Immunohistochemical localization of active caspase-3 in the mouse ovary: growth and atresia of small follicles

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Caspase-3 belongs to a family of highly conserved cysteine proteases that mediate the course of apoptotic cell suicide. It is recognized that ovarian follicular atresia is associated with apoptosis, a process that has been characterized mainly in larger antral follicles. The aims of this study were to investigate the expression of caspase-3 in the mouse ovary, and determine whether active caspase-3 is present within smaller follicles, which may constitute the resting pool. The inactive enzyme was expressed as a 32 kDa band on a western blot of tissue extracts, whereas the active form was localized immunohistochemically. Bromodeoxyuridine (BrdU) was administered to mice ($n = 7$) during a 12 h period and subsequently localized to identify potentially quiescent follicles. Measurements of BrdU-positive cells in

the mouse ovary were extrapolated with data obtained by morphometric analyses of small follicles using the nucleator technique. BrdU was incorporated into the granulosa cells of follicles regardless of size and the number of cells they contained, but was absent in a large proportion (89%) of small, single layered follicles. Active caspase-3 was localized to both the oocyte and granulosa cells of follicles that were considered to be undergoing atresia, but was not localized to the granulosa cells of any small, single layered follicles. The results of this study indicate that, in small follicles, granulosa cell proliferation occurs independently of the size of follicles and the number of constituent cells, and that follicles of this type may be inherently less susceptible to the normal physiological factors that induce atresia.

Introduction

The mammalian ovary contains follicles that are destined to undergo atresia unless rescued by endocrine or local paracrine survival factors (Kaipia and Hsueh, 1997). Atresia occurs as a consequence of apoptosis (programmed cell death) of constituent oocyte or follicle cells, or both, and is a highly structured sequence of biochemical events initiated in response to a variety of stimuli in the ovary, including growth factor deprivation (Kaipia and Hsueh, 1997), γ -irradiation (Lee *et al.*, 2000), accumulation of metabolites (Tilly, 1996) and induction via receptor mediated signals, such as the Fas ligand (Sakamaki *et al.*, 1997). Furthermore, the specific apoptotic events that take place in response to these stimuli are now becoming more distinct, including the Bcl-2 (and related protein) pathway (Tilly *et al.*, 1995) and those that are independent of mitochondrial disruption, such as the Fas receptor pathway (Hakuno *et al.*, 1996). The earliest known molecular convergence point for most of these cascades is the effector enzyme, caspase-3 (Cohen, 1997). This enzyme, when activated, exerts its effect downstream by cleaving essential nuclear proteins, including DNA fragmentation factor, poly (ADP-ribose) polymerase (PARP) and the structural nuclear lamins (Earnshaw *et al.*,

1999). The expression and activation of caspase-3 induce apoptosis in a number of different types of cell (Earnshaw *et al.*, 1999) and its expression has been found in the ovary of hens (Johnson and Bridgham, 2000), quails (Van Nassauw *et al.*, 1999), rats (Boone and Tsang, 1998) and mice and humans (Matikainen *et al.*, 2001). The localization of the active form of caspase-3 not only implicates it in follicular atresia but also serves as a useful and reliable marker for the identification of apoptotic cells.

The process of atresia can be recognized in follicles that have reached the multilayered–small preantral stage of development, a period when constituent granulosa cells express gonadotrophin receptors (Findlay and Drummond, 1999). Several reports indicate, however, that follicular atresia occurs at all stages of development, including smaller follicles classified as resting (Baker, 1963; Gougeon, 1996; Morita and Tilly, 1999; Perez *et al.*, 1999), which are not under the direct developmental control of gonadotrophins. As apoptosis is so prevalent in the ovary, some protective mechanisms may predominate in follicles that remain dormant throughout the course of the reproductive lifespan, thus making them more resistant to atresia.

Recruitment of healthy, resting follicles, however, involves the transformation of a squamous layer of pre-granulosa cells to a cuboidal phenotype (Eppig, 1991). It is often very difficult to distinguish the squamous and cuboidal cells morphologically in mice and, therefore, to

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determine whether granulosa cell division has started. A study in sheep (Lundy *et al.*, 1999) showed that proliferating cell nuclear antigen, a marker for DNA replication, is detectable in flattened granulosa cells of type 1 follicles. Similarly, Hirshfield (1991) demonstrated that flattened granulosa cells of rat follicles incorporate [³H]thymidine, indicating that granulosa cell proliferation precedes differentiation.

