Evidence of apoptosis in human primordial and primary follicles

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BACKGROUND: Apoptosis may operate the ‘selection’ between follicles destined for atresia and follicles that will remain available for ovulation. The aim of this study is to assess the expression of apoptosis in quiescent follicles.

METHODS: Ovarian cortex samples from women of reproductive age, fixed in formalin, were used for immunohistochemical and terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labelling (TUNEL) methods. In histological sections the follicles were classified as primordial, primary, secondary and antral. Follicle density was defined as the total number of follicles/0.5 cm² of ovarian cortical tissue. Mab DO-7 (anti-p53) and Mab 124 (anti-bcl-2) were used in the immunohistochemical study. RESULTS: TUNEL was positive in 23.4% of the primordial follicles, and in 23.2% of the primary follicles, both in oocytes and granulosa cells, whereas all secondary follicles were negative. Bcl-2 activity was expressed in 75% of secondary follicles. p53 was negative in all samples.

CONCLUSIONS: Apoptosis could be the process responsible for atresia of quiescent follicles and hence depletion of the ovarian germ stockpile. Follicular cells expressing Bcl-2 may therefore be the viable cells that escape the apoptotic process. Negative p53 patterns may be a favourable prognostic finding showing genome integrity in the replicating follicular cells of women of reproductive age.

Key words: apoptosis/Bcl-2/p53/primordial and primary follicles/TUNEL

Introduction

In female mammals, a finite number of follicle-enclosed oocytes sustain ovarian function up to the time of menopause. In humans, at birth there are 500 000 non-growing, or quiescent, follicles per ovary on the ovarian cortex, but only 400 of these follicles will develop to the pre-ovulatory stage (Gougeon, 1996). Very little is known regarding the loss of quiescent follicles in any mammalian species, although atresia of immature follicles may be a significant event in determining the female reproductive lifespan. The recent findings of the hallmark of apoptosis in atretic follicles constitute a step forward in the investigation of intraovarian hormonal regulation of follicle atresia (Billig et al., 1994; De Felici, 1997).

Apoptosis is an active form of programmed cell death. The characteristic structural and molecular events of apoptosis distinguish this type of cell death from necrosis, where a group of cells dies simultaneously (Tilly, 1996), whereas apoptosis always occurs in a single cell surrounded by viable cells (Kuan and Passaro, 1998). It has become evident that the physiological removal of cells through apoptosis in multicellular organisms is a mandatory process in the maintenance of homeostasis of the individual. The most prominent characteristics of apoptosis appear to be DNA cleavage between regularly spaced internucleosomal units by endonucleases, resulting in the generation of DNA fragments. A terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labelling (TUNEL) in-situ detection test can be used to evaluate DNA fragmentation on histological sections (Tilly, 1996).

Specific genes have been identified that encode proteins responsible for the initiation, progression, and completion of cell death, particularly a large family of Bcl-2-related proteins. Those proteins, which interact to form hetero- and homodimers, can be subdivided into two classes based on function: those that delay or inhibit apoptosis and those that facilitate or induce apoptosis (Tilly, 2003). The ratio or balance of Bcl-related death repressors (e.g. Bcl-2) to Bcl family-related death inducers (e.g. bax) within the cell controls the susceptibility of that cell to any given apoptotic stimulus (Yang and Korsmeyer, 1996; Reed et al., 1997). Bcl-2, which promotes cell survival by inhibiting the adapters needed for protease activation (caspase) and dismantling of the cell, is a key regulator of apoptosis, and is powerful enough to block the process in the early phases.

The genetic control of apoptosis also depends on a transcription factor, p53, which is known to control cellular...
proliferation. Although the mechanisms subserving the action of p53 in this regard have still to be defined, it has been shown to induce transcription of the bax gene and repress transcription of the Bcl-2 gene, possibly triggering more bax and hence apoptosis (Ko and Prives, 1996; Evan and Littlewood, 1998). Being an anti-oncogene, p53 could be defined as a guardian of the genome. In cells undergoing replication, if there is damage at the DNA level that the cell is unable to repair, the p53 protein rapidly stabilizes and translocates to the nucleus. It is now well established that p53 serves a dual role in cells, its other major function being the induction of apoptosis in those cells possessing irreparable damage (Ding and Fisher, 1998).

