The genes of cell death and cellular susceptibility to apoptosis in the ovary: a hypothesis

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Abstract

Recent advances in the field of cell death, primarily derived from gene-transfer experiments and manipulation of tumor cell lines *in vitro*, have identified key genes responsible for determining whether or not a given cell will initiate apoptosis. However, comparatively less is known of the role that the products of these genes play in physiological settings of cell death. In the ovary, a tremendous level of normal cell death takes place in the germline throughout the later stages of fetal development. This process is responsible for setting the absolute number of oocytes ('eggs') available for subsequent development and ovulation during adult life. Interestingly, death remains the fate of the vast majority of oocytes that survive the waves of attrition during fetal life and are endowed in the post-natal ovary as primordial follicles. This pool of oocytes is lost indirectly as a consequence of the death of the somatic (granulosa) cells that, in the case of a small percentage of the total follicles, support and nourish the oocyte until its release at ovulation. Due to the magnitude of cell death that occurs normally within the female gonad during both fetal development and post-natal life, the ovary has proven to be an excellent model to study the role of cell death genes in a physiological setting of endocrine-regulated apoptosis.

It is now known that a diverse spectrum of pro- and anti-apoptosis susceptibility genes, including members of the *bcl-2* and *CASP* (*ced-3/Ice*) gene families, are expressed in germ cells and/or somatic cells of the ovary. Many, but not all, of these genes are regulated by specific survival factors, such as gonadotropins and growth factors, and changes in the temporal patterns of cell death gene expression suggest an intimate association exists between the products of these genes and activation of cellular suicide. Moreover, pathological oocyte destruction, such as that triggered by exposure of female germ cells to chemotherapeutic compounds or environmental toxicants, may also be dependent upon gene-driven apoptosis. As such, this review will discuss data supporting the hypothesis that the susceptibility of ovarian cells to death induction is dependent upon the pattern of cell death gene expression occurring within those cells prior to and/or concomitant with receipt of the stimulus for apoptosis. Elucidation of the relationship between germ cell loss and cell death genes may allow future intervention into the process of oocyte depletion associated with normal and pathophysiological reproductive senescence.

Apoptosis in the ovary: a brief historical account

Numerous studies have now established that physiological cell death plays a fundamental role in the endowment of the primordial follicle stockpile during the peri-natal period and the subsequent depletion of the follicle pool by the degenerative process of atresia throughout prepubertal and reproductive life. The earliest known report of apoptosis in the ovary was made by Flemming in 1885 in a meticulously documented morphological study of how inner epithelial (granulosa) cells of rabbit ovarian follicles degenerate *in vivo*. The process of 'chromatolysis' was proposed as an underlying feature of this phenomenon, and in retrospective analysis the morphological characteristics described by Flemming (1885) perfectly match the criteria set forth by Kerr *et al* (1972) 87 years later for the identification of apoptotic cells. Despite this significant series of observations made over a century ago, the relationship between apoptosis and ovarian function essentially remained dormant, with the exception of several scattered morphological descriptions of cell turnover in the ovary (see, for example: Quatacker, 1971; Kerr *et al*, 1972; Byskov, 1974; Hirshfield and Midgley, 1978a; O'Shea *et al*, 1978; Braw *et al*, 1981), until the early part of this decade.

In light of the now well-known association between internucleosomal DNA fragmentation and apoptosis (Williams *et al*, 1974; Wyllie, 1980; Arends *et al*, 1990; Schwartzman and Cidlowski, 1993; Tilly, 1994), Zeleznik and co-workers (1989) identified the presence of a cation-dependent endonuclease activity in rat ovarian granulosa and luteal cell nuclei capable of eliciting such a pattern of DNA cleavage. Furthermore, the presence of this endonucleolytic activity appeared to be endocrine-regulated, suggesting that the demise of granulosa cells during follicular atresia and of luteal cells during luteolysis, may