The aim of the present study was to use morphological characteristics, coupled with the incorporation of a thymidine analogue, bromodeoxyuridine (BrdU), to identify follicles considered to be quiescent, and to ascertain whether these follicles could be correlated with a biochemical marker of apoptosis, namely active caspase-3. This study highlights some important attributes relating to the growth and atresia of small follicles in the mouse ovary.

Materials and Methods

Animals

Normal outbred C57BL/6 mice were purchased from the Department of Laboratory Animal Sciences, University of Otago, Dunedin. The mice were housed under standard (12 h light:12 h dark) conditions and provided with commercial pelleted food and water *ad libitum*. Mice were killed by cervical dislocation and ovaries designated for morphometric analyses were collected from mice at 1 week ($n = 5$) and 6 weeks of age ($n = 6$). Caspase-3 expression and BrdU incorporation were examined in ovaries removed from separate groups of mice at 6 weeks of age ($n = 6$ and 7, respectively). The experimental use of these animals was approved by the University of Otago Animal Ethics Committee AEC No 73/99, 35/01.

Morphological identification of small ovarian follicles

Whole ovaries were fixed in Bouin's fluid, dehydrated and polymerized in glycolmethacrylate resin. Blocks were positioned isotropically using the 'orientator' (Mattfeldt *et al.*, 1990). Serial sections, 30 μ m in thickness, were generated using systematic random sampling strategies and then stained with periodic acid-Schiff. Follicles classified as small according to Pedersen and Peters (1968), and the absence of a discernible zona pellucida and/or thecal layer, were selected randomly under a $\times 100$ oil immersion objective and viewed under an Olympus AX70 microscope. Follicle and oocyte diameters were recorded when the section was focused through the oocyte nucleolus (SON) and measurements were obtained for an unbiased estimate of the number of granulosa cells using the 'nucleator' technique originally described by Bagger *et al.* (1993) and applied in sheep ovary by Lundy *et al.* (1999). In brief, the SON of a randomly selected follicle was brought into focus and the image captured on a computer monitor. As the image was focused through the section (at a predefined depth), only granulosa cell nuclei that came into focus but were not in focus at the bottom of the section were counted.

The real distances between each of these sampled nuclei and the oocyte nucleolus were measured with the NIH microscale software and entered into the following equation: $N_{NU} = 1/f_2 \cdot 2 \cdot \Sigma d/h$, where $1/f_2$ represents the fraction of the dissector in the field of view; 2 is a constant for isotropically orientated sections; and $\Sigma d/h$ is the sum of the distances recorded for each sampled follicle divided by the depth of sampling (z axis).

The BrdU-treated mice received an i.p. injection (0.2 ml) of 5 mg BrdU ml⁻¹ sterile saline (Sigma Chemical Co, St Louis, MO) three times in a 12 h period to correlate these attributes with growth or granulosa cell inactivity. A control mouse received sterile saline only. Small BrdU-positive follicles were localized using immunohistochemistry, and measurements through the SON were recorded.

Immunohistochemistry

All ovaries retrieved for immunohistochemistry were fixed in freshly prepared 4% (w/v) paraformaldehyde in 0.1 mol PBS l⁻¹ and processed overnight in paraffin wax. A systematic random sampling strategy (across all tissue) was used to yield > 130 and 100 sections of 4 μ m for BrdU and caspase-3 analysis, respectively. In brief, the paraffin wax was removed and the sections rehydrated and exposed to antigen unmasking in 0.1 mol Tris-HCl buffer l⁻¹ (pH 10) with 5% (w/v) urea by heating for 20 min in a Sharp 1000 watt microwave oven for caspase-3 or 0.1 mg proteinase K ml⁻¹ (Sigma) in 0.1 mol Tris-EDTA buffer l⁻¹ (pH 7.4) followed by 4 mol HCl l⁻¹ for BrdU. Peroxidase activity was quenched with 0.3% (v/v) H₂O₂. Sections were blocked with a 1:200 dilution of donkey serum and incubated at 37°C for 1 h with 2 μ g rabbit anti-active caspase-3 monoclonal antibody ml⁻¹ (Research Diagnostics Inc, Flanders, NJ) followed by biotinylated anti-rabbit IgG (Amersham Pharmacia Biotech, Bucks) for caspase-3 immunostaining. Sections were incubated with 1:50 anti-BrdU (Dako, Carpinteria, CA) followed by biotinylated anti-mouse IgG (Amersham) for BrdU detection. Signals were amplified with streptavidin-biotinylated horseradish peroxidase complex (Amersham), developed with 3-amino-9-ethylcarbazole (Sigma), counterstained with haematoxylin and analysed under a light microscope. In the control sections, the primary antibody was replaced with an equivalent amount of primary antibody dilution buffer or Ig isotype serum.

