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Analysis of apoptosis and expression of *bcl-2* gene family members in the human and baboon ovary

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Abstract

Recent data support a role for apoptosis, under tight regulatory control by *bcl-2*, oxidative stress response, tumor suppressor, and *CASP* gene family members, in mediating granulosa cell demise during follicular atresia in the rodent and avian ovary. Herein we evaluated the occurrence of apoptosis in the human and baboon ovary relative to follicular health status, and analyzed expression of several cell death genes in these tissues. *In situ* localization of DNA strand breaks in fixed human and baboon ovarian tissue sections indicated that apoptosis was essentially restricted to granulosa cells of atretic antral follicles. Biochemical analysis of DNA oligonucleosomes in individual follicles isolated from baboon ovaries during the ovulatory phase revealed the presence of apoptotic DNA fragments in subordinate but not dominant follicles, thus substantiating the *in situ* labeling studies. Messenger RNA transcripts encoded by the *bax* death susceptibility gene, the *bcl-x$_{long}$* survival gene, the *bcl-x$_{short}$* pro-apoptosis gene, the *p53* tumor suppressor gene, and two members of the *CASP* gene family (*CASP-2/Ich-1, CASP-3/CPP32*), were detected by Northern blot analysis of total RNA prepared either from human ovaries or from Percoll-purified granulosa-lutein cells obtained from patients undergoing assisted reproductive technologies. Lastly, immunohistochemical localization of the BAX death-susceptibility protein in the human ovary revealed abundant expression in granulosa cells of early atretic follicles, whereas BAX protein was extremely low or non-detectable in healthy or grossly-atretic follicles. We conclude that apoptosis occurs during, and is probably responsible for, folicular atresia in the human and baboon ovary. Moreover, apoptosis in the human ovary is likely controlled by altered expression of the same cohort of cell death regulatory factors recently implicated as primary determinants of apoptosis induction or suppression in the rodent ovary.

Keywords: apoptosis; BCL-2; BCL-X; BAX; p53; caspase; ICH-1; CPP32; atresia; granulosa cell; ovary

Abbreviations: BAX, BCL-2-associated X-protein; BCL-2, B-cell lymphoma/leukemia-2 protein; BCL-X, BCL-2-related protein-X; caspase, cysteine aspartic acid-specific protease (*CASP*, designation of the gene); CPP32, cysteine protease p32 (caspase-3); ddATP, dideoxy-ATP; GLC, granulosa-lutein cell; ICE, interleukin-1β-converting enzyme (caspase-1); ICH-1, ICE/CED-3 homolog-1 (caspase-2); zVAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; zDEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone

Introduction

Using sensitive biochemical and molecular biological analyses, the occurrence of apoptosis in female germ cells, follicular granulosa cells, and luteal cells during ovarian development and cyclic function in laboratory and domestic animal species has been established (reviewed in Tilly, 1996; Martimbeau and Tilly, 1997). Moreover, identification of apoptosis as the underlying mechanism responsible for controlled cellular deletion in the ovary has sparked numerous investigations into the intracellular effector pathways that are either activated or repressed during execution of the cell death command. Consistent with a solid foundation of research data derived from analysis of apoptosis regulation in non-gonadal tissues and tumor cell lines (reviewed in Stellar, 1995; Wyllie, 1995; Ko and Prives, 1996; Reed *et al*, 1996; Yang and Korsmeyer, 1996; Patel *et al*, 1996; Kumar and Lavin, 1996), recent reports have documented the possibility that a highly-conserved pathway of cell death modulators plays a prominent role in determining cell fate in the ovary (reviewed in Tilly, 1996; Tilly *et al*, 1997). For instance, proteins encoded by members of the *bcl-2* gene family, including both positive (BAX, BCL-X$_{short}$) and negative (BCL-2, BCL-X$_{long}$) regulators of apoptosis (reviewed in Reed *et al*, 1996; Yang and Korsmeyer, 1996), have been proposed as key mediators in the control of cell death in the rodent, avian and bovine ovary (Knudson *et al*, 1995; Ratts *et al*, 1995; Tilly *et al*, 1995a; Johnson *et al*, 1996, 1997; Rueda *et al*, 1997; reviewed in Tilly *et al*, 1997). Along

these lines, nuclear accumulation of the p53 tumor suppressor protein, a transcriptional regulator of the *bcl-2* and *bax* genes (Miyashita *et al*, 1994; Miyashita and Reed, 1995), has also been demonstrated in rat granulosa cells destined for apoptosis (Tilly *et al*, 1995b). Despite the current lack of data formally linking p53 to altered expression of target cell death genes in the ovary, forced expression of p53 in rat granulosa cells does in fact lead to rapid apoptosis (Keren-Tal *et al*, 1995).

Other effectors not directly related to the BCL-2 family have also been proposed to regulate apoptosis in granulosa cells of the rodent ovary. For instance, members of the *CASP* (*ced-3/Ice*) gene family, a cohort of apoptosis-inducing proteases that function at the penultimate stage of cell death committal (Martin and Green, 1995; Kumar and Lavin, 1996; Patel *et al*, 1996), are believed to participate in granulosa cell death. To date, data have been presented that suggest a role for caspase-2 (ICH-1) and caspase-3 (CPP32) in follicular atresia in the rat ovary, albeit the function of caspase-1 (ICE) *per se* remains uncertain (Flaws *et al*, 1995). In addition, apoptosis in granulosa cells of mouse ovarian follicles cultured *in vitro* under serum-free conditions is associated with proteolysis of the caspase substrate, fodrin, and is prevented by the two specific peptide inhibitors of caspases, zVAD-FMK and zDEVD-FMK (Maravei *et al*, 1997). Taken collectively, these data have served as the basis for a hypothesis that apoptosis and altered expression of cell death genes are events associated with ovarian cellular deletion in all vertebrate species (Tilly *et al*, 1997). Consequently, it was our objective to investigate the occurrence of apoptosis in the human and non-human primate (baboon) ovary, and to determine the expression of cell death-associated genes in the human ovary. Furthermore, the spatial distribution of BAX, a protein whose expression is well-correlated with and may be required for apoptosis in granulosa cells of the rodent ovary (Knudson *et al*, 1995; Tilly *et al*, 1995a), was assessed in healthy and atretic follicles of the human ovary by immunohistochemical localization.