be dependent upon acquisition of this enzyme during development of the respective tissues. This study was followed in 1991 by two reports linking apoptosis to the process of follicular atresia in three different vertebrate species *in vivo* (Hughes and Gorospe, 1991; Tilly *et al*, 1991), with both studies relying on the occurrence of oligonucleosome formation as an indicator of apoptotic death. With the interest in apoptosis and the ovary re-fueled by these investigations, the past several years have seen an exponential increase in the number of publications documenting the occurrence of apoptosis in granulosa cells during atresia in all animal species studied to date (reviewed in: Guthrie *et al*, 1995; Tilly, 1996; Tilly and Ratts, 1996). In addition, many studies utilizing cultured granulosa cells or intact follicles have identified important roles for a wide spectrum of endocrine, paracrine and autocrine factors, such as gonadotropins (Tilly *et al*, 1992, 1995a; Chun *et al*, 1994; Tilly and Tilly, 1995), growth factors (Tilly *et al*, 1992, 1995b; Chun *et al*, 1994; Luciano *et al*, 1994), and neuropeptides (Flaws *et al*, 1995a), in regulating granulosa cell apoptosis during the later stages of follicular development.

In contrast to the now generous supply of research data supporting a role for apoptosis in follicular atresia, comparatively little is known of the pathways responsible for attrition of oogonia and oocytes in the fetal and early post-natal ovary of vertebrate species (Beaumont and Mandl, 1961; Borum, 1961; Pinkerton *et al*, 1961; Baker, 1963; Forabosco *et al*, 1991). Recent flow cytometric analysis of germ cells isolated from fetal mouse gonads has demonstrated that apoptosis is at least one mechanism underlying prenatal oogonium and oocyte loss (Coucouvanis *et al*, 1993). These *in vivo* investigations have been supported by several *in vitro* studies documenting the occurrence of apoptosis by many criteria in primordial germ cells (Pesce *et al*, 1993; Pesce and De Felici, 1994) and oogonia/oocytes (Martimbeau *et al*, 1996) following deprivation of serum or cytokines needed for survival in culture. Based on the findings that the survival advantage conveyed by at least one of the cytokines tested *in vitro*, namely SCF (Dolci *et al*, 1991; Godin *et al*, 1991; Pesce *et al*, 1993; Martimbeau *et al*, 1996), is supported by *in vivo* assessments of defects in gametogenesis and fertility in mice lacking expression of functional SCF or SCF receptor (Mintz and Russell, 1957; Motro *et al*, 1991; Besmer *et al*, 1993), it is reasonable to assume that data derived from future analyses of apoptosis in these *in vitro* models of germ cell attrition will have significant physiological relevance.

The bcl-2 : bax rheostat: a matter of life and death in the ovary?

With the understanding that apoptosis is so critical to germ cell and granulosa cell fate came the second wave of investigations, that being identification of the intracellular effectors responsible for activating or repressing the order for cellular suicide in the ovary. The first set of genes evaluated in this context, as with most other paradigms of apoptosis, was *bcl-2* and its family members (Reed, 1994; Korsmeyer, 1995).

Since the majority of available data pertaining to the ovary concern changes in *bcl-2*, *bax* and *bcl-x* expression (see below), the following discussion will be limited to these three family members. Presently, it is believed that proteins encoded by members of the *bcl-2* gene family interact via specific conserved domains (Yin *et al*, 1994) to form both homo- and heterodimers (Sato *et al*, 1994; Sedlak *et al*, 1995). Moreover, it has been proposed from manipulations of tumor cell lines that the relative ratio of the anti-apoptosis proteins, Bcl-2 and Bcl-x_L, to the pro-apoptosis factors, Bax and Bcl-x_S, in a given cell functions as a 'rheostat' to determine that cell's susceptibility to apoptosis following exposure to a death-promoting stimulus (Boise *et al*, 1993; Oltvai *et al*, 1993; Oltvai and Korsmeyer, 1994). For example, transfection of certain cells with *bcl-2* provides protection from such insults as tropic hormone deprivation, whereas gene-transfer of *bax* accelerates the rate at which these cells undergo apoptosis in response to the same stimulus (Oltvai *et al*, 1993; Reed, 1994; Korsmeyer, 1995). However, there is evidence that Bcl-2 and Bcl-x_L may also act independent of each other and of Bax heterodimerization to promote cell survival (Cheng *et al*, 1996; Gottschalk *et al*, 1996), albeit these mechanisms remain ill-defined and are beyond the scope of this review.