In the ovary, apoptosis has been demonstrated to be a sign of atresia of follicles in the various growth stages (Kondo et al., 1996; De Pol et al., 1997), while the gonadotropins have a role in favouring or inhibiting apoptotic mechanisms (Hsueh et al., 1994; Chun, 1996). Instead, the mechanism responsible for depletion of the stockpile of germ cells in quiescent primordial follicles throughout adult life has not yet been clarified. We suggest that apoptosis, the programmed death of oocytes and granulosa cells (GC), may operate the ‘selection’ between follicles destined to undergo atresia and follicles that will remain viable for ovulation.

In this study the apoptotic process was investigated by means of the TUNEL assay, together with immunohistochemistry for Bcl-2, and p53, in primordial and primary follicles in the ovarian cortex from women of reproductive age.

Materials and methods

Ovarian material

Ovarian cortical biopsies were obtained after informed consent from 13 women (mean age 33.6 ± 9.6, range 20–48) undergoing laparoscopy for benign gynaecological disease. Thin ovarian slices (1×5 mm) were washed three times in phosphate-buffered saline (bioMérieux) to remove debris and clots, and immediately fixed in buffered formalin. Four sections of each sample were cut for haematoxylin–eosin (HE) staining. In histological sections, follicles were classified, according to Gougeon (1996), as primordial, primary, secondary, and antral. A primordial follicle is defined as an oocyte surrounded by a flat, single layer of GC (follicle diameter 0.03 mm); a primary follicle, as an oocyte surrounded by a cuboidal layer of GC (follicle diameter 0.04 mm); a secondary follicle, as an oocyte surrounded by three to six layers of GC (follicle diameter 0.1–0.2 mm). Finally, in antral follicles an antral cavity appears, starting with the development of small fluid-filled cavities measuring 40 μm in diameter (Gougeon, 1996).

Follicle density was defined as the total number of follicles/0.5 cm² of ovarian tissue, including oocytes with and without a nucleus.

Preparation for immunohistochemistry

Sections 4 μm thick were cut for immunohistochemical detection. The immunohistochemical panel encompassed anti-p53 (MAB DO-7; Dako, Denmark) and anti-bcl-2 (MAB 124; Dako) antibodies. After routine deparaffinization and rehydration including an endogenous peroxide block with methanol–peroxidase for 30 min, the sections were microwaved for non-enzymatic epitope retrieval at 750 W for 3×5 min. The immunostaining was performed using the avidin–biotin–peroxidase amplification system in an automatic immunostainer (Techmate 500; Dako). Appropriate immunohistochemical controls were run in parallel, consisting of colon cancer specimen sections as the positive p53 control and follicular lymphoma sections as the positive bcl-2 control. Negative controls were performed by replacing the primary antibodies with the same concentration of normal mouse IgG (Dako). Staining for p53 and bcl-2 was considered positive when a brown stain was evident in the nucleus and cytoplasm of granulosa cells and/or oocytes respectively.

TUNEL procedure

Sections 3 μm thick were used for detection of DNA fragments of apoptotic cells by the TUNEL method (kit; Enzo Life Sciences, USA). After deparaffinization and rehydration, tissue sections were digested with proteinase K (20 μg/ml) at 37°C for 15 min. Following the application of an equilibration buffer, the sections were incubated in a working strength TdT enzyme that contained deoxyuridine triphosphate (d-UTP)-biotin under a coverslip for 30 min at 37°C. After removing the coverslip, the slides were incubated with streptavidin–conjugated alkaline phosphatase for 30 min at 37°C and then with nitroblue tetrazolium for 30 min at 37°C. Sections of normal lymph nodes were used as positive controls of the TUNEL method. In negative controls the TdT enzyme was omitted from the nucleotide mixture. The apoptotic signal was recorded as positive when either a diffuse-type or a granular-type dark blue staining of the nucleus was apparent. Follicles with positive TUNEL staining of the oocyte and/or ≥50% of the granulosa cells were considered apoptotic.

Statistical analysis

Data were expressed as mean ± SE. Statistical analysis was done using StatView™ SE+ Abacus Concepts, Inc., 1998. The results were evaluated by linear regression analysis.

Results

No surgical complication or bleeding occurred during biopsy procedures. Histological evaluation of the ovarian cortex slices revealed primordial and primary follicles, and secondary follicles in 12 of the 13 samples obtained. No follicles were evident in the biopsy of a 48 year old woman. The number of quiescent follicles in the ovarian sections, excluding one
patient with an outlier value, was inversely proportional to the patient’s age ($r^2 = 0.5832$) (Figure 1).

