0014-4754/96/070647-05$1.50 + 0.20 © Birkhäuser Verlag Basel, 1996

Research Articles

Apoptosis occurs in granulosa cells but not cumulus cells in the atretic antral follicles in pig ovaries

N. Manabe*, Y. Imai, H. Ohno, Y. Takahagi, M. Sugimoto and H. Miyamoto

Department of Animal Science, Kyoto University, Kyoto 606-01 (Japan), Fax +81 75 753 6340
Received 3 July 1995; received after revision 23 August 1995; accepted 9 November 1995

Abstract. The porcine antral follicles, 3–6 mm in diameter, were dissected from the ovaries of mature pigs, and then granulosa and cumulus cells were isolated from each follicle. In atretic follicles, high activity of neutral Ca^{2+}/Mg^{2+}-dependent endonuclease and DNA ladder formation, estimated by electrophoresis, were noted in granulosa cells but not in cumulus cells. Extremely low activity of the endonuclease and no DNA ladder formation were observed in both types of cells obtained from healthy follicles. Moreover, apoptotic cells were observed histochemically among granulosa cells only. A good correlation ($r = 0.987$) between the endonuclease activity of granulosa cells and the progesterone/estradiol ratio of follicular fluid in each follicle was found. These results suggest that apoptosis occurs in granulosa cells but not cumulus cells in the atretic antral follicles in pigs.
Key words. Apoptosis; Ca^{2+}/Mg^{2+}-dependent endonuclease; cumulus cell; granulosa cell; pig.

Apoptotic cell death of granulosa cells of rabbit Graafian follicles with involution or atresia was first observed in 1885 by Flemming[1], who called it chromatolysis. Recent studies of follicular atresia in the pig have shown that the degeneration of the atretic follicles can be explained in part by apoptotic death of granulosa and theca interna cells[2,3], but degenerative changes in cumulus cells during follicular atresia are unknown. Cells undergoing apoptosis exhibit a condensed nuclear structure, compacted cytoplasmic organelles, decreased cell size and DNA fragmentation that may be regulated by an endogenous neutral Ca^{2+}/Mg^{2+}-dependent endonuclease[4]. The aim of the present study was to define the degenerative changes in cumulus cells and to observe changes in the neutral Ca^{2+}/Mg^{2+}-dependent endonuclease activity in granulosa and cumulus cells during atresia in porcine antral follicles. In addition, steroid hormone concentrations in follicular fluid were determined, and in situ analysis of DNA fragmentation was performed on histological sections of follicles using the DNA 3′-end labelling technique.

Materials and methods

Animals and recovery of follicular granulosa and cumulus cells. Ovaries recovered at a local slaughterhouse from gilts were rinsed in Ca^{2+}/Mg^{2+}-free Hanks balanced salt solution (HBSS) + 10 mM EDTA disodium salt (modified HBSS) (Gibco BRL, Grand Island, NY), while individual preovulatory antral follicles 3–6 mm in diameter were dissected free from extraneous tissue. The follicles were punctured over a 1.5-ml centrifuge tube to collect follicular fluid. Follicular fluid from each follicle was separated from possible cellular material by centrifugation at 600 × *g* at 4 °C for 5 min, and then stored at −80 °C until assay for estradiol and progesterone. According to a modification of the method of Guthrie et al.[5], granulosa and cumulus cells were isolated from each follicle. Briefly, each follicle was placed in modified HBSS in a plastic dish and cut into two pieces with small scissors. Under a surgical dissecting microscope, the oocyte-cumulus cell complex was released into modified HBSS by scraping the inner surface of follicles with forceps, and then oocytes were removed with a Pasteur pipette. Granulosa cells were scraped from the follicle wall into modified HBSS. Follicles were classified as morphologically atretic or healthy by the presence or absence of cellular debris in their isolated granulosa cells[6]. Granulosa and cumulus cells of each follicle were transferred into individual 1.5-ml centrifuge tubes, suspended in 1 ml of modified HBSS + 6.8 mM EGTA (Sigma Chemical Co., St. Louis, MO), and incubated for 15 min at room temperature (RT). The cells were centrifuged at 600 × *g* for 5 min at RT. The supernatant was decanted and the cells were resuspended in 1 ml of modified HBSS. Aliquots of the cells were counted using a hemocytometer plate.