From the perspective of ovarian cell death, the presence and changes in expression of *bcl-2*, *bax* and *bcl-x* in the ovary have been documented in several species (Rodger *et al*, 1995; Tilly *et al*, 1995a, 1996a,b; Johnson *et al*, 1996; Rueda *et al*, 1997). In the rodent ovary, the most studied model system for ovarian cell death research, Bax has emerged as the principal player in regulating both germ cell and granulosa cell demise. The first report along these lines evaluated the *in vivo* effects of gonadotropins on ovarian expression of *bcl-2*, *bax* and *bcl-$x_{L/S}$* relative to the inhibition of granulosa cell death and follicular atresia, and further extended these observations by an analysis of these three genes in ovarian follicles deprived of tropic hormone support *in vitro* (Tilly *et al*, 1995a). The primary conclusion reached was that there exists a strong positive correlation between enhanced expression of *bax*, in the face of relatively constitutive *bcl-2* and *bcl-x* expression, and the induction of apoptosis in granulosa cells of follicles during atresia. This series of observations has been reinforced by a more recent analysis of ovaries collected from mice harboring a targeted disruption in the *bax* gene (Knudson *et al*, 1995). In accordance with the hypothesized role of Bax in granulosa cell apoptosis, follicles in Bax-deficient females contain granulosa cells that are apparently resistant to the induction of normal cell death during atresia (Knudson *et al*, 1995). These data, taken with recent preliminary observations that increased levels of Bax protein detected by immunohistochemistry in granulosa cells precede morphological evidence of follicle degeneration in the human ovary (Tilly *et al*, 1996b; manuscript submitted), collectively suggest that a rheostat mechanism involving a shift towards greater Bax levels is a critical step in the process of follicular atresia.

Interestingly, the fate of germ cells in the ovary during fetal development may also be driven by the status of the Bcl-2 : Bax rheostat. Although there exists scant data describing expression of any *bcl-2* gene family members

in germ cells, morphologic and morphometric results derived from gene knock-out mice have proven quite informative. In the case of Bcl-2-deficient female mice, significantly fewer primordial follicles are endowed in the post-natal ovary as compared with follicular endowment in wild-type sister-littermates (Ratts *et al*, 1995). Since the number of follicles endowed can be used as an indicator of the degree of peri-natal germ cell loss, these data would argue that a shift in the rheostat to favor Bax (due to the absence of Bcl-2) in germ cells may lead to greater numbers of oogonia/oocytes lost during fetal development. By comparison, Bax-deficient female mice have a three-fold greater number of primordial follicles endowed in the ovary at 42 days of age post-partum (Knudson *et al*, 1996). These data would support the hypothesis that a shift in the rheostat away from Bax, possibly favoring greater Bcl-2 (or Bcl-x$_L$?) bioavailability, reduces death susceptibility in germ cells. Another potentially important implication of the results from the *bcl-2* and *bax* gene knock-out experiments is that the Bcl-2 : Bax rheostat can be shifted with dramatic consequences in the magnitude of cell death by altering the expression of only one of the genes. As such, future evaluations of *bcl-2* family members in ovarian germ and somatic cells should consider that the absence of a change in expression one family member (such as *bcl-2* or *bcl-x$_L$*) may still be important for setting the rheostat if expression of another opposing family member (such as *bax*) is altered.