Results

Detection of apoptosis in the human and baboon ovary

To first identify the specific cell types that undergo cell death, *in situ* localization of DNA strand breaks demonstrated that apoptosis was prevalent in granulosa cells of atretic, but not healthy, follicles in the human and baboon ovary (Figure 1). To substantiate the fact that the DNA fragmentation detected *in situ* was the result of internucleosomal cleavage associated with apoptotic cell death, genomic DNA was extracted from individual dominant (healthy) and subordinate (atretic) antral follicles dissected from late ovulatory phase baboon ovaries. Autoradiographic analysis of DNA integrity revealed the presence of varying degrees of internucleosomal cleavage in subordinate follicles (Figure 2). In contrast, DNA prepared from dominant follicles of the same ovaries did not exhibit evidence of internucleosomal cleavage (Figure 2).

Isolation of cDNAs for human cell death regulatory genes

Using the RT–PCR technique, we next obtained full-length (*bcl-x*$_{long}$) or partial (*bax*, bases 85–530; *p53*, bases 206–730; *caspase-1*, bases 660–1117; *caspase-2*, bases 684–1110 of the long isoform; *caspase-3*, bases 311–949) cDNAs from human ovarian RNA. Sequence analysis confirmed 100% identity to the corresponding sequences of the human cDNAs previously reported (data not shown; see GenBank Accessions Z23115/L20121, L22473, X02469/M60950, M87507, U13021, and U13737, respectively).

Expression of cell death genes in the human ovary and in granulosa-lutein cells (GLC)

After cloning of the human cDNAs, Northern blot analyses were conducted to determine if these genes were expressed in human ovaries and in Percoll-purified GLC. Autoradiographic analysis of RNA blots hybridized with the various probes revealed the presence of *bax*, *bcl-x*, *p53*, *caspase-1*, *caspase-2* and *caspase-3* mRNA transcripts in human ovarian RNA samples collected during the follicular phase of the cycle (Figure 3). Percoll-purified GLC were also found to express all of the genes examined (Figure 4), with the exception of *CASP-1* (data not shown). In addition, RT–PCR analysis indicated that purified GLC apparently expressed more of the 'short' form of *bcl-x* mRNA when compared with the relative levels of *bcl-x*$_{long}$ *versus bcl-x*$_{short}$ mRNA in total ovarian homogenates (Figure 4).

Immunolocalization of BAX protein in granulosa cells destined for apoptosis

In the final set of experiments, we evaluated the spatial distribution of the BAX death-susceptibility protein in the human ovary as recent studies have provided convincing proof that *bax* expression is associated with (Tilly *et al*, 1995a), and may be required for (Knudson *et al*, 1995), granulosa cell apoptosis in the rodent ovary (reviewed in Tilly *et al*, 1997). Intense immunostaining for BAX was detected in granulosa cells of early atretic follicles (Figure 5A,B), although BAX immunostaining was absent in the residual granulosa cells of grossly atretic follicles that displayed high levels of DNA fragmentation (Figure 5C; see Figure 1 for *in situ* DNA labeling/apoptosis). In early atretic follicles, BAX immunoreactivity was most intense in peri-antral granulosa cells, but was also diffusely expressed throughout the theca-interstitial cell layer (Figure 5A,B). The BAX protein was not detected in granulosa cells of healthy developing antral folicles (Figure 5D), and was similarly low or absent in interfollicular stromal cells (Figure 5).

Discussion

Initial studies of rat, avian and porcine ovaries documented a role for apoptotic cell death in the loss of granulosa cells during follicular atresia (Hughes and Gorospe, 1991; Tilly *et al*, 1991, 1992). The occurrence of apoptosis in granulosa cells appears to be a fundamental event conserved among