Neutral Ca^{2+}/Mg^{2+}-dependent endonuclease assay and DNA analysis. The neutral Ca^{2+}/Mg^{2+}-dependent endonuclease activity was assessed as described by Zeleznik et al.[7]. Briefly, nuclear pellets were highly purified from isolated granulosa and cumulus cells, and resuspended in either reaction buffer (10 mM Tris-HCl, 25 mM NaCl and 0.34 M sucrose, pH 7.0) + 1 mM $CaCl_2$

* Corresponding author.

or reaction buffer + 1 mM $CaCl_2$ and 5 mM $MgCl_2$. Incubations were terminated at time 0 by addition of stop solution (0.15 mM EDTA and 35 mM EGTA), or continued at 37 °C for 10 min and terminated thereafter. Low and high molecular weight DNA fractions were separated from each nuclear pellet by centrifugation at 9000 × *g* for 20 min at 4 °C, and DNA contents were determined by fluorescence assay using 4,6-diamidino-2-phenylindole (Sigma)[8]. DNA standards were prepared from calf thymus DNA (Sigma). The percentage of DNA degraded in each nuclear incubation was calculated as a percentage of the amount of low molecular weight DNA divided by the total DNA content (low plus high molecular weight DNA). DNA samples of the endonuclease assay and cellular total DNA prepared as described previously[9] were electrophoresed in 2% agarose gels with 40 mM Tris-acetate (pH 8.1) containing 2 mM EDTA, 18 mM NaCl and 10 µg/ml ethidium bromide at 60 V for 90 min. Gels were photographed with ultraviolet transillumination.

RIA of follicular fluid steroid hormones. Concentrations of steroid hormones were determined in aliquots of follicular fluid using the procedures described by Guthrie et al.[10]. Briefly, frozen follicular fluid from each follicle was diluted 100-fold with Medium 199 solution (Gibco BRL). Levels of estradiol-17β and progesterone in diluted fluid samples were quantified in duplicate without solvent extraction via double antibody [^{125}I] ligand RIA kits (BioMérieux, Marcy-l'Etolle, France).

In situ end labelling of fragmented DNA and histology. Apoptotic cells were demonstrated histochemically as described by Billig et al.[11]. Briefly, paraffin-embedded sections of healthy and atretic follicles mounted on glass slides precoated with 3-amino-propyltriethoxysilane (Aldrich Chemical Co., Milwaukee, WI) were deparaffinized in xylene, and rehydrated through a graded alcohol series. Tissue sections were incubated with proteinase K (10 µg/ml) for 10 min at RT, washed in distilled water and then treated with 2% H_2O_2 in methanol for 10 min at RT to block endogenous peroxidase. The sections were incubated with terminal deoxynucleotidyl transferase (1 U/µl; TDT, Boehringer Mannheim, Indianapolis, IN) solution containing 45 µM ddATP and 5 µM digoxigenin-11-2′,3′-deoxyuridine-5′-triphosphate (DIG-ddUTP, Boehringer Mannheim) for 1 h at 37 °C, and then immersed in stop solution (double-strength salt sodium citrate buffer: 0.3 M NaCl, 30 mM trisodium citrate, pH 7.0). After washing with PBS, the sections were incubated with peroxidase-labelled anti-digoxigenin antibody solution for 30 min at RT, and then washed in PBS. The slides were incubated in a solution of 3,3′-diaminobenzidine (0.1% in 0.05 M Tris-HCl, pH 7.2) and 0.002% H_2O_2 for 5 min at RT. They were then rinsed in distilled water and counterstained with 0.2% methyl green solution, dehydrated through a graded ethanol series, cleaned in xylene and mounted. The following positive and negative controls were included in each experimental run: as negative controls, the sections were incubated with the omission of either TDT or DIG-ddUTP. As a positive control, tissue sections were treated with DNase I (1 µg/ml; Boehringer Mannheim) for 10 min at RT before exposure to TDT, and paraffin sections prepared from young adult rat testis were used as physiological positive controls[12]. Adjoining sections from each specimen were stained with hematoxylin and eosin for morphological analysis.