Western immunoblot analysis

Pairs of whole mouse ovaries were homogenized at 4°C in lysis buffer consisting of 50 mmol Tris-HCl l⁻¹ (pH 7.5), 5% (w/v) sodium dodecyl sulphate, 5% (v/v) glycerol, 6 mol urea l⁻¹ and 10 μ l protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 13 000 *g* for 30 min and the supernatants collected. Total protein was estimated with the Pierce BCA protein assay (Rockford, IL). Thirty micrograms of protein was loaded on to a 10% (w/v) SDS-polyacrylamide gel and separated under reducing

Table 1. Characteristics of small follicles in the mouse ovary

	Total number of small follicles studied	GCSON	Nucleator	Diameter of oocyte (μm)	Diameter of follicle (μm)	Number of granulosa cell layers
Postnatal (1 week)	59	4.1 (0, 12)	26.6 (0, 106.3)	23.5 (16.4, 39.4) ^a	31.7 (18.5, 52.7) ^a	1
Peripubertal (6 weeks)	70	4.0 (0, 18)	22.1 (2.5, 136.5)	17.5 (9.7, 36.2) ^b	25.6 (14.8, 57.6) ^b	1
BrdU-positive	18/178	N/A	N/A	> 14.5	> 23.3	≥ 1
Active caspase-3-positive	0/161	N/A	N/A	Not measured	Not measured	≥ 2

GCSON: values for the number of granulosa cells in the section through the oocyte nucleolus; nucleator: the total number of granulosa cells per follicle determined by the nucleator; BrdU: bromodeoxyuridine; N/A: not applicable.

The GCSON, nucleator, oocyte and follicle diameters are presented as geometric means and ranges (in parentheses).

^{ab}Values within the same column with different superscripts are significantly different ($P < 0.001$).

conditions using a BioRad MiniProtein II unit. Proteins were electrotransferred with a BioRad Mini TransBlot cell on to a nitrocellulose membrane, blocked with 3% (w/v) non-fat milk powder and 0.05% (v/v) Tween 20, and probed with 1:100 anti-caspase-3 polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA) for 3 h at room temperature. Membranes were incubated with an anti-goat horseradish peroxidase conjugated secondary antibody (Sigma) and developed with ECL chemiluminescence according to the manufacturer's instructions (Amersham). This procedure was repeated three times to confirm consistency within and between samples.

Statistical analysis

The data for follicle characteristics were represented as geometric means and ranges. All follicles within a treatment group were pooled for statistical purposes and therefore animal-to-animal variation was not considered. Relationships between the number of granulosa cells and oocyte or follicle diameters were analysed by regression analyses.

Results

Follicle characteristics

Small follicles were characterized by morphological measurements (Table 1). The number of constituent granulosa cells per follicle was consistent between the two age groups as confirmed by both the 'nucleator' and the number of granulosa cells in the section through the oocyte nucleolus. The number of granulosa cells recorded in the SON of small follicles was ≤ 18 in the postnatal and peripubertal age groups, which is consistent with the classification of small follicles reported by Pedersen and Peters (1968).

The size of follicles (diameter of oocytes and follicles) was significantly smaller in the peripubertal age group compared with the postnatal age group ($P < 0.001$). There

Fig. 1. Relationship between the diameter of small, single-layered follicles and the number of granulosa cells per follicle as determined by the 'nucleator' in mice. The diameters of bromodeoxyuridine (BrdU)-positive follicles (sectioned through the oocyte nucleolus) are represented along the y axis by diamond symbols.

was a positive correlation between the number of granulosa cells and the size of the follicle (Fig. 1), which was independent of age ($P < 0.001$). The diameter of the oocytes of all follicles measured showed a positive correlation with the diameter of their constituent follicles ($r^2 = 0.91$).