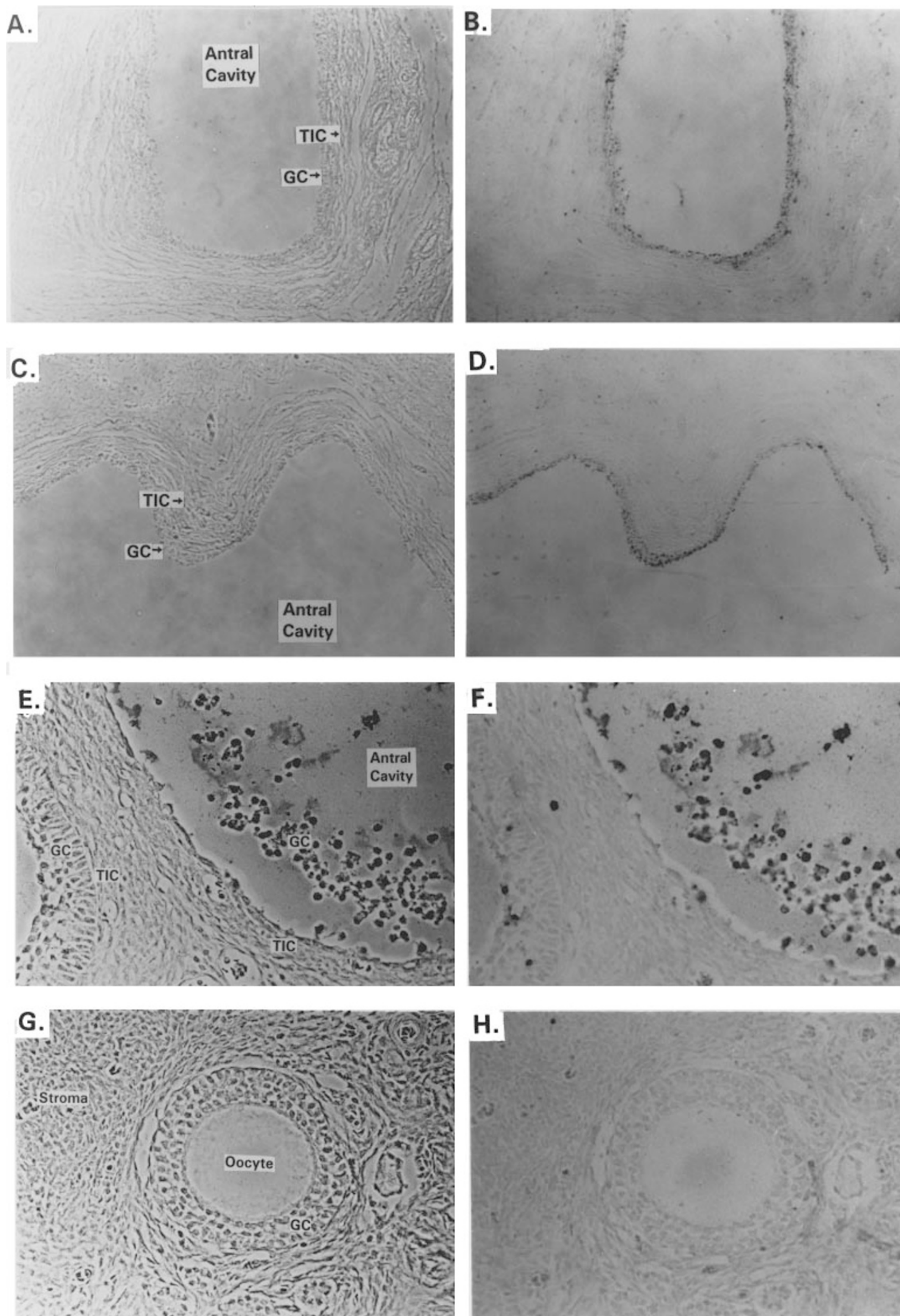

Figure 1 *In situ* localization of apoptosis-associated DNA strand breaks in granulosa cells of human and baboon ovarian atretic follicles. Panels **A – D** represent brightfield (**A,C**) and phase-contrast (**B,D**) photomicrographs of two different grossly-atretic antral follicles in the human ovary (Panel **A**, approximate follicle diameter=7 – 8 mm; Panel **C**, approximate follicle diameter=10 – 12 mm). Note the intense labeling of essentially all remaining granulosa cells (GC) for apoptotic DNA breakdown (indicated by the darkly-stained cells in Panels **B** and **D**), and the relative absence of DNA labeling in theca-interstitial cells (TIC) and the surrounding interfollicular stroma. These data are representative of results observed with ovaries from five different patients. These findings were confirmed by a similar analysis of ovaries from cycling female baboons (Panels **E – H**), which also revealed the occurrence of apoptosis in GC of atretic antral follicles (note the darkly-stained cells in Panel **F**; **E**, brightfield view of the tissue shown in **F** by phase-contrast). By comparison, TIC and cells in the surrounding stroma were not labeled. The specificity of the detection of apoptosis by this approach was further shown by the lack of DNA labeling in any cells of a healthy preantral follicle (**G,H**; brightfield and phase-contrast photomicrographs, respectively) present within the same ovarian section as the antral follicles depicted in Panels **E,F**. These data are representative of results from analysis of ovaries from four different baboons (see also Tilly, 1993)

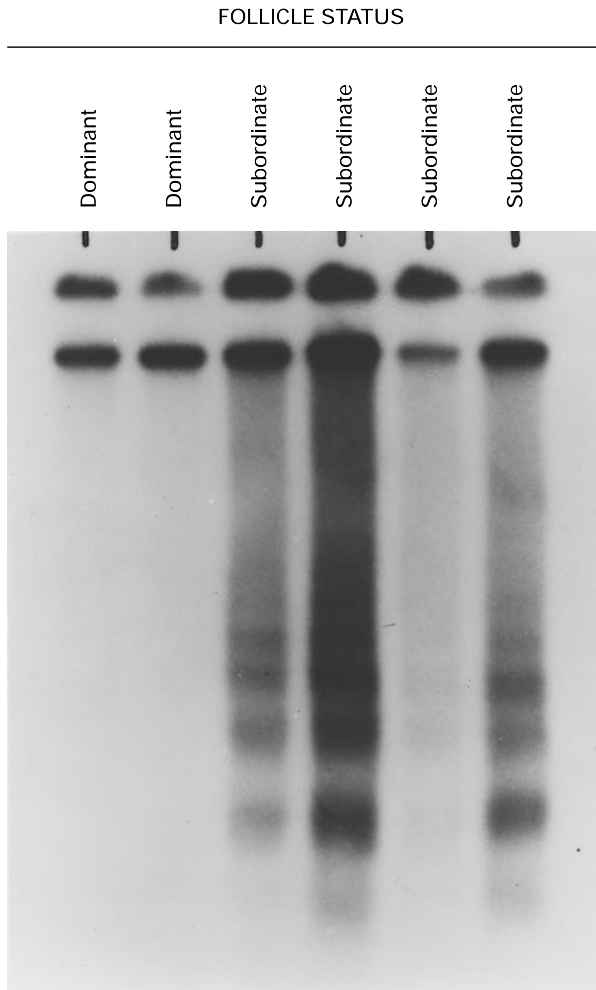

Figure 2 Biochemical analysis of internucleosomal DNA cleavage in extracts of individual follicles isolated from ovulatory phase baboon ovaries. Genomic DNA was extracted from each follicle, radiolabeled with [α^{32}P]-ddATP using the terminal transferase reaction, and resolved by agarose gel electrophoresis. After drying, gels were exposed to autoradiographic films at -80°C to visualize the absence or presence of oligonucleosomal DNA fragments characteristic of cell death via apoptosis. Data from one dominant follicle (>10 mm) and two subordinate antral follicles (4–7 mm) are shown from each of two baboons in the late follicular phase of the cycle

