. When the isolated granulosa cells were cultured without any additive for 3 hr, only round weakly fluorescent healthy granulosa cell nuclei were observed. When the cells were co-cultured with at least 0.01 μg/ml PFG-5 for 3 hr, many small condensed fluorescent signals (apoptotic bodies, a morphological hallmark of apoptosis) were

observed. After incubation, DNA samples from these isolated granulosa cells were electrophoresed in 2% agarose gels, and the DNA displayed a ladder pattern (biochemical hallmark of apoptosis). However, DNA samples of the cells co-cultured without any additive displayed no such pattern on electrophoresis. The granulosa cells were co-cultured with 0.1 μg/ml PFG-5 for 3 hr, and then the percentages of cells with degraded DNA were determined by FACS analysis. No degraded DNA was observed in cells co-cultured without any additive. However, high percentages of degraded DNA, representing apoptotic bodies, were demonstrated in cells co-cultured with PFG-5.

We confirmed that granulosa cells undergo apoptosis and that no apoptotic cell death occurs in cumulus cells in pig atretic follicles[1]. However, it has not been determined which trigger molecules induce granulosa cell apoptosis, or how intercellular apoptotic signals are transmitted in the granulosa cells. 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 pathways in granulosa cells. We generated PFG-5 that reacted against two cell-surface proteins. In rodents, the Fas/APO-1/CD-95, which is a transmembrane glycoprotein that belongs to the TNF/NGF receptor family and mediates apoptosis in a variety of lymphoid and tumor cells[2], can mediate granulosa cell apoptosis in ovarian follicle atresia and luteal cell degeneration[3]. However, it has not been clarified whether this protein mediates apoptosis in pig ovaries. Our preliminary comparative studies of the progression of granulosa cell apoptosis in the atretic follicles revealed histochemically that there are species-specific differences in the apoptotic process in granulosa cells[4]. Briefly, apoptosis demonstrated histochemically by TUNEL staining was seen in scattered granulosa cells located on the inner surface of the follicular wall of porcine ovaries. In contrast, such apoptotic granulosa cells were demonstrated on the outer surface of the follicular wall of bovine ovaries. In rodents, such apoptotic cells were seen randomly throughout the follicular epithelial wall. These observations indicated that local regulation mechanisms of granulosa cell apoptosis are different among mammalian species. Tumor necrosis factor (TNF) also induces apoptosis in a variety of tumor cells, and the TNF-receptor 1 (TNFR1) can also mediate apoptosis[5]. The molecular weights of Fas and TNFR1 are 45 and 65 kD, respectively. Fas was histochemically detected in the granulosa cells of both healthy and atretic follicles in the ovaries of rodents[3], but TNFR1 was not detected in the ovarian follicular cells. The molecular weight of the granulosa cell-surface antigen recognized by PFG-5 is 55 kD, and the antigen visualized histochemically by PFG-5 was only detected in the granulosa cells. Such differences in localization dependent on the stage of follicle development are important when considering the physiological function of the cell death receptors.

We concluded that the antigens recognized by PFG-5 may belong to the TNF/NGF receptor family but not Fas or TNFR1. PFG-5 antibody will be useful as a probe to investigate the cell death receptor on the granulosa cell membrane and its natural ligands, and to define the intercellular

pathway of apoptotic signal transduction in granulosa cells of porcine ovaries. Biochemical details of this cell death receptor of the granulosa cells should be elucidated in future studies.

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