Before closing this section, two additional points concerning the topic of Bcl-2-related proteins and ovarian function require brief discussion. First, enhanced expression of *bax* has also been recently identified in other ovarian cell types during instances of apoptosis not related to germ cell attrition or follicular atresia (e.g. luteolysis; Tilly *et al*, 1996b; Rueda *et al*, 1997), further supporting a fundamental role for this specific death-susceptibility factor in multiple aspects of ovarian development and cyclic function. Second, activation of anti-oxidant defense pathways has been implicated as a primary survival mechanism for granulosa cells in developing follicles (Tilly and Tilly, 1995), consistent with the reported anti-apoptosis actions of Bcl-2 being at least partly mediated via the effects of this protein on stabilizing the reduction-oxidation state of the cell and thus preventing apoptosis induced by accumulation of reactive oxygen species or their intermediates (Hockenberry *et al*, 1993; Kane *et al*, 1993; Steinman, 1995).

The p53 tumor suppressor protein: a cytoplasmic link to the nucleus?

The studies reviewed in the previous section establish that, in accordance with data derived from work with tumor cell lines and genetic manipulation, members of the *bcl-2* gene family likely function as key participants determining the susceptibility of ovarian cells to a death stimulus. However, as with other cell systems, the mechanisms responsible for altering the Bcl-2 : Bax rheostat in ovarian cells are currently unknown. The first glimpse into one potential signaling pathway comes from studies of the p53 protein. As a preface to these discussions, it is critical to first cite three recent investigations

from Reed and colleagues (Miyashita *et al*, 1994a,b; Miyashita and Reed, 1995) that served as the impetus for evaluating the potential role of p53 in ovarian cell demise. These experiments, the first to establish an important link between p53 and transcriptional regulation of cell death genes, identified a p53-enhancer element in the *bax* gene promoter (Miyashita and Reed, 1995) and a p53-repressor element in the *bcl-2* gene promoter (Miyashita *et al*, 1994a). Moreover, using a combination of *in vivo* and *in vitro* approaches, a positive correlation between p53 levels and *bax* expression, and a negative correlation between p53 levels and *bcl-2* expression, in cells of various lineages was documented (Miyashita *et al*, 1994b). In the ovary, p53 protein has been localized by immunocytochemistry to the nuclei of granulosa cells destined for apoptosis during follicular atresia (Tilly *et al*, 1995c). Moreover, provision of exogenous gonadotropin support *in vivo* abolishes p53 immunoreactivity in the ovary (Tilly *et al*, 1995c), concomitant with a loss of *bax* expression (Tilly *et al*, 1995a) and a complete absence of apoptosis (Tilly *et al*, 1995a) in the same cell populations. These data, taken with evidence demonstrating p53-mediated apoptosis in granulosa cells by gene-transfer techniques (Keren-Tal *et al*, 1995), support the concept that ovarian survival factors may somehow prevent nuclear translocation of p53 as one means to shift the Bcl-2 : Bax rheostat away from Bax predominance in granulosa cells.

Of final note in this section, there is recent evidence that the germ cell survival factor, SCF, can prevent p53-induced apoptosis in erythroleukemia cells (Abrahamson *et al*, 1995). Although the presence and function of p53 in female germ cells during the peri-natal waves of attrition is currently unknown, these findings suggest that one of the anti-apoptosis actions of SCF in the fetal ovary may be at the level of interfering with p53-mediated pathways of cell death induction in oogonia/oocytes. Collectively, the data discussed above underscore the importance of future work to continue to decipher the role of p53 in regulating apoptosis in germ cells and somatic cells of the ovary, studies that may be greatly facilitated with the current availability of p53-deficient mice.

Caspases: the penultimate step to irrevocable cell death?

