Anat. Histol. Embryol. **33,** 23–27 (2004)

ISSN 0340–2096

Dipartimento di Scienze Biomediche Comparate, Università degli Studi di Teramo, Piazza A. Moro, Teramo, Italy

Colocalization of DNA Fragmentation and Caspase-3 Activation During Atresia in Pig Antral Follicles

P. Berardinelli*, V. Russo, A. Martelli, D. Nardinocchi, O. Di Giacinto, B. Barboni and M. Mattioli

Address of authors: Dipartimento di Scienze Biomediche Comparate, Università degli Studi di Teramo, Piazza A. Moro, 1, 64100 Teramo, Italy; *Corresponding author: e-mail: berardinelli@ianve.vet.unite.it

With 4 figures

Received January 2003; accepted for publication May 2003

Summary

Apoptosis is the cellular mechanism of ovarian follicular atresia. The major downstream effector of this phenomenon in many tissues is caspase-3 but little is known about its role in pig ovarian apoptosis. In the present study, we detected the localization of caspase-3 in parallel with nuclear fragmentation (TUNEL) on healthy and early atretic antral follicles. In healthy antral follicles caspase-3 and TUNEL positivity were occasionally recorded within theca layer. The incidence of DNA fragmentation, as indicated also by the biochemical detection, increased mainly in the granulosa layer of early atretic follicles. Quantitative analysis revealed, besides, that atresia was accompanied by a higher incidence of caspase-3 (57.20 ± 20.05 versus 3.64 ± 0.61 positive cells in atretic versus healthy follicles, respectively; $P < 0.05$), of TUNEL positivity (20.13 ± 9.33 versus 0.42 ± 0.12; $P < 0.05$) and simultaneous immunostaining for caspase-3 and TUNEL (15.02 ± 6.95 versus 0.31 ± 0.05; $P < 0.05$) in the granulosa layer. In detached granulosa cells isolated from the follicular fluid of early atretic follicles a further significantly increase was recorded in the percentage of TUNEL positivity and in the incidence of cells that showed colocalization of caspase-3 activity and DNA fragmentation. Granulosa cells of early atretic follicles exhibited a higher positivity for caspase-3 localized in the cytoplasm and occasionally in the nucleus area of granulosa cells. These results indicate that capsase-3 was involved and precociously activated during the process of atresia. Finally, the progressively higher incidence of TUNEL positivity and of double immunostaining in atretic cells collected within the follicular fluid seems to indicate that proteases activity leads only tardily in a detectable DNA fragmentation.

Introduction

Apoptosis is a type of programmed cell death recognized as a widespread physiological phenomenon that participates in tissue regression and remodelling (Palumbo and Yeh, 1994). This process occurs cyclically also in the ovary and is implicated in the mechanism of follicular atresia and luteal regression. In the ovarian physiology, follicular atresia is a key phenomenon through which the ovary eliminates those follicles that are not selected for ovulation. During ovarian follicular development, only a number of dominant follicles reach maturation, whereas the remaining majority become atretic in a process characteristic of physiological cell death or apoptosis (Tilly et al., 1991; Palumbo and Yeh, 1994). Two major stages of cell involution can be distinguished within the ovary: degeneration of germ cells (attrition), which account for the largest loss of oocytes and occurs mainly prenatally, and follicle regression (atresia), which occurs during postnatal reproductive life (Kaipia and Hsueh, 1997). Apoptotic cells are identified by DNA fragmentation triggered by the activation of specific Ca^{2+}/Mg^{2+}-dependent endonucleases such as a family of cysteine aspartate specific proteases called caspases, which have a demonstrated role in the execution of apoptosis in a number of different cell types (Villa et al., 1997; Porter and Janicke, 1999). These enzymes participate in a cascade that is initiated in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell. These cytoplasmatic proteases can be classified as initiators (caspases-8, -9, and -10) or effectors (caspases-3, -6 and -7). Under the appropriate stimulus, initiators caspases activate effector caspases that, in turn activate the endogenous endonucleases, which are involved in degrading DNA (Thornberry and Lazebnik, 1998); in particular, caspase-3 is the principal downstream effector enzyme of cell death and its role results in the activation of the endogenous endonucleases. Knowledge on the molecular mechanism that takes part in the suicide programme becomes crucial, both to have a precocious enzymatic marker to detect the beginning of the process (Liu et al., 1997; Porter and Janicke, 1999) and to hypothesize pharmacological strategies to prevent apoptosis (Kim et al., 2000; Tatemoto et al., 2000). For this reason the purpose of the present work was to examine the involvement of caspase-3 in the process of atresia in pig antral follicles, as demonstrated in other species (Boone and Tsang, 1998; Van Nassauw et al., 1999; Johnson and Bridgham, 2000). To achieve this objective we first analysed a part of the follicle wall to demonstrate DNA fragmentation (Tilly et al., 1991) with a widely used technique, such as gel electrophoresis, and to confirm the presence of apoptosis. The remaining follicle wall was analysed by a combination of *in situ* end labelling of nuclear fragment DNA (TUNEL) and an immunohistochemical analysis for caspase-3, to identify whether in the atretic follicle it is possible to find some correlation between DNA fragmentation and the activation and location (cytoplasm or nucleus) of caspase-3.