Overall, 64 primordial follicles, 43 primary follicles and eight secondary follicles were evaluated (Figures 2 and 3).

Apoptotic DNA fragmentation (TUNEL positive) was found in 23.4% of the primordial follicles, and in 23.2% of the primary follicles (Figures 3 and 4); the distribution of TUNEL positive reactions and DNA fragmentation in oocytes and GC in primordial and primary follicles is illustrated in the Table I. No TUNEL positive oocytes or GC were found in secondary follicles.

No positive statistical correlation was found between patient age and apoptosis, whereas there was a positive statistical correlation between follicular stage and apoptosis ($r^2 = 0.60$), demonstrating that apoptosis is most evident in primordial and primary, i.e. quiescent, follicles. Bcl-2 was expressed in 75.0% of secondary follicles but was negative in all primary and primordial follicles (Figure 5). A negative Bcl-2 reaction in primordial and primary follicles was statistically correlated with apoptosis (TUNEL positive) ($r^2 = 0.92, P < 0.001$). All samples were negative for p53.

**Discussion**

The results of this study suggest that apoptosis could be the process responsible for atresia of quiescent follicles and hence
depletion of the human ovarian germ stockpile. TUNEL staining has been widely used to identify apoptotic cells, although p53 and Bcl-2 detections may cause underestimation of the true extent of the apoptotic process, and complementary studies of annexin V binding and caspase activity may be used to assess the overall morphological modifications of apoptosis (Pino et al., 2003).

In the present study, abundant apoptotic DNA fragmentation was observed in most oocytes in the primordial and primary follicles, suggesting that apoptosis in quiescent follicles may occur initially in the oocyte and subsequently in the GC, contrasting with atresia in maturing or fully mature follicles, in which apoptosis of the granulosa cell precedes oocyte degeneration (Kondo et al., 1996; Tilly, 1996). These data suggest that immature follicles may be particularly vulnerable to apoptosis induced by intrinsic intra-oocytic signals, although local factors may also play a role, while growing follicles are more sensitive to the signals sent out from the stromal cells toward the follicular structure.

Our results also demonstrate that Bcl-2 was expressed in the GC of secondary but not of primordial follicles, and that there is a significant positive correlation between Bcl-2 and the absence of apoptosis. We may hypothesize that quiescent follicles, after entering the growing follicle pool, acquire anti-apoptosis protection factors, and that Bcl-2 could be one of these factors. Therefore, follicular cells expressing Bcl-2 may be the viable cells that will be available for ovulation. In this case, Bcl-2 could constitute a marker of the follicular ovarian reserve.

Finally, we assessed expression of p53, which was found to be constantly negative in the cells of follicles with apoptotic alterations. It can therefore be hypothesized that apoptosis of immature cells is a physiological follicular selection event, and that there is probably genetic control of apoptosis. Negative p53 findings are a favourable prognostic index of genome integrity in replicating follicular cells, and hence of the functional viability of the follicle.

Several studies have already shown that apoptosis is present in human ovaries even before birth: in human fetal ovaries, apoptosis was identified in fetuses aged 13 to 28–32 weeks gestation (Vaskivuo et al., 2001; Abir et al., 2002; Markstrom et al., 2002). The studies by Vaskivuo et al. (2001) and Abir et al. (2002) both showed that most of the apoptotic activity was in the germ cells and not in the GC. In adult human ovaries, apoptosis was detected in follicles in the various phases of pre-ovulatory growth, mainly in the GC (Tilly, 1999; Mikkelsen, et al., 2001; Vaskivuo et al., 2001). Moreover, it has been reported that DNA fragmentation associated with apoptotic death in human mature oocytes is age-related (Wu et al., 2000).

Bcl-2 immunostaining in the adult human ovary has mainly been observed in GC of secondary and antral follicles but not in quiescent follicles (Vaskivuo et al., 2001). In different studies of fetal ovaries, Bcl-2 expression was observed: (i) only in fetuses aged 13–14 gestational weeks (Vaskivuo et al., 2001); (ii) in all components of the ovaries from fetuses of all ages (Abir et al., 2002); (iii) in supporting cells but not in the oogonia (Quenby et al., 1999); or (iv) restricted to somatic cells (Hartley et al., 2002).

As for p53, it has been shown that gonadotrophin suppresses p53 messenger RNA expression in the immature rat ovary and that the p53 protein is present in apoptotic cell nuclei of atretic follicles (Tilly et al., 1996). Moreover, in the rat, over-expression of p53 protein is closely coupled to extensive apoptosis in the GC of large antral follicles (Kim et al., 1999). However, the dynamic changes in p53 protein content during follicular development and atresia remain to be elucidated.