For the sake of completeness, it is important to touch on the role of proteases, specifically those encoded by members of the *CASP* (Alnemri *et al*, 1996) or *ced-3/Ice* gene family, in ovarian cell death. This concept is of particular interest in light of a recent report that caspases function downstream of Bcl-2-like proteins in the regulation of cell death in vertebrates (Chinnaiyan *et al*, 1996), analogous to that described for the respective Bcl-2 and caspase homologs in *Caenorhabditis elegans*, Ced-9 and Ced-3 (Hengartner and Horvitz, 1994). This proposed hierarchy between Bcl-2 family members and caspases has recently been supported by two additional reports describing the ability of Bcl-2 to prevent activation of caspase-3 (CPP32) in various paradigms of apoptosis (Estoppey *et al*, 1997; Perry *et al*, 1997). At this point, however, the mechanism(s) by which Bcl-2 acts in this regard remains to be established (Kumar, 1997). Nonetheless,

members of the *CASP* gene family in vertebrates, of which there are now at least ten, are believed to function at a committal point(s) in cell death execution as the targets cleaved by these proteases are primarily homeostatic proteins found in both the cytoplasm and nucleus (Martin and Green, 1995; Fraser and Evan, 1996; Patel *et al*, 1996). To date, there exists only a single report of caspases in the ovary that demonstrated a reduction in expression of *CASP-2* (Ich-1, the long isoform; Wang *et al*, 1994) and *CASP-3* (CPP32; Fernandes-Alnemri *et al*, 1994) following *in vivo* gonadotropin stimulation of immature rats (Flaws *et al*, 1995b). However, in this same study, various inhibitors of serine and cysteine proteases did not alter the onset of apoptosis in granulosa cells of follicles incubated *in vitro* without hormonal support. The one exception was an

inhibition of internucleosomal DNA fragmentation, but not morphological evidence of apoptosis, achieved with the putative cysteine protease inhibitor, sodium aurothiomalate (Flaws *et al*, 1995b).

Subsequent preliminary experiments with incubated follicles have now shown that sodium aurothiomalate is also a potent inhibitor of the nuclease(s) responsible for oligonucleosome formation in granulosa cells (Trbovich *et al*, 1996a). Thus, the discrepancy in the biochemical (absence of DNA ladders) versus morphological (presence of pyknosis, cellular condensation, budding) evidence can be attributed to the occurrence of high molecular DNA cleavage not prevented by this inhibitor in granulosa cells (Trbovich *et al*, 1996b). Although these data argue both for (expression and gonadotropin down-regulation of
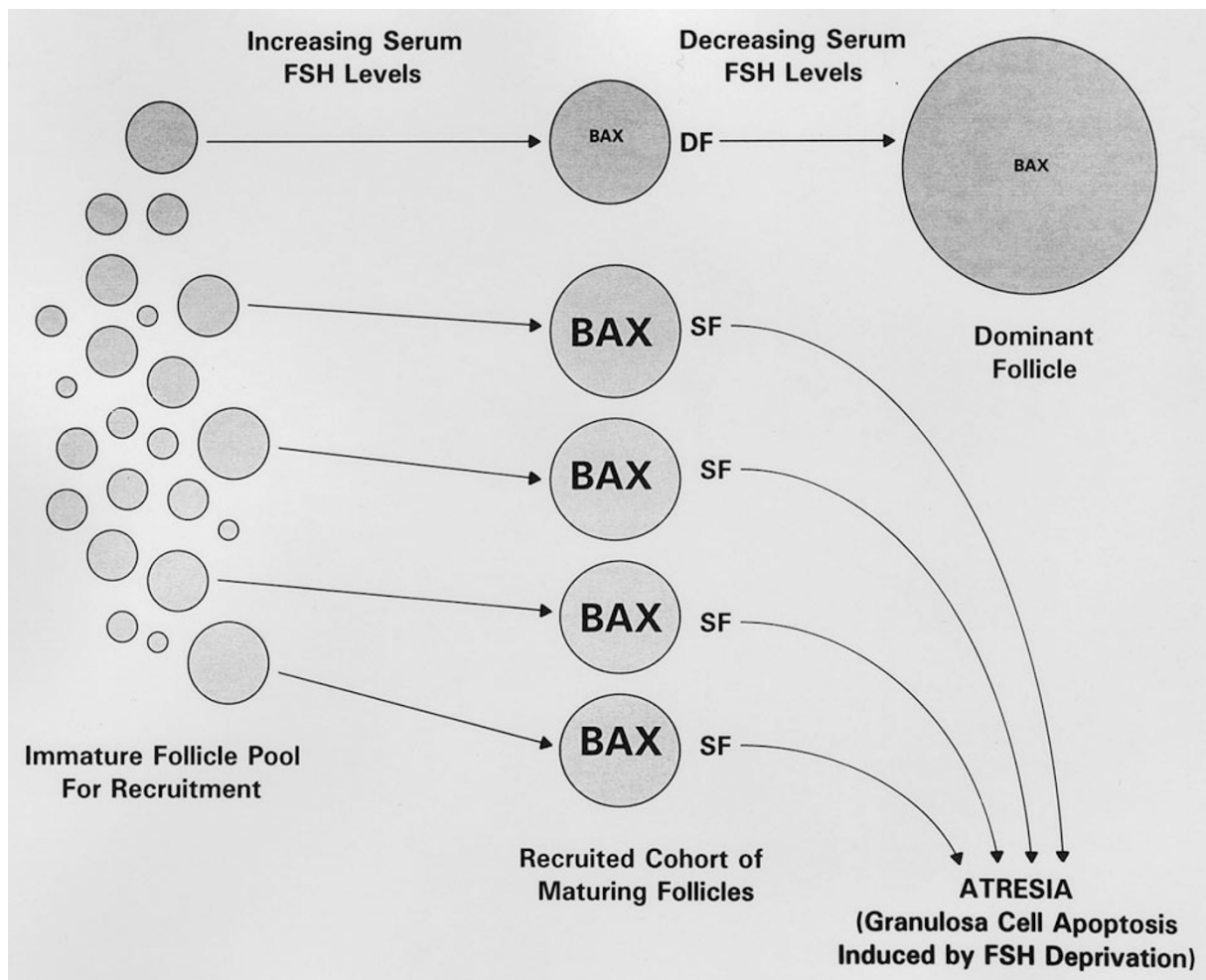