Materials and Methods

Ten ovaries of five prepubertal Large White gilts of 6 months old were collected at the local abattoir within 20 min of slaughter. The tissue specimen were transported at controlled temperature (20–25°C) and further processed in laboratory

where single follicles were isolated in dissection medium (Dulbecco's phosphate buffer medium supplement with 0.4% BSA). After measuring the diameter with a calibrated grid, only 4–5 mm antral follicles were recruited. With the aid of a stereomicroscope the isolated follicles were then classified as healthy as indicated by their translucent appearance, limpid follicular fluid and extensive vascularization or early atretic as indicated by the dark opaque aspect and the looseness of granulosa layer. Each antral follicle was opened on the bottom of a Petri dish and the follicle walls obtained from each structure were cut with a razor blade in two halves. Each half of follicle walls (which will be indicated as emifollicle) was separately analysed for the DNA evaluation or for immunohistochemical procedures. The follicular fluid of early atretic follicles was further collected to isolate the granulosa detached cells by centrifugation, which had completely lost any contact with the granulosa layer.

DNA fragmentation analysis

Before performing the DNA extraction, theca and granulosa compartments of healthy and early atretic follicles were separated under a stereomicroscope by scraping the internal face of the follicle wall as previously described (Barboni et al., 2000).

Total DNA was extracted separately from theca and granulosa cells with the extraction buffer (Tris-HCl 5 mM, pH 8; EDTA 20 mM; 0.5% TritonX-100). The cellular lysate was collected by centrifugation (27 000 ***g***, 30 min.) and DNA extracted in a phenol solution. DNA concentration was determined by spectrophotometry (A_{260}) and analysed by 2% agarose gel electrophoresis with ethidium bromide staining; 10–30 μg of DNA was loaded in each lane.

DNA fragmentation analysis was not carried out on detached granulosa cells because not enough amounts of DNA could be extracted from this kind of cells.

Localization of DNA fragmentation and caspase-3 in follicle sections and cell suspensions

Follicle walls were fixed in 10% neutral buffered formalin (NBF) for 12 h at 4°C and subsequently embedded in paraffin wax. The emifollicle walls were then serially sectioned (5 μm), placed on poly-L-lysine-coated slides, deparaffinized, rehydrated and used for immunohistochemical (caspase-3) and *in situ* end labelling of fragment DNA (TUNEL) apoptotic cell detection. Detached granulosa cells were fixed in 10% NBF for 1 h at 4°C and stained for caspase-3 and TUNEL treated as cell suspensions.