Statistical analysis. Analysis of variance was carried out with the StatView IV program using a Macintosh computer. Differences with a probability of $p < 0.05$ were considered significant.

Table 1. Ca^{2+}/Mg^{2+}-dependent endonuclease activities[a] in granulosa and cumulus cells of healthy and atretic follicles of pigs.

	Granulosa cells	Cumulus cells
Healthy follicles	1.6 ± 0.5	1.1 ± 0.6
Atretic follicles	44.4 ± 13.0[b]	1.5 ± 0.4

[a]Ca^{2+}/Mg^{2+}-dependent endonuclease activities are represented as percentages of DNA degraded during incubation. Each value is the mean of 20 follicles and expressed as mean ± SD.
[b]$p < 0.001$ vs healthy follicles.

Table 2. Follicular fluid progesterone and estradiol concentrations, and progesterone/estradiol ratios in healthy and atretic follicles of pigs[a].

	Progesterone (ng/ml)	Estradiol (ng/ml)	Progesterone/ estradiol ratio
Healthy follicles	126.5 ± 7.1	19.2 ± 1.9	6.6 ± 0.7
Atretic follicles	284.4 ± 30.4[b]	2.5 ± 0.6[b]	117.6 ± 23.4[b]

[a]Each value is the mean of 20 follicles and expressed as mean ± SD.
[b]$p < 0.001$ vs healthy follicles.

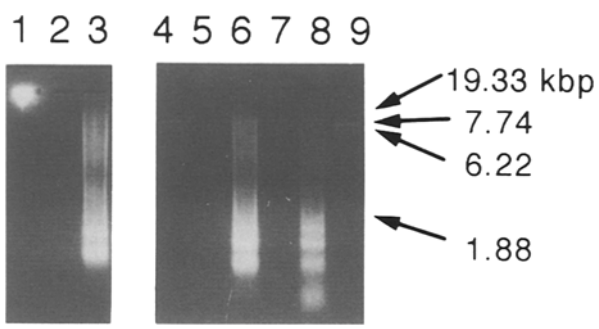

Figure 1. Electrophoretic analysis of DNA fragments in granulosa and cumulus cells obtained from atretic follicles. Total DNA prepared from cumulus and granulosa cells (lanes 1 and 3, respectively), low molecular weight DNA samples of the endonuclease assay prepared from cumulus (lanes 5 and 7) and granulosa (lanes 6 and 8) cells in each follicle, and size markers (lane 4 and 9) are shown. DNA fragmentation was observed only in granulosa cells, and not in cumulus cells.