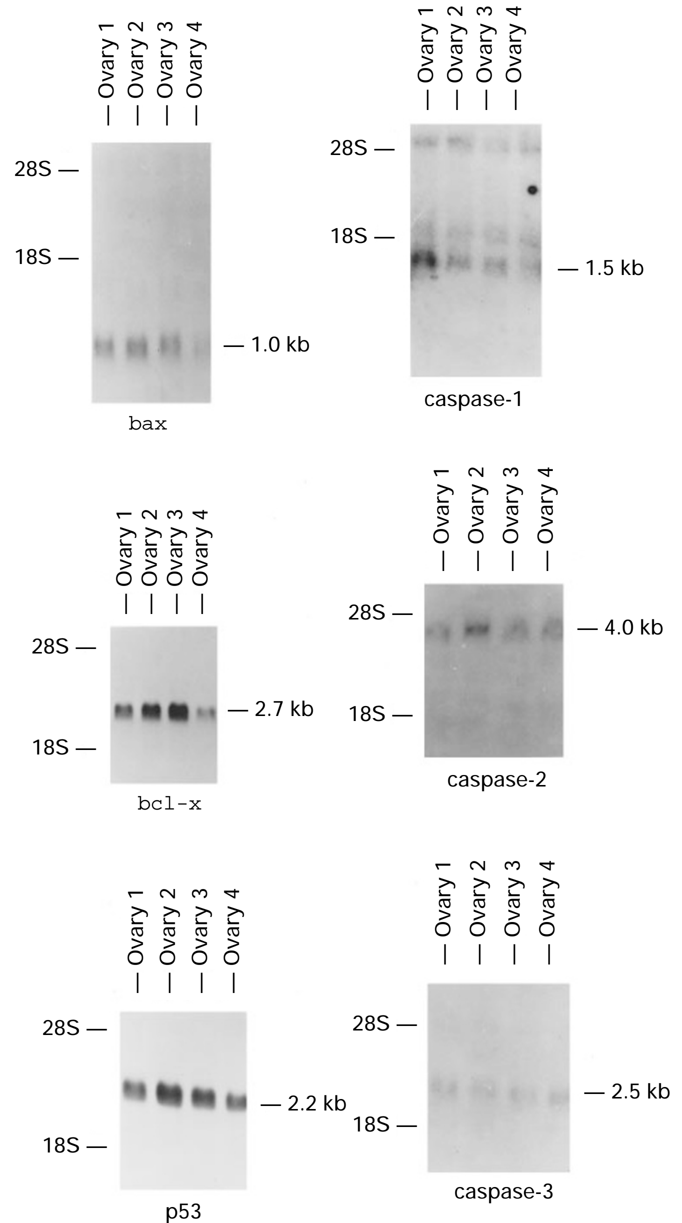

Figure 3 Northern blot analysis of cell death gene expression in the human ovary. Total RNA extracted from ovaries of four different patients (indicated as Ovary 1–4 over each blot) was resolved by denaturing gel electrophoresis (10 μg/lane), blotted to nitrocellulose and hybridized to radiolabeled antisense RNA probes against human *bax*, *bcl-x*, *p53*, *caspase-1*, *caspase-2* or *caspase-3*. The estimated size of each primary transcript for each gene studied (in kilobases, kb), and the migration distances of the 18S and 28S rRNA species, are indicated

vertebrate animal species as this form of physiological cell death has been identified in atretic follicles of mouse, rabbit, bovine and ovine ovaries as well (Gavrieli *et al*, 1992; Dharmarajan *et al*, 1994; Jolly *et al*, 1994, 1997; Maravei *et al*, 1997). Despite these advances in our understanding of the mechanisms responsible for ovarian cellular deletion in animal models, comparatively little is known of the occurrence and regulation of physiological cell death in human follicular cells. In this investigation, extensive levels of DNA fragmentation indicative of apoptotic cell death were detected, *in situ*, in granulosa cells of atretic antral follicles of the human and baboon ovary. These data, which were fully supported by demonstration of oligonucleosomal DNA fragments in individual subordinate but not dominant follicles isolated from baboon ovaries in the ovulatory phase of the cycle, are consistent with the identification of apoptosis in granulosa cells of atretic follicles in all other species examined to date.

Confirming that apoptosis was prevalent in granulosa cells of human atretic follicles, we next pursued investigations into the potential mechanisms involved in activating this physiological death pathway in the human ovary. Recent data derived from studies of apoptosis regulation

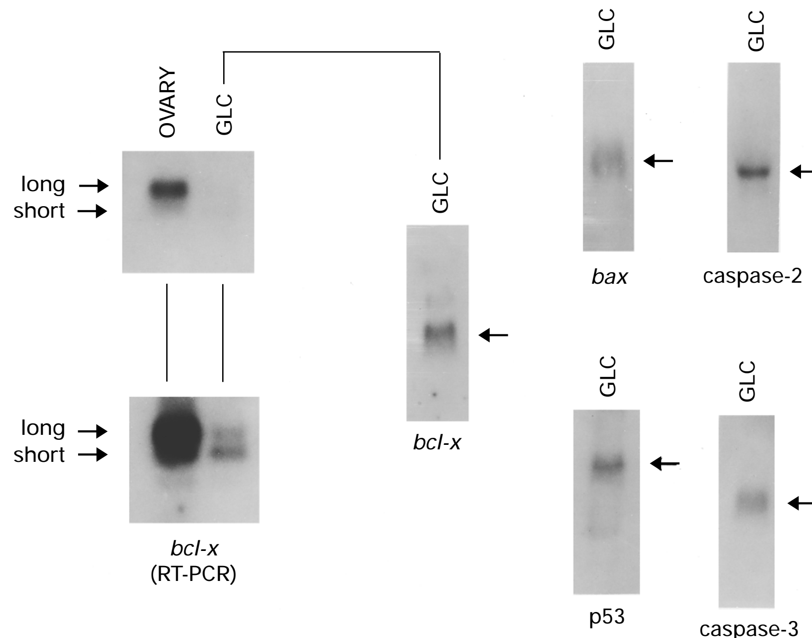

Figure 4 Cell death gene expression in human granulosa-lutein cells (GLC). Total RNA extracted from Percoll-purified GLC was analyzed as described in the legend to Figure 3 for the presence of *bax*, *bcl-x*, *p53*, *caspase-1*, *caspase-2* and *caspase-3* mRNA transcripts using radiolabeled antisense RNA probes and Northern blot analysis (arrows indicate the transcripts as defined in Figure 3). Under these conditions, *caspase-1* mRNA could not be detected in GLC (data not shown). In addition to the Northern blot analysis of *bcl-x* mRNA in GLC, the far left panel depicts the results obtained from RT–PCR/Southern blot hybridization analysis of the 'long' *versus* 'short' forms of the message in total RNA prepared from whole ovary or from GLC. Due to differences in the relative abundance of *bcl-x* mRNA in the two preparations, two different autoradiographic exposures were required and are presented (upper panel, 6 h; lower panel, 18 h). As revealed by RT–PCR, note that GLC apparently possess roughly equivalent amounts of the long and short forms of the *bcl-x* transcript, whereas whole ovary contains primarily the long form. These data are representative of results obtained with ovary and GLC RNA prepared from at least four different patients per transcript analyzed

in the ovary of rodents have revealed that the death of granulosa cells during atresia is likely controlled by the protein products of several recently characterized apoptosis-associated genes. For example, several members of the *bcl-2* gene family are expressed in granulosa cells of the rat ovary (Tilly *et al*, 1995a), and enhanced expression of the *bax* death-susceptibility gene (Oltvai *et al*, 1993) has been reported to herald demise of granulosa cells during follicular atresia *in vivo* and *in vitro* (Tilly *et al*, 1995a; Tilly, 1996). The role of BAX in ovarian cell death, implied by data obtained from analysis of changes in *bax* mRNA levels, has been reinforced following evaluation of mice harboring a targeted disruption in the *bax* gene. Histologic analysis of ovaries from BAX-deficient mice revealed an apparent defect in the ability of granulosa cells to undergo apoptosis during follicular atresia (Knudson *et al*, 1995).