Histological sections were treated to carry out a double immunohistochemical detection to reveal nucleosomal fragmentation products and to localize caspase-3 in the follicle wall of healthy and early atretic antral units. On the same section the ApopTag *in situ* Apoptosis detection kit (Intergen Company, Oxford, UK), following the manufacturer's instructions, and a rabbit polyclonal antibody CPP32, which recognizes the proenzyme as well as the active form (Novocastra Laboratories Ltd, Newcastle-upon-Tyne, UK) were applied. Briefly, tissue sections were incubated with proteinase K (20 μg/ml; Sigma Chemical Company, St Louis, MO, USA) for 15 min at room temperature. The sections were incubated with the equilibration buffer first and then with terminal deoxynucleotidyl transferase (working strength TdT enzyme) and the anti-CPP32 antibody (1 : 150) at 37°C for 3 h. Then, the slides were incubated with Fluorescein anti-digoxigenin conjugate (FITC) and with a secondary biotinylated anti-rabbit antiserum (1 : 200) for 1 h at room temperature. After washing, sections were incubated for 1 h in Avidin-TRITC (1 : 200; Sigma) in order to visualize the anti-CPP32 (caspase-3). As negative controls the sections were incubated with the omission of TdT and anti-CPP32 (caspase-3). The cells isolated by centrifugation from follicular fluid of early atretic follicles were treated just as the histological sections except for the incubation with proteinase K. At the end of the reactions these cells were applied on a microscope slide ready to be inspected.

Statistical analysis

Sections and detached cells were inspected using an Axioscop 2 plus (Zeiss, München, Germany) epifluorescence microscope equipped with an Axiovision Cam (Zeiss) and with a set of excitation filters of 488 and 568 nm for the excitation of FITC and Avidin-TRITC, respectively. The Ks300 computed image analysis system (Zeiss) was used for a quantitative evaluation of the apoptotic cells. Individual cells were counted on a ×200 field (i.e. ×20 objective lens and ×10 ocular lens; 442 368 μm^2 per field). At least four randomly selected fields were counted per section to quantify the number of positive cells to TUNEL or caspase-3 (CPP32) or positive to both. The counting of the cells was carried out blind, in fact the investigator performing the image analysis did not have any knowledge of the examined slides as they were prepared and then coded by a different person before the cells were counted.

Quantitative data of caspase-3 or Tunel, and caspase-3/TUNEL positive cells in antral and atretic follicles were analysed for statistical significance by Student's *t*-test; $P < 0.05$ was considered to be statistically significant.

Results and Discussion

DNA fragmentation of granulosa cells and theca shells was assayed separately on agarose gels in individually healthy or early atretic emifollicles (4–5 mm in diameter). Ethidium bromide staining (Fig. 1) indicated that fragmentation of DNA was undetectable in both granulosa and theca compartments of healthy follicles while increases in low molecular weight DNA were always detected in follicles morphologically classified as early atretic on the basis of the appearance of the follicle wall (see material and methods). The extensive intranucleosomal cleavage of DNA, which gave a typical ladder pattern on gel electrophoresis, in early atretic follicles involved the granulosa layer (Fig. 1) while no signs of DNA fragmentations were recorded in the theca compartment. These biochemical analysis were previously carried out to demonstrate, in the present research, that apoptosis was the form of cell death involved in ovarian follicle regression, as TUNEL, the TdT-mediated dUTP nick end-labelling technique, could not distinguish between apoptosis and necrosis (Boone and Tsang, 1998; Plasier et al., 1999). For this reason, after the biochemical characterization, the remaining portion of the other half of the follicle walls (emifollicle) was sectioned and immunostained to localize simultaneously caspase-3 and DNA. Double staining method for *in situ* TUNEL and caspase-3 immunohistochemistry experiments showed three