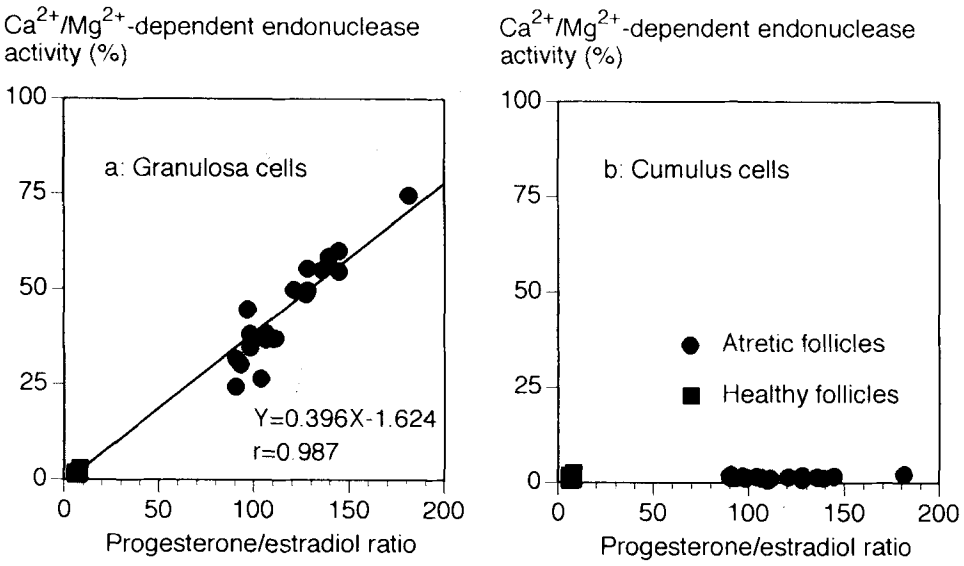

Figure 2. Correlations between the progesterone/estradiol ratio of follicular fluid and the neutral Ca^{2+}/Mg^{2+}-dependent endonuclease activity of granulosa (*a*) and cumulus cells (*b*). A good correlation ($r = 0.987$) between the progesterone/estradiol ratio and the endonuclease activity was found only in granulosa cells.

Results and discussion

In atretic follicles, high activity of neutral Ca^{2+}/Mg^{2+}-dependent endonuclease (in the presence of 1 mM Ca^{2+} and 5 mM Mg^{2+}) was noted only in granulosa cells and not in cumulus cells ($p < 0.001$) (table 1). Low activities ($< 1.6\%$) of the endonuclease were noted in both granulosa and cumulus cells prepared from healthy follicles. Extremely low activities ($< 1.0\%$) of Ca^{2+}-dependent endonuclease (in the presence of 1 mM Ca^{2+}) were demonstrated in all cases. As shown in figure 1, total DNA prepared from granulosa cells of atretic follicles displayed a ladder pattern (hallmark of apoptosis) on gel electrophoresis, but DNA prepared from cumulus cells of the same atretic follicles displayed no such pattern. In addition, using the endonuclease assay, DNA ladder formation was observed in the low molecular weight DNA fraction prepared from granulosa cells only, but not from cumulus cells. The most widely used marker for apoptosis has been the unique feature of internucleosomal cleavage of cellular DNA, catalyzed by Ca^{2+}/Mg^{2+}-dependent endonuclease activity[2,7]. This enzyme attacks linker DNA located between the nucleosomal units spaced every 185 bp along the DNA molecule, resulting in a characteristic ladder of DNA fragments. Recent studies have shown that apoptotic fragmentation of DNA occurs in granulosa cells of atretic but not of healthy porcine follicles[2,3]. In atretic rat follicles, neutral Ca^{2+}/Mg^{2+}-dependent endonuclease activity increases in granulosa cells[7]. However, no detailed information concerning of the demise of cumulus cells has yet been reported. The present results indicate that no apoptotic cell death occurs in cumulus cells during follicular atresia in pigs.

The ranges of concentrations for estradiol (1.7 to 23 ng/ml), and progesterone (115 to 335 ng/ml) in follicular fluid correspond well with previously reported values[13,14] (table 2). Follicular fluid progesterone concentration was 2.25-fold higher ($p < 0.001$) in atretic follicles than in healthy follicles. In contrast, estradiol concentration was lower in atretic than in healthy follicles ($p < 0.001$). Sex steroids are important for intraovarian regulation of follicle growth and atresia, and the profile of sex steroid production differs between healthy and atretic follicles[10,14,15]. In pigs, estradiol production by atretic follicles is decreased, but production of progesterone is increased[10]. Estrogen is essential for follicle growth and differentiation, and the absolute number of atretic follicles decreases after estrogen treatment[16]. The progesterone/estradiol ratio of follicular fluid in each follicle provides an index of follicular growth or atresia[10]. A good correlation ($r = 0.987$, $n = 40$, $p < 0.001$) between neutral Ca^{2+}/Mg^{2+}-dependent endonuclease activity of granulosa cells and the progesterone/estradiol ratio of follicular fluid was found in each follicle (fig. 2a). However, there was no correlation between the endonuclease activity of cumulus cells and the progesterone/estradiol ratio of follicular fluid (fig. 2b). These findings are highly suggestive of a lack of apoptosis in cumulus cells obtained from porcine atretic follicles.