The present study investigated a quiescent follicular population obtained from human ovarian cortex and was therefore not susceptible to variations in gonadotrophins. It was found that apoptosis was present mainly in primordial and primary follicles, both at the oocyte and the GC level. It seems possible that the apoptotic process may follow a particular pattern during different phases: first in the fetus mainly in germ cells, then in the adult, in quiescent cortical follicles in both oocytes and GC, and finally in growing follicles, mainly in the GC. Moreover, in quiescent follicles in both fetal and adult ovaries, the oocyte may possess an intrinsic mechanism for the activation of apoptosis. In quiescent follicles, the process could begin in the oocyte and then extend to the surrounding pre-granulosa cells, inducing atresia of the whole follicle. Instead, in antral follicles the process could start at the level of the granulosa cells and then spread to the oocyte (Tilly, 1996). In any case, by the end of the process, the whole follicular structure has become atretic, while the surrounding stromal cells remain viable. Thus, there seems to be a structural plan inside the follicle that communicates the atresia signals from a single cell to all the others in the structure.

However, evidence of one or a few granulosa cells with signs of apoptosis does not necessarily mean that the whole follicle is destined to undergo atresia, as this may belong to the normal processes of follicular damage and regeneration.

In growing follicles the regulation of apoptosis is under endocrine control, and the primary factor responsible for the maintenance of granulosa cell survival appears to be FSH (Chun et al., 1996). It could be that growing follicles, being gonadotrophin-dependent, are vulnerable to metabolic and environmental changes and that alterations in the hormone and ovarian paracrine milieu may induce follicular atresia (Armstrong and Webb, 1997). In addition to FSH, several other factors have been reported to function either as inhibitors of apoptosis in granulosa cells, e.g. intraovarian insulin-like growth factor (IGF), or as inducers, e.g. transforming growth factor-β, GnRH, etc. (Chun and Hsueh 1998). It is likely that there is a complex communication network between the stromal and granulosa cells and the oocyte, and that this will send out the signals that decide the fate of the follicle (Sakata et al., 2000).

In maturing follicles, the control of apoptosis in the oocyte and granulosa and theca cells is complex because they have an antral cavity and a very complex structural morphology, requiring intense metabolic and hormonal activity to maintain cell proliferation, the final processes of oocyte maturation and steroidogenic activity of the granulosa and theca cells (Salha et al., 1998). Moreover, the presence or absence of survival
factors plays a role in regulating the apoptotic process, so that it is the balance between these competing activities that will determine the cell fate.

Various authors have investigated gonadotrophin control of primary and secondary follicles (Gougeon, 1996; McGee and Hsueh, 2000) and some reports show that in quiescent follicles, FSH does not act as a survival factor but various locally produced growth factors are involved in paracrine, autocrine and genetic control mechanisms (Markstrom et al., 2002). It has also been suggested that the signal might emanate from the oocyte enclosed in the granulosa cells, and that the different response of granulosa cells to microenvironmental factors and signals from their encapsulated oocytes may be the basis for the selective fate commitment (Picton et al., 1998; Hardy et al., 2000).

In conclusion, follicular atresia may occur at all stages of follicle growth and development, but quiescent follicles are the most susceptible to apoptosis-induced atresia. In agreement with Morita et al. (2000), our results suggest that in primordial and primary follicles, which are gonadotrophin independent, apoptosis is a cell-autonomous physiological event (germ line intrinsic), which is independent of changes in the ovarian hormonal milieu and is not a consequence of changes or alterations in the cell genome.

The complexity of programmed cell death signalling indicates that there are multiple points in the apoptosis pathway. A better understanding of the mechanisms responsible for apoptosis in quiescent follicles will help to elucidate the events inducing premature ovarian failure (early menopause) or the failure to respond to exogenous gonadotrophins with ovulation in patients undergoing assisted reproductive technology. Moreover, the possibility of controlling early intra-cellular events that signal for the activation of more downstream steps in the program of apoptosis may hold great promise for therapeutic development (Chun and Hsueh, 1998). These data could open new vistas of clinical importance as regards planning approaches to prevent the decrease of the ovarian germ cell population, controlling ovarian longevity and the menopause, as well as protecting the ovaries of cancer patients from damage due to treatment side-effects.

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References


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