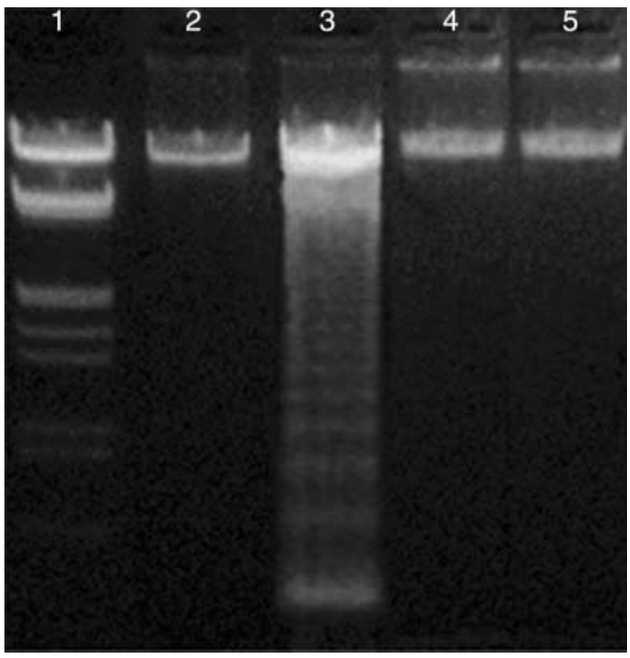

Fig. 1. Analysis of extracted DNA from granulosa and theca layers of 4–5 mm antral and atresic follicles by 2% agarose gel electrophoresis with ethidium bromide staining. Lane 1: control (λ Eco/Hind); lane 2: healthy antral granulosa; lane 3: atretic granulosa; lane 4: healthy antral theca; lane 5: atretic theca.

different patterns of fluorescence as demonstrated in Fig. 2: pattern I red cells that resulted positive for caspase-3 (TRITC); pattern II TUNEL positive cells with green nuclei (Fluorescein) and pattern III cells with simultaneous immunostaining for caspase-3 and TUNEL. The analysis of half of healthy follicle walls revealed few cells positive for caspase-3 or TUNEL (Fig. 3). On a field of 442 368 μm^2 (no. of fields = 32) a mean number of 15 cells resulted immunostained for caspase-3 (pattern I) of which 12.24 ± 3.34 localized in the theca layer and 3.64 ± 0.61 in the granulosa compartment. As shown in Fig. 2a, in the theca layer were also detected 9.33 ± 2.63 positive TUNEL cells (pattern II) and a low number of double immunostained cells (6.02 ± 2.01; pattern III). In half of the atretic follicle walls caspase-3 staining and the TdT-mediated dUTP nick end labelling involved mainly granulosa layer (Fig. 3). The theca compartment continued to exhibit a low number of scattered immunopositive cells as in healthy emifollicles (5.96 ± 2.82 versus 9.33 ± 2.63 cells with pattern II and 9.98 ± 4.56 versus 12.24 ± 3.34 with pattern I and 3.01 ± 2.18 versus 6.02 ± 2.01 with pattern III in atretic versus healthy follicles, respectively). By contrast, granulosa layer displayed a higher number of caspase-3 (57.20 ± 20.05 versus 3.64 ± 0.61 positive cells in atretic versus healthy follicles, respectively; $P < 0.05$, Student's *t*-test) and TUNEL positive cells (20.13 ± 9.33 versus 0.42 ± 0.12; $P < 0.05$). As showed in Fig. 3 immunohistochemistry analysis revealed a major incidence of pattern III with a simultaneous localization of caspase-3 and TUNEL (15.02 ± 6.95 versus 0.31 ± 0.05; $P < 0.05$). In more detail, the single cell analysis (Fig. 2b) revealed in early atretic follicle either granulosa cells that show a caspase-3 activity localized in the cytoplasm or in the whole cell compartment. In fact, caspase-3 staining did not reveal any interference with the green fluorescence recorded in the nucleus by TUNEL technique in some granulosa cells (see the arrow in Fig. 2b), indicating a clear separation between cytoplasm and nucleus fluorescence or, by contrary, this double immunostaining was completely superimposed as indicated by the asterisk in Fig. 2b. When the immunohistochemistry analysis was carried out on the detached granulosa cells, collected within the follicular fluid of early atretic follicles, a significantly higher incidence of the pattern II (34.94 ± 7.21 versus 20.13 ± 9.33 in detached versus layered granulosa cells of early atretic follicles; $P < 0.05$) and of the pattern III (31.68 ± 7.09 versus 15.02 ± 6.95; $P < 0.05$) was recorded (Fig. 3). The double immunostained cells showed an intense immunoreactivity for TUNEL, always localized in the nuclear region, while caspase-3 also in detached granulosa cells could result localized either in the cytoplasm or occasionally over the whole cell compartment (Fig. 4). By contrast, detached granulosa cells showed a lower positivity for caspase-3 (21.12 ± 6.02 versus 57.20 ± 20.05 detached and layered granulosa cells of early atretic follicles, respectively; $P < 0.05$).