Our present findings from the mRNA and protein analyses indicate that expression of the *bax* gene in the human ovary also predominates in granulosa cell populations that are on the verge of apoptosis, but is absent in granulosa cells of healthy follicles. Furthermore, in the same early atretic follicle we noted discrete immunolabeling of peri-antral granulosa cells without evidence BAX immunostaining in the adjacent granulosa cells lying more proximal to the basal lamina. This apparent 'gradient' of BAX within the follicle mirrors a similar 'gradient' of apoptosis that is known to occur in granulosa cells during atresia of antral follicles (reviewed in Tilly and Ratts, 1996). Therefore, it is possible that the intense BAX immunoreactivity detected in peri-antral granulosa cells prior to marked morphological changes indicative of pending atresia can be used to demarcate a subordinate follicle destined for degeneration. Interestingly, BAX protein was non-detectable in grossly-atretic follicles, suggesting that once BAX has served to predispose the granulosa cells to apoptosis activation, the protein is lost during completion of the cell death process.

Using Northern blot analysis, we also observed robust expression of the *bcl-x* gene in human ovarian homogenates as well as in Percoll-purified GLC. Further analysis of the *bcl-x* messages by RT–PCR and Southern blot hybridization confirmed that, similar to data reported for the rat (Tilly *et al*, 1995a) and avian (Johnson *et al*, 1996) ovary, the long or 'death repressor' form of *bcl-x* (Boise *et al*, 1993) was the primary transcript expressed in human ovarian homogenates. However, purified human GLC apparently expressed more of the short or 'death inducer' form of *bcl-x* (Boise *et al*, 1993) as compared with the levels of this mRNA species detected in whole ovarian preparations by RT–PCR/Southern blot hybridization. Although the reason for this is unclear at present, we have recently shown that Percoll-purified GLC obtained from individual follicles of patients undergoing assisted reproductive technologies display variable levels of DNA oligonucleosomes (Piquette *et al*, 1994). Thus, our previous proposal that cellular reorganization within the dominant

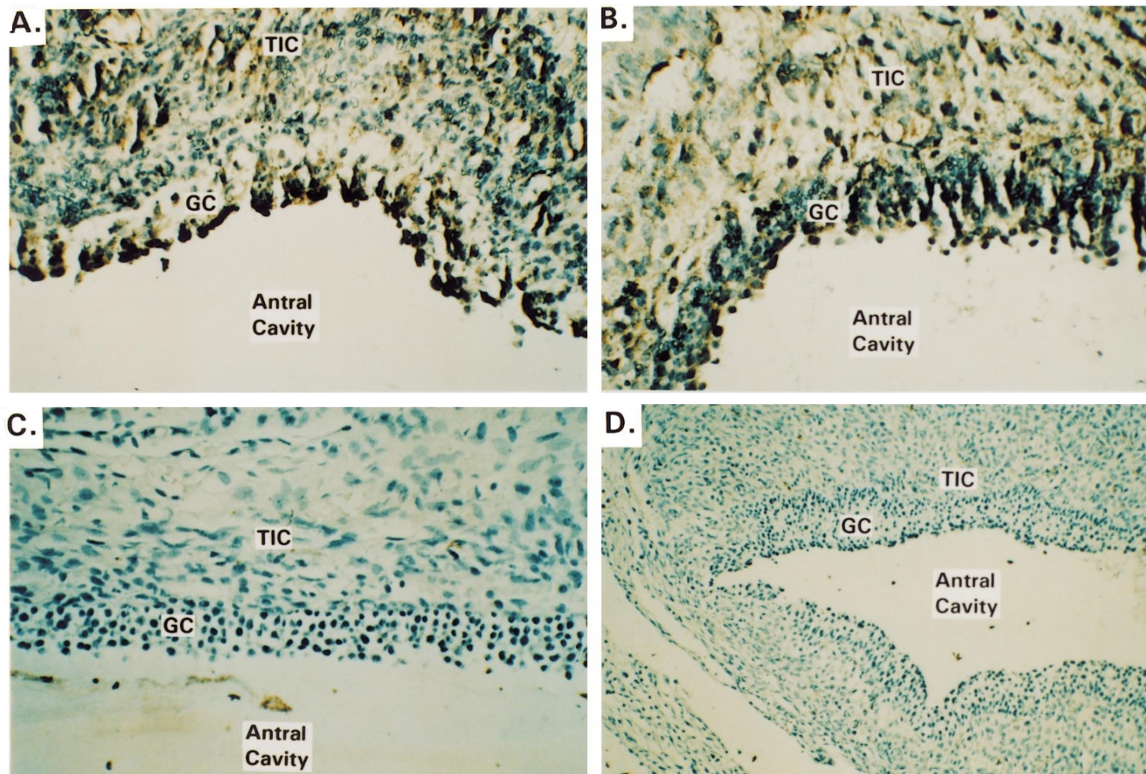

Figure 5 Immunohistochemical localization of the BAX death-susceptibility protein in human ovarian follicles. Panel **A** depicts an early atretic antral follicle (approximate follicle diameter=5–7 mm) demonstrating BAX immunoreactivity (brown-staining) in theca-interstitial cells (TIC) and in granulosa cells (GC). Note that the most intense BAX immunolabeling is found in the peri-antral GC. This was a consistent observation as shown by a similar pattern of BAX immunoreactivity in a different early atretic antral follicle of approximately the same size (Panel **B**). In contrast, BAX immunostaining was extremely low or absent in GC and TIC of grossly atretic antral follicles (Panel **C**; approximate follicle diameter=10–12 mm). The BAX protein was also absent in GC and TIC of healthy antral follicles (Panel **D**; approximate follicle diamter=6–8 mm). The occurrence of atresia was confirmed by *in situ* DNA labeling of adjacent sections (see Figure 1 for examples), which revealed that approximately 11%, 13%, >85% and 0% of the granulosa cells were apoptotic in the follicles depicted in Panels **A**, **B**, **C** and **D**, respectively. These data are representative of results obtained with ovaries from two different patients

