—Research Note—

Deficient Proliferation and Apoptosis in the Granulosa and Theca Interna Cells of the Bovine Cystic Follicle

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Abstract. The aim of the present study was to examine the frequencies of cell proliferation and death of granulosa and theca interna layers during development of cystic follicles in order to understand the mechanisms of cystic follicle formation. Paraffin sections of cystic follicles were immunostained with antibodies against proliferating cell nuclear antigen (PCNA) and cleaved caspase-3 in order to observe proliferating and apoptotic cells, respectively. The concentrations of estradiol-17 β and progesterone in the follicular fluid of these follicles were measured by ELISA. The granulosa and theca interna layers contained both PCNA- and caspase-3-positive cells, although their numbers were limited. There was significant negative correlation between the estradiol-17 β and progesterone concentrations in the follicular fluid. Regression analysis revealed no significant correlation, except for that between the PCNA-positive cells in the theca interna and the caspase-3-positive cells in the granulosa layer. These results indicate that the granulosa and theca interna cells of the cystic follicle show weak proliferative activity and low apoptotic frequency; this implies that the cystic follicle grows slowly and then maintains a static condition without degeneration, which leads to long-term persistence of the follicle.

Key words: Apoptosis, Cell proliferation, Cow, Cystic follicle, Granulosa

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Ovarian cysts (≥ 25 mm in diameter) are a significant cause of reproductive failure in cattle. The disease process is a consequence of failure of a mature follicle to ovulate at the appointed time during the estrous cycle [1]. Follicular cysts are reportedly caused by an endocrine imbalance in the hypothalamo-hypophyseal-gonadal axis [2–4]. Inappropriate productions of LH, steroid hormones and steroid synthesizing enzymes have been observed in cows with cystic follicles [3–10]. Delay of follicle regression after ovulation disorder is alternative cause of cysts [4] because a preovulatory follicle that cannot be ovulated will not grow further if it regresses immediately; therefore, no cystic

follicle will be formed.

Granulosa and theca interna cells proliferate in the healthy growing follicle, whereas cell proliferative activity decreases in the atretic follicle [15]. In the previous report, the frequencies of cell proliferation in the granulosa and theca interna layers were much lower in the cystic follicle than in the atretic follicle [16]. However, the authors of this report classified cystic follicles into only three categories, and no other reports have examined the cell proliferation of cystic follicles in more detailed stages.

In atretic follicles, apoptosis occurs to eliminate granulosa and theca interna cells [11, 12]. The frequencies of apoptotic cells in the granulosa and theca interna layers increases during the atretic process [11, 12]. Similarly, cystic follicles contain granulosa cells during the early stage, whereas apo-

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ptosis results in disappearance of granulosa layers as the cystic stage progresses [12]. However, apoptotic cells were seen frequently in the late stage (without granulosa layer) than the early stage (with granulosa layer) of cystic follicle [12]. Isobe and Yoshimura [12] classified cystic follicles into only two groups, those with or without granulosa layers. More precise investigation of the frequency of apoptosis in the cystic follicle during development is required. The major downstream effector of apoptosis is caspase-3, which in turn activates endogenous endonucleases responsible for DNA fragmentation [13]. Therefore, induction of caspase-3 is a useful indicator of apoptosis during the early stage compared with the TUNEL method [14]. In the present study, caspase-3 antibody was employed to identify the apoptotic cells of cystic follicles.

A cystic follicle containing granulosa cells is characterized by a high concentration of estradiol-17 β and low concentration of progesterone in its follicular fluid [7, 17]. However, these concentrations are changed drastically by disappearance of the granulosa cells [7, 17]. Since estradiol-17 β originates in granulosa cells, elimination of the granulosa cells results in a reduction in the estradiol-17 β concentration. Therefore, measurement of the estradiol-17 β concentration enables determination of the stage of cystic follicles [17].

The aim of the present study was to examine the cell proliferation and cell death of the granulosa and theca interna layers during the developing stage of the cystic follicle. We investigated the frequency of cell proliferation and cell death of their granulosa and theca interna layers of cystic follicles with immunohistochemistry and explored relationship with the steroid hormone profiles of the follicular fluid.