The hallmark of apoptosis is the presence of a typical intranucleosomal DNA fragmentation that is identified by the appearance of a ladder pattern on gel electrophoresis (Hughes and Gorospe, 1991). The presence of a typical ladder pattern in DNA in early atretic follicles indicated, in the present work, that apotosis in swine ovary is associated with the atresia of antral follicles by indicating that the cells involved are for the most part granulosa cells. Only by using a single cell analysis, like TUNEL, DNA fragmentation could be occasionally revealed in the theca layer. Furthermore, TUNEL analysis showed that DNA fragmentation involved cells within the theca layer without any correlation with the process of atresia suggesting that the two follicle compartments, granulosa and theca, are physiologically under different trophic control. These results seem to indicate that the stimulus, which leads to the apoptosis in theca and granulosa cells, may be different. Granulosa cells started to respond to Ca^{2+}/Mg^{2+}-dependent endonucleases activity probably as a consequence of a modified hormonal condition (Hay et al., 1976; Braw and Tsafriri, 1980; Kaipia and Hsueh, 1997) that, at the beginning, resulted totally unable to influence the theca compartment. A major sensitivity of the granulosa layer to the trophic or hormonal sustain may have a possible explanation in the blood vessel organization of the wall during follicle growing and regression. In fact, health depends, in antral follicles, on the presence of a rich network of capillaries, organized in two concentric networks an inner one close to the basal membrane and an outer one within the external theca connected to each other by anastomotical vessels. As vessels cannot cross the barrier represented by the basal membrane, the capillary network that develops within the internal theca, in strict contact with the basal membrane, becomes the only source of nutrients and gases for the avascular compartment represented by granulosa cells and the oocyte (Hay et al., 1976; Mattioli et al., 2001). For this reason, it is not surprising that the apoptotic process starts within the granulosa layer, as a consequence of the vascular changes that accompany the precocious signs of atresia that involve the regression of the inner network which is clearly of overriding importance to the survival or death of the adjacent avascular membrane granulosa (Moor and Seamark, 1986; Berardinelli et al., 2002). This may be consistent with the fact that most of the theca cells survive during the atretic process