BrdU incorporation

In all animals studied, BrdU was incorporated into the nuclei of both granulosa and thecal cells of growing follicles, including a proportion (18 of 178; 10%; Table 1) of

Fig. 2. Immunostaining of granulosa cells in the mouse ovary showing incorporation of bromodeoxyuridine (BrdU) in (a,d) large preantral and (b,c) small, single-layered mouse follicles. The arrow indicates a BrdU-negative follicle. Scale bars represent 50 μ m.

those classified as small (Fig. 2). Measurements of BrdU-positive small follicles taken throughout a range of sizes show that (Fig. 1) BrdU incorporation was detected in follicles of any size and containing any number of granulosa cells.

Expression of caspase 3

The inactive pro-form of caspase-3 was detected as a 32 kDa band in western blots of tissue extracts of mouse ovary (Fig. 3). The active form was localized in the granulosa cells of larger preantral and small antral follicles; staining was also evident in some oocytes of small preantral follicles (Fig. 4) and luteal cells of regressing corpora lutea (Fig. 4). Staining was never observed in thecal cells, including those that surrounded atretic follicles. Active caspase-3 was detected in follicles that were not considered to be morphologically atretic (that is, follicles in which no pyknotic or abnormal nuclei were observed). In some preantral follicles, an enlarged immunopositive oocyte was often associated with underdeveloped granulosa cell layers and an absence of surrounding thecal cells. Active

caspase-3 was never found in the granulosa cells of any small, single-layered follicles (Table 1) or large preovulatory follicles with healthy granulosa cells (Table 2). Immunostaining was not observed in the control sections, whether the primary antibody was replaced with dilution buffer or with Ig isotype serum.

Discussion

The main aim of the present study was to investigate the occurrence of atresia in non-growing follicles. It was necessary to discriminate quiescent follicles from follicles that had already started to develop by correlating morphological characteristics with granulosa cell proliferation. It was apparent that the number of granulosa cells was directly proportional to the size of the follicle, and that BrdU incorporation into granulosa cells of small follicles indicated evidence of granulosa cell proliferation. The fact that BrdU was incorporated into small follicles of any diameter indicates that BrdU incorporation occurs independently of both follicle size and number of granulosa

Table 2. Immunostaining for active caspase-3 in mouse ovarian follicles at different stages of growth

Follicular stage	Oocyte	Granulosa cells	Theca interna cells
Small (primordial and primary)	–	–	–
Small preantral	+	–	–
Large preantral	+	++	–
Small antral	–	++	–
Large antral	–	++	–
Preovulatory	–	–	–

The results are based on the proportion of follicles with immunopositive cells.

(–): no immunostaining observed (within the oocyte, granulosa or theca interna cells) of a particular stage of follicular development; (+): occasional but infrequent immunostaining; and (++) : relatively frequent immunostaining.

Fig. 3. Western blot analysis of the expression of caspase-3 in mouse ovarian tissue extracts. The inactive form (CPP 32) was detected at 32 kDa.

cells. Although small follicles are formed shortly after birth in mice (Hirshfield, 1991), the size and number of granulosa cells when the follicle is arrested may not have an effect on the order of follicle recruitment after birth.

Both the oocyte and follicle diameters were smaller in peripubertal mice compared with postnatal mice. This change in size was not proportional to the number of

constituent granulosa cells indicating that there is a fixed number of cells in the small follicles, despite the fact that size may be influenced by larger adjacent structures, such as corpora lutea or developing follicles. Consequently, it is likely that the supply of small follicles in the mouse ovary exists as a resting pool rather than a cohort of slow-growing follicles, in which case these follicles would have been likely to show an increase in the number of granulosa cells per follicle as the ovary matured. Although most (> 89%) of the small follicles that did not incorporate BrdU could be construed as resting, it is equally likely that BrdU-positive cells were simply not selected in the 5 µm plane of section. Furthermore, it is also possible that not every dividing granulosa cell passed through the S-phase of the cell cycle during the 12 h period examined. A previous study on follicle cell proliferation in the rat ovary using [³H]thymidine infusion (Hirshfield, 1989) indicated that the duration of the cell cycle in granulosa cells may exceed 7 days. In the same study, it was also noted that a proportion of very small follicles (with ≤ 4 granulosa cells in a cross-section) had at least one labelled cell. This finding further supports the contention that the smallest follicles in the rodent ovary have the potential to develop irrespective of size or number of cells.