Materials and Methods

Collection and preparation of tissue

Ovaries with cystic follicles (n=19) were collected from Holstein-Friesian cows at a slaughterhouse. Follicular cysts were diagnosed based on macroscopic characteristics (>25 mm in diameter [18]). The follicular walls of the cysts were thin and translucent in appearance. The cystic follicles used in this study showed no signs of luteinization. The walls of cystic follicle were cut with a razor blade in

order to collect the follicular fluid. The fluid from the follicle was collected into a beaker, centrifuged (1700 $\times$ g, 15 min) and stored at -30 C until hormone measurement. After removal of the follicular fluid, the walls of the cystic follicles were fixed with 10% (v/v) formalin in phosphate buffered saline (PBS; pH 7.4) and processed into paraffin sections. The sections (4- μ m thick) were air-dried on MAS-coated slides (Matsunami Glass Ind., Osaka, Japan) for immunohistochemistry.

Immunohistochemistry for PCNA and caspase-3

After deparaffinization, the sections were boiled in 0.01 M boric acid (pH=6.0) in an autoclave at 121 C for 1 min to retrieve the antigen for immunostaining with only caspase-3 antibody. Sections were incubated at room temperature for 2 h with mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA, Biomedex, Foster City, CA, USA) without dilution and with rabbit polyclonal antibody against cleaved caspase-3 (Cell Signaling Technology, Beverly, MA, USA) at 1: 800 dilution. After washing with PBS, the immunoreactions on the sections were detected using a histofine MAX PO (MULTI) kit (Nichirei, Tokyo, Japan) according to the manufacturer's protocol. Briefly, the sections were incubated with solution containing both peroxidase conjugated anti-rabbit and anti-mouse IgG (100 μ g/ml) for 60 min. Immunoreaction products were visualized by incubating the sections with a substrate solution consisting of 1 mg 3', 3'-diaminobenzidine and 10 μ l 2.5% H₂O₂ in 5 ml 0.05 M Tris-HCl (pH 7.5) and then counterstaining them with hematoxylin. Control slides were prepared in an identical manner except that the first antibody was replaced with 1 μ g/ml normal rabbit and mouse IgG (Vector Laboratories, Burlingame, CA, USA). Healthy and atretic follicles were occasionally seen within the cystic follicle wall. These healthy and atretic follicles contained PCNA-positive and caspase-3-positive cells in the granulosa layer, respectively (Fig. 1a and b). These results validate the present immunohistochemistry because PCNA and caspase-3 were found in the granulosa layers of healthy and atretic follicles, respectively [14, 16].

Enzyme immunoassay for estradiol-17 β and progesterone

After determination of the presence of a granulosa layer by immunohistochemistry, the

Fig 1. Light micrographs of sections immunostained with anti-PCNA (a, c) and caspase-3 (b, d) antibody in healthy (a), atretic (b) and cystic (c, d) follicles. Sections of negative control were incubated with normal rabbit IgG in place with the primary antibody (e). No staining is observed. G: Granulosa. TI: Theca interna. Bars = 50 μ m.

concentrations of estradiol-17 β and progesterone were measured in the follicular fluid of cystic follicle containing granulosa layer. The enzyme immunoassay procedure for progesterone and estradiol-17 β were performed as described previously [17, 19–23]. The antibody for progesterone was raised with rabbit [19] and that for estradiol-17 β was purchased from Cosmo Bio (Tokyo, Japan). The assay sensitivity and intra- and interassay

CVs were 5.5 pg/ml, 8.6–8.7% and 12.2–12.7% for progesterone and 16 pg/ml, 8.5–9.3% and 9.5–10.5% for estradiol-17 β , respectively.

Image analysis of immunopositive cells

Sections containing 13 follicles each were examined under a light microscope connected to a computerized image analysis system (Mac Aspect; Mitani, Fukui, Japan). The populations of PCNA-

Table 1. Correlations between steroid hormone concentrations in follicular fluid and frequencies of PCNA and caspase-3 immunoreactions in the bovine cystic follicles

		E ₂	P ₄	PCNA		Caspase-3	
				G	TI	G	TI
PCNA	E ₂	–	–0.47	–0.18	–0.30	–0.30	–0.29
	P ₄	0.04	–	–0.13	–0.37	0.06	0.18
	G	0.27	0.33	–	0.42	–0.17	0.43
	TI	0.15	0.10	0.07	–	0.54	–0.17
Caspase-3	G	0.14	0.42	0.27	0.02	–	–0.16
	TI	0.14	0.24	0.05	0.27	0.28	–

E₂: estradiol-17 β . P₄: progesterone. G: granulosa. TI: theca interna. Values in lower-right area: regression coefficients. Values in lower-left area: Probabilities.

and caspase-3-positive cells in the randomly chosen fields for the granulosa or theca interna layers were counted. The frequency of positive cells in the granulosa layer of a follicle was expressed as the percentage of positive cells per cells examined, and that in the theca interna was expressed as the number of positive cells per 10,000 μm^2 areas because there was a large amount of irregular intercellular space in the granulosa layer but not in the theca interna layer. The average of five counts was used as the number of positive cells in a follicle.