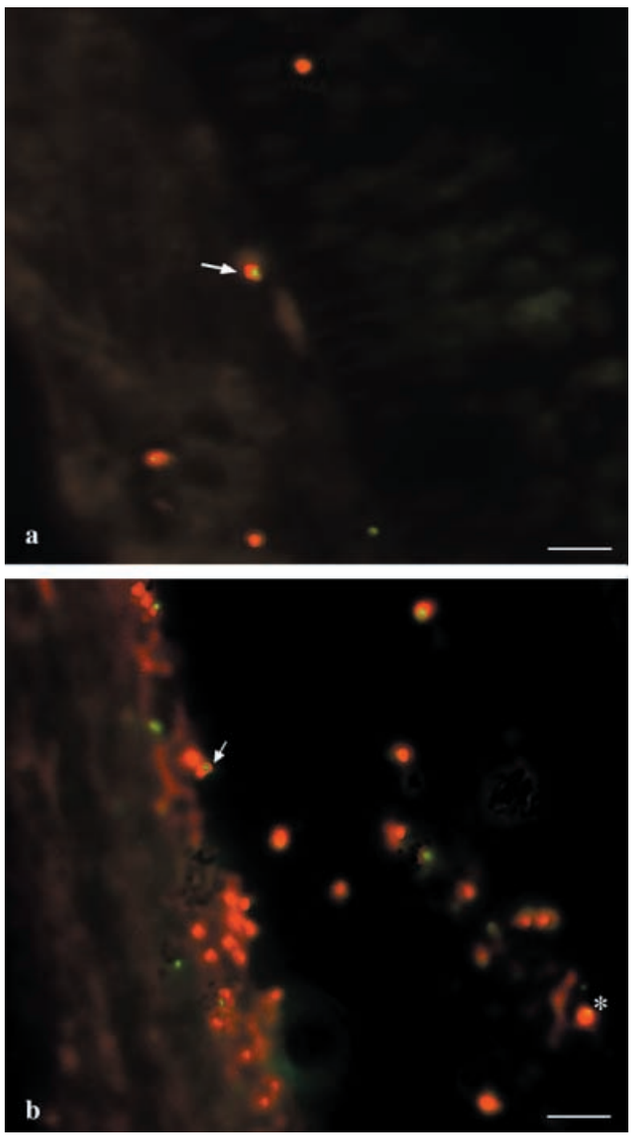

Fig. 2. Double staining method for *in situ* TUNEL and caspase-3 immunohistochemistry detection on sections of 4–5-mm healthy antral (a) and early atretic (b) follicles. Immunohistochemical analysis revealed three different fluorescence patterns: Pattern I red cells that resulted positive for caspase-3 (TRITC); pattern II TUNEL positive cells with green nuclei (Fluorescein) and pattern III cells with simultaneous immunostaining for caspase-3 and TUNEL. The arrow indicates a double immunostained cell with caspase-3 localized in the cytoplasm while the asterisk indicates a cell where caspase-3 and TUNEL positivity were colocalized into the nucleus as indicated by the yellow fluorescence signal. Bar = 50 μm.

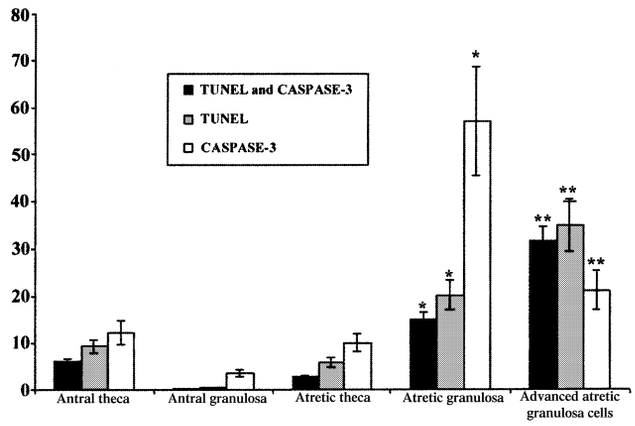

Fig. 3. Quantitative distribution of caspase-3, TUNEL and double immunostained (caspase-3/TUNEL) positive cells recorded on section of healthy and early atretic antral follicles or in detached granulosa cells isolated from the follicular fluid of early atretic structures. Statistical significance was carried out among each fluorescence pattern recorded in the different groups by using the Student's *t*-test. Asterisks indicate data significantly different for $P < 0.05$.