In the early stage of atresia the oocyte-cumulus cell complex is preserved (fig. 3a), but scattered granulosa cells with condensed nuclei and DNA fragmentation were observed on the inner side of the granulosa cell layer by conventional histology (fig. 3b) and by in situ DNA 3′-end labelling (fig. 3c). In the late stage of atresia, the oocyte-cumulus cell complex is suspended in follicu-

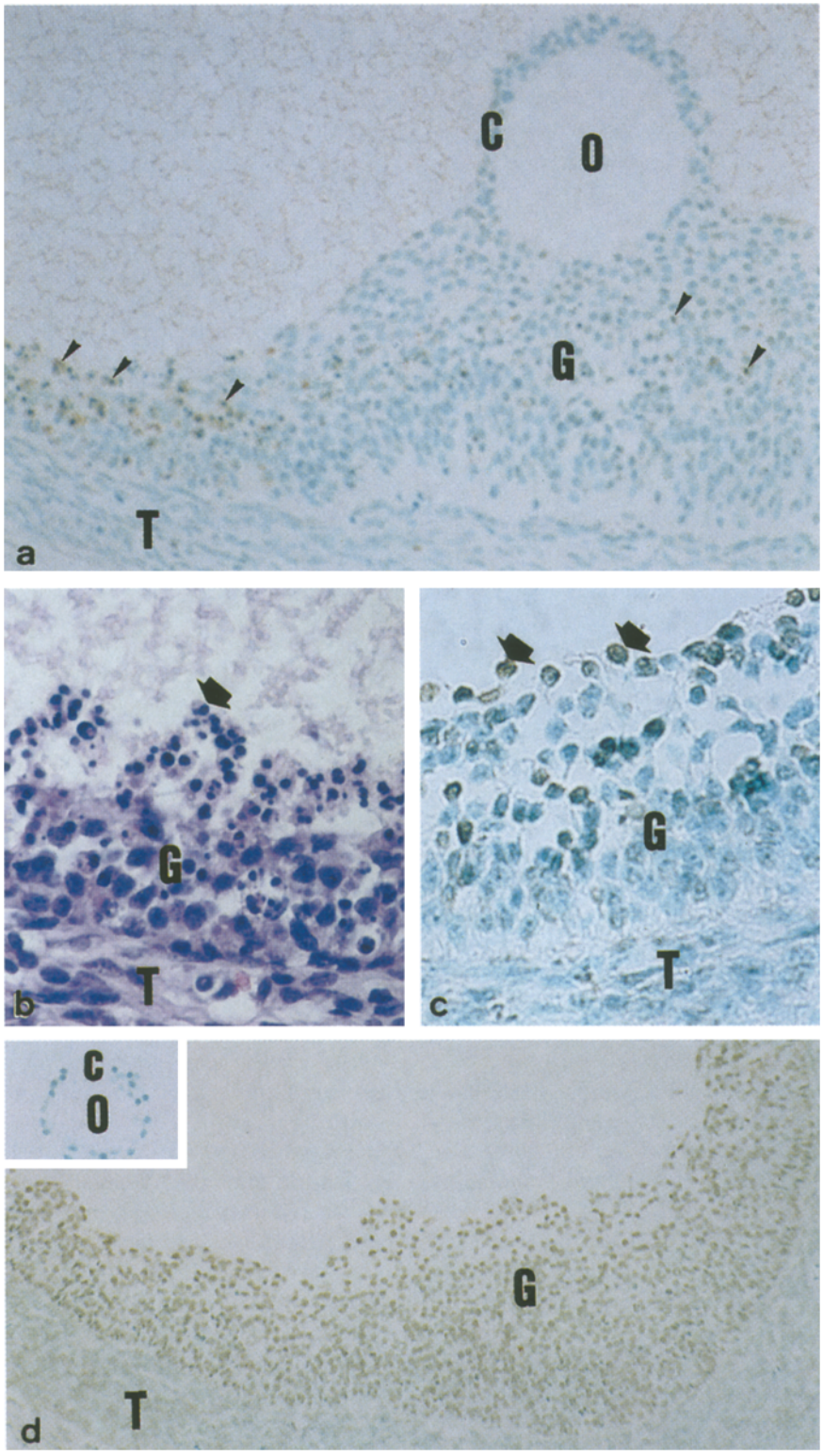

Figure 3. Porcine antral follicle sections stained for in situ detection of apoptosis by the histochemical DNA 3′-end-labelling technique (*a*, ×100; *c*, ×200; and *d*, ×50) and stained with hematoxylin and eosin (*b*, ×200). In the early stage of follicular atresia, DNA fragmentation in nuclei (arrows in *a* and *c*) was demonstrated in granulosa cells (G) scattered on the inner surface of the follicular wall, but not in cumulus cells (C) or theca interna cells (T). Scattered granulosa cells (G) with condensed nuclei (arrow) were observed on the inner side of the follicle (*b*). In the late stage of atresia, most granulosa cells (G) suspended in the follicular fluid were positive for genomic DNA fragmentation (*d*), but cumulus cells were negative (inset, ×100).

lar fluid. No apoptotic cumulus cells were observed by in situ labelling, and most granulosa cells with apoptosis were isolated and suspended in follicular fluid (fig. 3d). From these and our previous[17] results obtained using the histochemical DNA 3′-end labelling technique which allows the identification of apoptosis in situ on a cell-by-cell basis with preservation of follicular architecture[11], granulosa cells scattered on the inner surface of the follicular wall appear to be the first to undergo apoptosis. In an advanced stage of follicular atresia, detachment and degeneration of the granulosa cell layer and fragmentation of basal lamina[17] will occur. Remarkably, no chromatin degradation or apoptosis occurs in cumulus cells during porcine follicular atresia.