Under normal physiological conditions, morphological signs of atresia, such as nuclear pyknosis in large follicles, can easily be identified by the relatively large number of constituent granulosa cells and the propensity that these follicles have to be eliminated. Conversely, atresia of small follicles is more difficult to assess given their smaller size and considerably smaller number of constituent cells and, therefore, the requirement for an accurate marker of follicle cell apoptosis other than morphological criteria is essential and remains to be determined in small follicles. The active form of caspase-3 has been used as a marker for cell death in a number of tissues, particularly in tissues that show signs of degeneration as a consequence of disease or ageing (Hartmann *et al.*, 2000). Although localization of active caspase-3 was found to be a useful indicator of follicular atresia in larger follicles of the mouse ovary, active caspase-3 was never observed in granulosa cells of small follicles. These results are consistent with those of Matikainen *et al.* (2001) who also did not detect the processed form of caspase-3 in small follicles of the human ovary. Little is known about the half-life of biologically active caspase-3 in the ovary; however, given the number of small follicles observed, it is unlikely that the period of caspase-3 activation in either the oocyte or granulosa cells of small follicles was too short to be detected at any point. Lee *et al.* (2000) showed that primordial follicle degeneration that is induced by high doses of γ-irradiation proceeds at a much faster rate than that of larger follicles, and thus the time course of atresia of small quiescent follicles may be too rapid to be detected by current methods.

Morphologically 'atretic' primordial follicles have been described (Kondo *et al.*, 1996; Perez *et al.*, 1999; Lee *et al.*, 2000). However, studies investigating the demise of primordial follicles using *in situ* TdT-mediated dUTP nick-

Fig. 4. Immunostaining of active caspase-3 in the mouse ovary. (a,b,d) Follicles with many immunopositive cells were considered to be at the late stages of atresia, whereas (c) follicles with few positive cells may indicate early atresia. (a) The arrow shows an immunopositive oocyte; (b) the hatched line shows a regressing corpus luteum and (d) the arrowhead shows a small, single-layered immunonegative follicle. Scale bar represents (a,b,c) 100 μ m and (d) 50 μ m.

end labelling (TUNEL) or the localization of other apoptotic components have not been well documented in the normal ovary. Devine *et al.* (2000) discovered that, in the rat ovary, atresia of primordial follicles, as determined by ultrastructural observations, involved the loss of the oocyte, which was atypical of apoptosis and, thus, proposed that the mechanism of oocyte death may operate in a different manner to that of granulosa cell apoptosis. Similarly, Bergeron *et al.* (1998) described the functional requirement of caspase-2 for oocyte apoptosis, and this was supported by the findings of Matikainen *et al.* (2001), who determined that oocyte death was unaffected in *caspase-3* gene knockout mice. In the present study, active caspase-3 was localized in several oocytes of preantral follicles, indicating that the integrity of this pathway may still be preserved and activated under certain conditions, although the details of these conditions remain to be elucidated.

Processed caspase-3 may also be found in luteal cells of degenerating corpora lutea. It is well established that luteolysis occurs via apoptosis of luteal cells (Zelevnik *et al.*, 1989), which is in agreement with the evidence that cells of this origin are activating caspase-3 during apoptosis, and that this pathway of execution is conserved subsequent to

differentiation. Furthermore, in all atretic follicles observed, active caspase-3 was never detected in cells of thecal origin, which have been shown to undergo apoptosis during follicular atresia in other species, including sheep (O'Shea *et al.*, 1978), rats (Foghi *et al.*, 1997) and humans (Matikainen *et al.*, 2001), implying that apoptosis of cells of thecal origin is limited or may occur via a different mechanism in mice.

In summary, the results of the present study indicate that, in the mouse ovary, the pool of primordial follicles considered to be in a quiescent state are difficult to identify by morphology alone. Although BrdU may be incorporated into granulosa cells of follicles from a range of sizes, it is possible that these cells are spontaneously cycling independent of follicular growth and, therefore, maintaining a constant number of healthy support cells for the oocyte. However, this process is unlikely given that caspase-3 activity was not observed in these cells in any of the sections studied. Therefore, this finding indicates that the mechanism by which small follicles undergo atresia is independent of caspase-3 activation, or that these follicles express an increased resistance to the physiological factors that normally induce apoptosis.

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