Statistical analysis

Correlation analysis was carried out using Pearson's correlation analysis followed by analysis for significance of the correlation coefficient by Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Caspase-3-positive cells were observed in the granulosa and theca layers of cystic follicles (Fig. 1d). However, a limited number of caspase-3-positive cells were seen in both the granulosa and theca interna layers. Similarly, the granulosa and theca interna layers of cystic follicles contained PCNA-positive cells, although their numbers were small (Fig. 1c).

The frequencies of PCNA- and caspase-3-positive cells were 17.6% (3.3–28.3) and 0.2% (0–1.2) in the granulosa layer and 16.9 (9.2–46.4) and 0.2 (0–1.0) cells/10,000 μm^2 in the theca interna layer, respectively. The concentrations of estradiol-17 β and progesterone ranged between 0 and 339 and 15 and

186 ng/ml, respectively, in the follicular fluid of granulosa-containing cystic follicles. There was significant negative correlation between the estradiol-17 β and progesterone concentrations in the follicular fluid ($P = 0.04$, Table 1). When regression analyses were conducted for PCNA-positive cells, caspase-3-positive cells in the granulosa and theca interna cells and the estradiol-17 β and progesterone concentrations in the follicular fluid, no significant correlations were found except for that between PCNA-positive cells in the theca interna and caspase-3 positive cells in the granulosa layer.

Discussion

The objective of the present study was to examine the cell proliferation and death in cystic follicles at various stages. The frequencies of apoptotic cells in the granulosa and theca interna layers had no relationship with the steroid hormone concentrations in the follicular fluid of cystic follicles. Similarly, there was no relationship between the frequencies of PCNA-positive cells in the granulosa and theca interna layers and the steroid hormone concentrations in the cystic follicular fluid. These results indicate that the incidence of apoptosis and proliferation of granulosa and theca cells is not dependent on the stage of the cystic follicle. The frequencies of PCNA-positive cells in the cystic follicles were quite low compared with those of healthy follicles (17.6 vs. 80% in the granulosa layer and 16.9 vs. 80 cells/10,000 μm^2 in the theca interna layer, [16]). Atretic follicles contained 60 PCNA-positive cells/200 cells in the granulosa layer and 10–20 PCNA-positive cells/20,000 μm^2 in the theca

interna layer [24]. Therefore, the proliferative activities of the granulosa and theca cells of cystic follicles were remarkably low and resembled those of atretic but not healthy follicles. Although apoptotic cells are localized widely in atretic follicles [11, 12], the frequency of caspase-3-positive cells is low in cystic follicles, as it is in healthy follicles [12], indicating that apoptosis is rare in cystic follicles. This low frequency in the cystic follicles is different from atretic follicles, which possess a high frequency of apoptotic cells in the granulosa and theca interna layers. Thus, weak proliferative activity and a low apoptotic frequency are novel characteristics of the cystic follicle; these characteristics imply that the cystic follicle grows slowly and then maintain a static condition without degeneration, which leads to long-term persistence of the follicle.

The granulosa cells are lost in the cystic follicle during the late stage [12, 16]. However, the present results indicate that extremely few granulosa cells degenerate through the process of apoptosis. It remains unclear whether other mechanisms participate in removal of granulosa cells from the cystic follicle.

There was significant positive correlation between the frequency of caspase-3-positive cells in the granulosa layers and the frequency of PCNA-positive cells in the theca interna layers of the cystic follicles. Theca interna layer contains highly developed vasculature [17, 22, 25, 26]. Since theca

interna cells proliferate actively and vasculature develops remarkably in healthy follicles [27], the vasculature in the theca interna layer appears to be strongly associated with proliferation of cells. Therefore, the current results indicate that even though apoptosis occurs in the granulosa layer, the vasculature in the theca interna layer can grow, resulting in accumulation of follicular fluid by permeation of serum components into the follicle. Cystic follicle may be able to grow to full size even during the stage when granulosa cells are dying as a result of apoptosis.

In conclusion, weak proliferative activity and low apoptotic frequency are observed in the granulosa and theca interna layers of the cystic follicle regardless of the concentration of steroid hormones; this implies that the cystic follicle grows slowly and then maintains a static condition without degeneration, which leads to the long-term persistence of the follicle.

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