Figure 1 Hypothesized role of Bax in selection of the dominant follicle in the ovary of mono-ovulatory species. Each menstrual or estrous cycle, a cohort of follicles is recruited to begin the final steps of growth and maturation to the preovulatory stage of development. However, only one follicle will be selected as the dominant follicle (DF) for continued survival and ultimately ovulation, whereas the remaining subordinate follicles (SF) in the cohort undergo the degenerative process of atresia resulting from an induction of apoptosis in the follicular granulosa cells. From the data discussed in this review, it is hypothesized that granulosa cells within the different follicles possess varying levels of Bax protein, and that granulosa cells within the follicle that will be selected as dominant contain the lowest amounts of Bax (the amount of Bax is portrayed by the size of typography, with small letters indicating low Bax and large letters indicating high Bax). Upon declining FSH support, the likely trigger for atresia of the subordinate follicles, the granulosa cells possessing the lowest levels of Bax also exhibit the lowest susceptibility to tropic hormone (FSH) deprivation-induced apoptosis. As such, this feature may aid the dominant follicle in resisting activation of the atresia process, whereas the remaining follicles in the cohort initiate atresia due to a greater susceptibility of their granulosa cells to death induction, in the face of waning FSH levels.
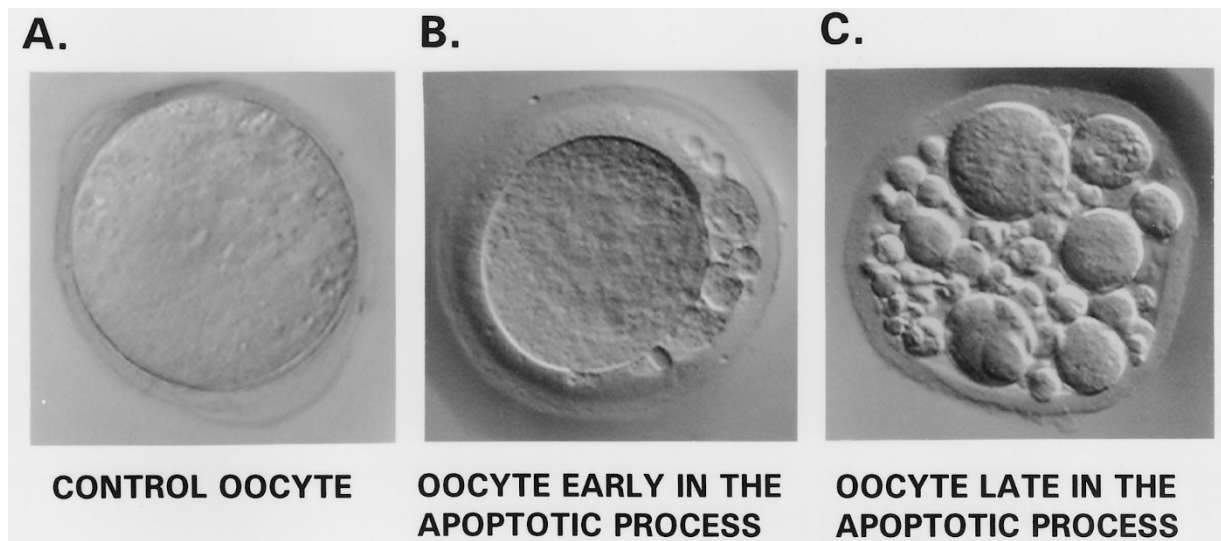
A. CONTROL OOCYTE