follicle prior to, during and after ovulation is associated with apoptosis may be further supported by the findings that a potential death inducer ($BCL\text{-}X_{short}$) is being upregulated in populations of granulosa cells during follicular rupture and the ensuing luteinization process. Unfortunately, our attempts to document expression of the *bcl-2* gene in the human ovary or in GLC by RT–PCR analysis proved unsuccessful. It should be noted, however, that previous studies on the presence of this death repressor gene in the human ovary have provided somewhat conflicting data. For example, some investigations failed to detect *bcl-2* mRNA in human GLC using Northern blot analysis (Jerome F. Strauss III, personal communication), whereas others have reported the presence of BCL-2 protein in the human corpus luteum (Rodger *et al*, 1995) and ovarian stroma (Lu *et al*, 1993) by immunohistochemistry.

In addition to *bcl-2* gene family members, we detected abundant expression of several other cell death regulatory genes in human ovaries and, importantly, in purified human GLC. These included p53, a tumor suppressor protein that may function upstream of BCL-2 family members via transcriptional regulation of the *bcl-2* and *bax* genes (Miyashita *et al*, 1994; Miyashita and Reed, 1995), and caspases, which likely function downstream of BCL-2-related proteins in pathways of cell death execution (Chinnaiyan *et al*, 1996; Estoppey *et al*, 1997; Perry *et al*, 1997; reviewed in Kumar, 1997). Interestingly, the levels of *caspase-1* mRNA in human ovarian homogenates were extremely low as reflected by the background level of non-specific hybridization to ribosomal RNA species that had to be maintained in order to visualize the 1.5 kb *caspase-1* mRNA transcript by Northern blot analysis. Moreover, only *caspase-2* and *caspase-3* transcripts were detectable in human GLC, even following prolonged autoradiographic exposure of blots hybridized with the *caspase-1* cRNA probe. The apparent absence of *caspase-1* mRNA in GLC may reflect the lack of involvement of this particular member of the *CASP* gene family in cell death signaling in this cell type, consistent with data recently reported for the rat ovary (Flaws *et al*, 1995).

In summary, we have provided evidence supporting the hypothesis that apoptosis and a cohort of conserved cell-death regulatory factors are fundamental to the cyclic growth and regression of ovarian follicles in the human ovary. Additionally, data implicating apoptosis in follicular atresia in the human are supported by parallel experiments

conducted with baboon ovaries (present studies), as well as a large number of previous studies reported for laboratory and domestic animal models (reviewed in Tilly, 1996). The knowledge gained by such investigations may prove useful for the future design of compounds that would function as contraceptives by selectively destroying target ovarian structures (such as preovulatory follicles for pre-conception, and the corpus luteum for post-conception) through controlled activation of physiological cell death pathways. Furthermore, a greater understanding of the events involved in mediating germ cell and follicle depletion from the human ovary may lead to novel approaches for delaying the menopause, as well as preventing premature reproductive senescence in females exposed to noxious stimuli such as radio- and chemotherapy and environmental toxicants (reviewed in Tilly and Perez, 1997; Tilly *et al*, 1997). Our current studies are addressing these hypotheses (Perez *et al*, 1997), as well as further characterizing the presence and spatial distribution of cell death regulatory proteins in the human female reproductive tract during menstrual cycle-related episodes of apoptosis (Tao *et al*, 1997).

Materials and Methods

Tissue samples

Human ovaries Ovarian tissues were collected from a total of five women between the ages of 32–43 undergoing oophorectomy and/or total abdominal hysterectomy for benign conditions. Wedges were cut from ovaries and either fixed immediately in 4% neutral-buffered paraformaldehyde (w:v in 1 × -concentrated Dulbecco's phosphate-buffered saline, D-PBS) for future histochemical manipulations, or were snap-frozen and stored at −80°C until processed for RNA extraction and analysis.

Human granulosa-lutein cells (GLC) Women between the ages of 22–40, undergoing assisted reproductive technological procedures with controlled ovarian hyperstimulation (*n*=10 patients), were treated with leuprolide acetate (Lupron; TAP Pharmaceuticals Inc., Deerfield, IL, USA) starting in the mid-luteal phase. On day 3 of the following cycle, patients were treated daily with human menopausal gonadotropins (Pergonal or Metrodin, Serono Laboratories, Norwell, MA, USA; Humegon, Organon, West Orange, NJ, USA) while maintained on leuprolide acetate treatment. When at least two follicles with diameters ⩾17 mm and serum estradiol levels of ⩾500 pg/ml were noted, patients were treated with 10 000 U of human chorionic gonadotropin (Pregnyl, Organon; Profasi, Serono Laboratories). Preovulatory follicles were aspirated and flushed 35 h later. Discarded follicular aspirates pooled from several follicles per patient were centrifuged (10 min, 700 × *g*, 20°C), and the top layer of cells enriched with GLC was carefully collected into Ham's F-10 medium (supplemented as described in Piquette *et al*, 1994). Following a second centrifugation step, the top GLC-enriched cell layer was collected in Ham's F-10 medium and then centrifuged through 50% Percoll (prepared in Ham's F-10 medium) for 30 min at 700 × *g*. Following Percoll separation, the GLC-enriched interphase layer was collected, washed in Ham's F-10 medium and centrifuged once again. The resultant GLC pellet was snap-frozen and stored at −80°C until RNA extraction and analysis.