In conclusion, the biochemical and histochemical results of the present study demonstrate that apoptosis occurs in granulosa cells but not in cumulus cells during ovarian follicular atresia in pigs.

Acknowledgements. We thank Drs T. Miyano and S. Kato (Kobe University, Kobe, Japan) for advice on the preparation of porcine ovarian cells. A part of this work was supported by a grant-in-aid to N.M. and H.M. from the Ministry of Education, Science and Culture, Japan.

1 Flemming, W., Arch. Anat. EntGesch. (1885) 221.
2 Tilly, J. L., Kowalski, K. I., Johnson, A. L., and Hsueh, A. J. W., Endocrinology *129* (1991) 2799.
3 Tilly, J. L., Kowalski, K. I., Schomberg, D. W., and Hsueh, A. J. W., Endocrinology *131* (1992) 1670.
4 Arends, M. L., Morris, R. G., and Wyllie, A. H., Am. J. Pathol. *136* (1990) 593.
5 Guthrie, H. D., Welch, G. R., Cooper, B. S., Zakaria, A. D., and Johnson, L. A., Biol. Reprod. *50* (1994) 1303.
6 Dailey, R. A., Clark, J. R., First, N. L., Chapman, A. B., and Casida, L. E., J. Anim. Sci. *41* (1975) 835.
7 Zeleznik, A. J., Ihrig, L. L., and Bassett, S. G., Endocrinology *125* (1989) 2218.
8 Kapuscinski, J., and Skoczylas, B., Anal. Biochem. *83* (1977) 252.
9 Tilly, J. L., and Hsueh, A. J. W., J. cell. Physiol. *154* (1993) 519.
10 Guthrie, H. D., Cooper, B. S., Welch, G. R., Zakaria, A. D., and Johnson, L. A., Biol. Reprod. *52* (1995) 920.
11 Billig, H., Furuta, I., and Hsueh, A. J. W., Endocrinology *133* (1993) 2204.
12 Arends, M. J., Wyllie, A. H., Int. Rev. expl Pathol. *32* (1991) 223.
13 Babalola, G. O., and Shapiro, B. H., J. Reprod. Fertil. *84* (1988) 79.
14 Coley, A. J., Howard, H. J., Slanger, W. D., and Ford, J. J., Biol. Reprod. *51* (1994) 655.
15 Maxson, W. S., Haney, A. F., and Schomberg, D. W., Biol. Reprod. *33* (1985) 495.
16 Harman, S. M., Louvet, J. P., and Ross, G. T., Endocrinology *96* (1975) 1145.
17 Imai, Y., Manabe, N., Ohno, H., Takahagi, Y., and Miyamoto, H., J. Reprod. Dev. (in press).