B. OOCYTE EARLY IN THE APOPTOTIC PROCESS

C. OOCYTE LATE IN THE APOPTOTIC PROCESS

Figure 2 Analysis of apoptosis in individual oocytes as a model to characterize events responsible for pathological conditions of ovarian germ cell depletion. (**A**) depicts a healthy single oocyte exposed to vehicle alone, whereas (**B**) and (**C**) show a progressive appearance of the apoptotic morphology in individual oocytes exposed to an ovotoxicant. Note the beginning of the budding phenomenon in the oocyte shown in (**B**), and the dramatic increase in the extent of membrane budding and cellular fragmentation of the oocyte depicted in (**C**).

caspases in the ovary) and against (lack of effect of general protease inhibitors on apoptosis in incubated follicles) the participation of proteases in ovarian cell death, more recent experiments using cell-permeable and specific peptide inhibitors of this class of proteases (zVAD-FMK and zDEVD-FMK) have further supported the concept that these gene products are in fact important for apoptosis in both ovarian granulosa cells (Maravei *et al*, 1996; manuscript submitted) and germ cells (G.I. Perez, C.M. Knudson, L. Leykin, G.A.J. Brown, S.J. Korsmeyer, J.L. Tilly, unpublished data). Conclusive proof of this hypothesis, however, may await the availability of data generated from the future in-depth analysis of ovarian germ cell and follicular dynamics in mice lacking expression of one or more of the caspases generated through gene-targeting approaches.

Cell death genes in the ovary: what does it all mean?

In summary, great strides have been made in understanding the pathways activated or repressed in ovarian cells that may alter their fate following local endocrine signaling events. For the most part, the data currently available in the literature support the concept that apoptosis in the ovary is dependent upon the expression of certain key cell death genes, such as *bax*, and that the susceptibility of any given cell to apoptosis is likely decided before receipt of the actual stimulus for death. The possible implications of these data are many, and the following scenario provides just one example. A problem faced by ovarian physiologists is understanding the basis of follicle selection, whereby in mono-ovulatory species (e.g. humans) one dominant follicle is selected from a cohort of