Baboon ovaries As a comparative model system, ovaries were collected from normal-cycling adult female baboons (*Papio anubis*) weighing between 12–16 kg. Animals were housed individually in large aluminium/stainless steel primate cages in air-conditioned rooms with a 12 h light : 12 h dark cycle. Baboons received high-protein monkey chow (Purina Mills, St. Louis, MO, USA) twice daily, fresh fruit and vitamins once daily, and water *ad libitum*. Menstrual cycles were monitored by records of cycle history and by turgescence of external sex skin (Albrecht, 1980). Ovaries were surgically removed from baboons anesthetized with a mixed gas of halothane (1–1.5%): nitrous oxide (0.5 liters/min): oxygen (2 liters/min). Depending upon the experiment, ovaries were either fixed immediately in 4% neutral-buffered paraformaldehyde (*n*=4 animals; *in situ* DNA labeling analysis) or were used for microdissection of individual follicles under a stereomicroscope (*n*=2 animals; biochemical analysis of DNA for internucleosomal cleavage). In the latter instance, isolated follicles obtained from late ovulatory phase ovaries were sized, snap-frozen in liquid nitrogen, and stored at −80°C until processed for DNA analysis. Dominant *versus* subordinate follicles were selected based on size, with the largest diameter follicle obtained from the late follicular phase ovaries in each animal designated as dominant.

Protocols All protocols involving human and baboon tissue collection were reviewed and approved by the appropriate institutional review committees (IRB/HSPC and IACUC). Studies with baboons were conducted in strict accordance with United States Department of Agriculture (USDA) regulations and with the protocols set forth in the NIH Guide for the Care and Use of Laboratory Animals (publication 85-23, 1985).

In situ labeling for apoptosis

Localization of apoptosis-associated DNA strand breaks in fixed ovarian tissue sections was performed using a non-radioactive DNA labeling technique, as previously described in detail (Tilly, 1994). Briefly, tissues were fixed in 4% neutral-buffered paraformaldehyde for 24 h, washed in sterile 7% sucrose (w:v in 1 × -concentrated D-PBS), embedded in paraffin, and sectioned (6 μm) for mounting on 3-aminopropyltriethoxysilane (AES, Aldrich Chemical, Milwaukee, WI, USA)-coated glass slides. Sections were treated for 30 min with 10 μg/ml proteinase-K (Boehringer-Mannheim, Indianapolis, IN, USA) to release intercalated proteins from the DNA, washed and labeled for 15 min with biotin-14-deoxy-ATP (dATP; Grand Island, NY, USA) using terminal transferase enzyme (Boehringer-Mannheim). Sites of incorporation of biotinylated-dATP were detected using streptavidin-alkaline phosphatase (Sigma Chemical Co., St. Louis, MO, USA) with nitroblue tetrazolium salt (NBT; 300 μg/ml) and 5-bromo-4-chlor-3-indolylphosphate toluidinium salt (BCIP; 248 μg/ml) as colorimetric substrates for the reaction. Negative controls included processing of samples in the absence of terminal transferase enzyme or the labeling nucleotide. In either case, no colorimetric reaction was observed (data not shown).

Biochemical analysis of DNA oligonucleosomes

Genomic DNA was extracted from individual follicles as originally described (Gross-Bellard *et al*, 1973) and modified (Tilly and Hsueh, 1993). The quantity and purity of each nucleic acid sample was assessed by measuring the optical density at A260 nm *versus* A280 nm. Purity estimates for each sample, as measured by the ratio of A260 : A280, were consistently 1.85 or greater. An equivalent amount of DNA (500 ng) from each sample was then labeled in parallel on 3′-ends with [α^{32}P]-dideoxy-ATP (ddATP; 3000 Ci/mmol; Amer-

sham, Arlington Heights, IL, USA) using terminal transferase enzyme (Boehringer-Mannheim), as detailed previously (Tilly and Hsueh, 1993; Tilly, 1994). For qualitative assessments of DNA integrity (the absence or presence of DNA oligonucleosomes characteristic of apoptosis), labeled samples were analyzed by autoradiography (Hyperfilm, Amersham) at −80°C following size fractionation through 2% agarose gels (Tilly, 1994).

Extraction of RNA

Total RNA was extracted and purified using the guanidinium thiocyanate-phenol-chloroform single-step procedure, as described (Chomczynski and Sacchi, 1987). The quantity and purity of each nucleic acid sample was assessed by measuring the optical density at A260 nm *versus* A280 nm.

Isolation and characterization of human cDNAs

Total RNA isolated from human ovarian tissue was reverse transcribed into first strand cDNA using random hexamer primers (Boehringer Mannheim) and avian myeloblastosis virus reverse transcriptase (AMV-RT) (Promega, Madison, WI, USA). After computer analysis of appropriate cDNA sequences using the OLIGO program, primers were synthesized (DNA International, Lake Oswego, OR, USA) based on human *bcl-2* (forward: 5′-TAT-AAG-CTG-TCG-CAG-AGG-GG-3′, bases 61 through 80; reverse: 5′-TGA-CGC-TCT-CCA-CAC-ACA-TG-3′, bases 490 through 471; Cleary *et al*, 1986), *bcl-x*$_{long}$ (forward: 5′-TTG-GAC-AAT-GGA-CTG-GTT-GA-3′, bases −39 through −20 of the 5′-untranslated region; reverse: 5′-GTA-GAG-TGG-ATG-GTC-AGT-G-3′, bases 6 through 24 of the 3′-untranslated region; Boise *et al*, 1993), *bax* (forward: 5′-GGT-TTC-ATC-CAG-GAT-CGA-GAC-GG-3′, bases 85 through 106; reverse: 5′-ACA-AAG-ATG-GTC-ACG-GTC-TGC-C-3′, bases 530 through 509; Oltvai *et al*, 1993), *p53* (forward: 5′-TGC-GTG-TGG-AGT-ATT-TGG-ATG-AC-3′, bases 206 through 228; reverse: 5′-ACT-GAC-CCT-TTT-TGG-ACT-TCA-GG-3′, bases 730 through 708; Matlashewski *et al*, 1984), *caspase-1/Ice* (forward: 5′-TGC-CCA-GAG-CAC-AAG-ACT-TCT-GAC-3′, bases 660 through 683; reverse: 5′-CGA-ACC-TTT-CTG-AAA-ATG-TCC-TCC-3′, bases 1117 through 1094; Cerretti *et al*, 1992), *caspase-2/Ich-1* (forward: 5′-TGG-CAT-ATA-GGT-TGC-AGT-CTC-GG-3′, bases 518 through 540; reverse: 5′-TGT-TCT-GTA-GGC-TTG-GGC-AGT-TG-3′, bases 880 through 858; Wang *et al*, 1994) or *caspase-3/CPP32* (forward: 5′-ACA-TGG-AAG-CGA-ATC-AAT-GGA-CTC-3′, bases 287 through 301; reverse: 5′-AAG-GAC-TCA-AAT-TCT-GTT-GCC-ACC-3′, bases 973 through 950; Fernandes-Alnemri *et al*, 1994) mRNA coding sequences, and were used for polymerase chain reaction (PCR) amplification of the corresponding human cDNA sequences as follows. The first strand cDNA was subjected to 35 cycles of PCR amplification using one of the primer sets (1 min denaturation at 94°C, 1 min annealing at 50°C, and 2 min extension at 72°C). The amplified products were resolved through 1.5% agarose gels, isolated, purified (Gene Clean; Bio 101, La Jolla, CA, USA) and subcloned into the pCRII vector (Invitrogen, San Diego, CA, USA) for large-scale plasmid preparation and automated DNA sequence analysis (version 2.0.15; Applied Biosystems, Foster City, CA, USA).