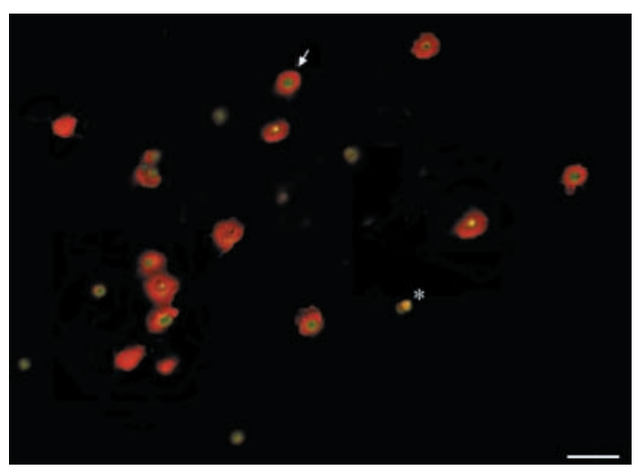

Fig. 4. An example of immunohistochemical analysis carried out on detached cells collected from the follicular fluids of early atretic follicles. Green fluorescence indicated the DNA fragmentation detected by TUNEL methods and the red signal evidentiated the positivity for caspase-3. Arrow = double immunostaining for caspase-3 and TUNEL, with caspase-3 localized into the cytoplasm; asterisk = double immunostaining for caspase-3 and TUNEL, with caspase-3 translocated in the nucleus (in yellow). Bar = 20 μm.

and, as hypothesized by O'Shea et al. (1978), they can be reincorporated in the ovarian interstitium. However, this observation that emphasizes the importance of the interaction between capillary bed and cells function in antral follicles does not ultimately demonstrate direct and unequivocal evidence as the primary cause of atresia is still being sought. The biochemical identification of nucleosomal DNA fragmentation during atresia in swine ovaries demonstrated that apoptosis is a basic mechanism associated with ovarian follicular regression in physiological conditions. DNA fragmentation, although, is an ultimate step in the apoptosic pathway cascade; this process is induced by proapoptotic signals and involves caspases proteins. Among the caspases identified so far, caspase-3 is probably the best correlated with apoptosis (Krajewska et al., 1997). Recent studies have also documented the expression of caspase-3 within the mammalian and avian ovary (Boone and Tsang, 1998; Johnson and Bridgham, 2000; Tatemoto et al., 2000). In the present study, it has been demonstrated an involvement of this protease in the apoptotic process of atresia in swine antral follicles. Intense caspase-3 immunoreactivity was, in fact, principally observed in the granulosa cells of early atretic follicles. Caspase-3 positivity involved a very high number of cells in comparison with those that exhibit DNA fragmentation (TUNEL positivity) in parallel. Colocalization quantitative studies indicated that in the granulosa layer only

a low percentage of cytoplasmatic caspase-3 staining cells showed also TUNEL positive cells, while these results completely changed when the observation was carried out on the cells that have lost any relation with the granulosa layer as a consequence of their advanced status of atresia. In the last case, the cells that exhibited either double immunostaining (DNA fragmentation and caspase-3 activity) or TUNEL positivity grew, suggesting that this protease activity was able to complete only tardively the process of apoptosis. Finally, it is interesting to consider that caspase-3 could result localized either in the cytoplasm or, occasionally, within the nucleus implying that this protease resides primarily in the cytosol but there is also translocation of active caspase-3 to the nuclei of at least some of the cells undergoing apoptosis (Kim et al., 2000). It is not clear, therefore, what role caspase translocation plays in apoptosis. The high proportion of granulosa cells positive to the antibody anti-caspase-3 within the granulosa layer could be due to the fact that this enzyme is upregulated in these cells as part of the apoptotic process (Boone and Tsang, 1998) and this caspase-3 immunostaining pattern reveals an upstream step of the apoptosis inducing pathway that permits a precocious identification of the programmed cell death in atretic follicles better than with the classical DNA fragmentation detection methods. The increased expression of this protease may overcome the inhibitory effects of endogenous cell survival factors such as the Bcl-2 family and inhibitors of apoptosis proteins.

Acknowledgements

This work was supported by cofinanziamento Es. Fin. 2001 and ex 60% 2002.

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