follicles at a comparable developmental stage for continued survival, growth and ovulation (Hodgen, 1982; Ginther *et al*, 1996). Although it is known that the remaining follicles are lost by atresia, the reasons that one follicle possesses a 'survival' advantage over other follicles, some of which even lie proximal to the dominant follicle, remain to be fully elucidated. It is known that a loss of FSH support, achieved via negative feedback loops involving among other things increased estradiol and inhibin secretion from the ovary during dominant follicle selection (Zeleznik, 1981; Hodgen, 1982; Zeleznik *et al*, 1985; Ginther *et al*, 1996), is likely the fundamental event involved in triggering demise of the subordinate follicles. This argument is supported by data indicating that gonadotropins do in fact maintain a suppression of apoptosis in granulosa cells of antral follicles both *in vivo* and *in vitro* (Hirshfield and Midgley, 1978b; Braw *et al*, 1981; Tilly *et al*, 1992, 1995a; Chun *et al*, 1994; Tilly and Tilly, 1995). However, in theory all of the follicles in the developing cohort receive the same ovarian blood supply that carries this important survival factor from the pituitary gland (albeit differences in blood flow or vascularization on a per follicle basis may be an important variable; Zeleznik *et al*, 1981), and thus a switch from dependency on FSH to luteinizing hormone (LH), as reflected by increased acquisition of functional LH receptors in the granulosa cells in the dominant follicle, has been proposed as a basis for why the dominant follicle continues to grow and survive in the presence of declining FSH (Ginther *et al*, 1996).

However, based on the data reviewed above, an alternate hypothesis is offered. It is suggested that certain follicles, by virtue of the patterns of cell death genes expressed in their granulosa cells, possess a predetermined survival advantage in the face of waning FSH

support. In the case of the Bcl-2 : Bax rheostat, this concept is supported by evidence showing that expression of *bax* in granulosa cells precedes apoptosis and is inhibited by gonadotropins, and that the absence of functional Bax causes defects in the normal induction of granulosa cell death during atresia. Thus, it may be that the relative levels of Bax in granulosa cells of different follicles, all of which appear morphologically and developmentally similar, is an important factor in determining whether or not a given follicle will undergo atresia upon a decrease in the level of FSH support seen by all follicles in the later half of the follicular phase (Figure 1). A similar hypothesis could be proposed for the massive attrition of germ cells in the developing ovary during the peri-natal period, with SCF substituting for FSH as the primary survival signal present in limited quantities. In this case, the status of the Bcl-2 : Bax rheostat in any given germ cell may be a primary determinant of which oogonia/oocytes live and which oogonia/oocytes die in the presence of suboptimal levels of SCF derived from the surrounding somatic cells.

Regardless of the outcome of future experiments to test the validity of these hypotheses, it remains that the concepts of apoptosis and cell death genes, as they pertain to ovarian function, are intriguing and are here to stay. The use of knowledge gained by these investigations has already permitted recent breakthroughs in understanding pathological conditions of germ cell loss. For example, it has been reported that oocyte loss following exposure of the ovary to environmental toxicants occurs via an induction of apoptosis (Mattison, 1980; Springer *et al*, 1996a) and this process is preceeded by increased expression of *bax* in those follicles or oocytes destined for destruction (Springer *et al*, 1996b; G.I. Perez, X.-J. Tao and J.L. Tilly, unpublished data). Furthermore, using an *in vitro* culture system to analyze apoptosis in individual oocytes under defined conditions (Figure 2), we have recently observed that oocytes collected from Bax-deficient female mice are resistant to apoptosis induced by exposure to a variety of ovotoxicants (G.I. Perez, C.M. Knudson, L. Leykin, G.A.J. Brown, S.J. Korsmeyer and J.L. Tilly, unpublished data). As such, data derived from future studies of apoptosis and cell death genes in the ovary will likely continue to open new avenues for research in reproductive physiology, as well as to provide a unique model for assessing the function of known, and as yet unknown, cell death regulatory factors in a physiologically-relevant setting of fetal and post-natal cell death.

Acknowledgements

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