Preparation of radiolabeled probes and Northern blot analysis

We were not successful in the isolation of a *bcl-2* cDNA from human ovarian RNA by RT–PCR amplification; however, all other cDNA sequences were isolated. Antisense RNA probes complementary to human *bcl-x*, *bax*, *p53*, *caspase-1*, *caspase-2* and *caspase-3* mRNA coding sequences were synthesized by *in vitro* transcription from linearized plasmid templates using RNA polymerase, [α^{32}P]-CTP (3000 Ci/mmol; Amersham) and the Gemini II Riboprobe Core System (Promega), as described (Melton *et al*, 1984). For the Southern blot hybridization analysis, the human *bcl-x* cDNA probe was radiolabeled with [α^{32}P]-deoxy-CTP (3000 Ci/mmol; Amersham) using the random priming method (Feinberg and Vogelstein, 1983), and purified from unincorporated radionucleotides by column chromatography (NucTrap Push Columns, Stratagene, La Jolla, CA, USA).

Total RNA samples prepared from human ovarian tissues or from Percoll-purified human GLC were fractionated through 1.2% denaturing agarose gels (10 μg RNA/lane), visualized with ethidium bromide staining and UV transillumination (to confirm RNA integrity and sample loading equality), and blotted to pure nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) by overnight capillary transfer using 20×-concentrated sodium chloride/sodium citrate solution (20×-concentrated SSC: 3 M sodium chloride, 0.3 M sodium citrate) as the transfer buffer. The RNA samples were then UV cross-linked to the membranes (Stratalinker, Promega), and hybridized to radiolabeled antisense RNA probes (3×10^6 cpm/ml hybridization buffer) under highly stringent conditions at 65°C for 18–20 h, as described (Tilly *et al*, 1995a,b; Flaws *et al*, 1995). Following a 10 min wash at 20°C in 2×-concentrated SSC/0.1% sodium dodecyl sulfate (SDS) and extensive washing (20–40 min) at 65°C in 0.1×-concentrated SSC/0.1% SDS, autoradiography was carried out by exposure of membranes to film (Hyperfilm, Amersham) for 2–8 days at −80°C.

RT–PCR/Southern blot analysis of alternatively-spliced *bcl-x* transcripts

To determine the relative expression of the 'long' (death repressor) *versus* 'short' (death inducer) forms of *bcl-x* mRNA (Boise *et al*, 1993), total RNA samples prepared from human ovarian tissue or from Percoll-purified human GLC were subjected to RT–PCR analysis using human *bcl-x* primers (see 'Isolation and Characterization of Human cDNAs' above) that span the 189 bp region deleted in *bcl-x*$_{short}$ (Tilly *et al*, 1995a). Following a 25-cycle amplification, one-tenth of the resultant PCR reaction containing the amplified cDNA products was resolved by gel electrophoresis and blotted to nylon-reinforced nitrocellulose membranes (Schleicher and Schuell) by overnight capillary transfer using 10×-concentrated SSC. Southern blot hybridization was then conducted with a radiolabeled human *bcl-x* cDNA probe at 42°C for 18–20 h (Tilly *et al*, 1995a), after which membranes were washed under highly stringent conditions (see above). Autoradiography was then carried out by exposure of blots to film (Hyperfilm, Amesham) for 2–18 h at −70°C. Identity of the two amplified PCR products was further confirmed by sequence analysis.

Immunohistochemistry

Paraffin-embedded fixed human ovarian tissue sections, mounted on glass slides as described above (see '*In Situ* DNA Labeling Analysis' above), were subjected to immunohistochemical staining for the presence and distribution of the BAX death-susceptibility protein using a rabbit polyclonal antiserum raised against a human BAX peptide sequence, as detailed previously (Krajewski *et al*, 1994). Briefly, high temperature antigen unmasking was first performed by microwaving the sections for 5.5 min in 10 mM sodium citrate. After cooling, sections were treated with 0.5% hydrogen peroxide (in methanol) for 5 min at 20°C to quench endogenous peroxidase activity, pre-blocked with TNK buffer

(100 mM Tris-HCl (pH 7.6), 550 mM NaCl, 10 mM KCl, 2% bovine serum albumin, 0.1% Triton X-100, 1% normal goat serum) for 45 min at 20°C, and then incubated for 18 h at 4°C with the primary antibody diluted 1 : 500 in TNK buffer, After washing, sections were incubated with a biotinylated goat anti-rabbit IgG (2.8 μg/ml) for 1 h at 20°C, and then washed again. Localization of the primary antibody-biotinylated second antibody complex was then performed using streptavidin-horseradish peroxidase with diaminobenzadine as the chromogenic substrate (Krajewski *et al*, 1994). To permit visualization of the tissue architecture, slides were lightly counter-stained with hematoxylin before coverslipping. As negative controls, sections were prepared and analyzed as described with primary antibody replaced by normal rabbit serum. Under these conditions, no immunoreaction was observed (data not shown).

Data analysis and presentation

For studies of human ovaries and GLC, all experiments were repeated with tissues or cells collected from at least five or ten different patients, respectively. Comparative studies conducted with baboon ovarian tissues were done with four different animals, with the exception that the dissection and analysis of apoptosis in dominant *versus* subordinate follicles was conducted with ovaries from two different animals in the ovulatory phase of the cycle. For all studies, photomicrographs (*in situ* DNA analysis, immunohistochemistry) and autoradiograms (biochemical DNA analysis, Northern and Southern blot analyses) representative of results obtained with different patient or baboon samples are provided (see also Figure Legends).

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