Apoptosis and ovarian function

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For decades, the mechanisms responsible for germ cell depletion from the ovary, either directly during the perinatal period or indirectly via follicular atresia during postnatal life, have remained relatively obscure. The recent application of sensitive biochemical techniques for the study of cell death to the analysis of ovarian function has revealed that these two events, as well as a third instance of ovarian cell degeneration (luteolysis), are dependent upon the activation of physiological cell death mechanisms. It is therefore hypothesized that the controlled deletion of ovarian cell populations is accomplished via activation of a ‘universal’ pathway of cellular suicide involving altered expression of a conserved cohort of genes. The identity of the hormonal and intracellular effectors responsible for the coordination of life and death decisions made by ovarian cells during development as well as the biological and clinical implications of gene-directed cell death in the ovary are explored in this review.

Over a century ago, at a time when the terms ‘apoptosis’ and ‘programmed cell death’ were unknown, a detailed morphological characterization of inner epithelial (granulosa) cells within degenerating rabbit ovarian follicles offered the first glimpse into the pathway by which follicular atresia is probably accomplished. Only recently, however, has the role of physiological cell death in ovarian function re-emerged as an intriguing and exciting research area that encompasses almost every aspect of ovarian physiology, from early oogenesis to the demise of the corpus luteum. Several lines of evidence now support the possibility that there is a final common ovarian cell death pathway composed of genes encoding intracellular effector proteins that have been conserved, both structurally and functionally, across species and cell types. This article presents a brief description of the identifying features of physiological versus pathological cell death in multicellular organisms, and then reviews our current understanding of the phenomenon of cell death in the ovary. In addition, new possibilities for future analysis of the regulatory pathways that dictate the fate of ovarian cells during development are explored.

The morphology and biochemistry of apoptosis

The earliest descriptions of physiological cell death recognized as distinct from pathological tissue destruction were derived from morphological evaluations (Majno and Joris, 1995). In 1972, a paper was published, considered now by many to contain a landmark series of observations by light microscopic morphological examinations, which essentially set the precedents for identification of cells undergoing apoptosis (Kerr et al., 1972). The criteria by which apoptosis is characterized include a loss of cell volume (cytoplasmic condensation) accompanied by nuclear pyknosis resulting from margination of the chromatin and its redistribution against the nuclear envelope. In addition, many of the cytoplasmic organelles are maintained intact until the final stage of cell death, which is generally demarcated by the formation and release of plasma membrane-bound vesicles (apoptotic bodies) containing the cellular constituents of the dying cell which are then phagocytized by neighbouring cells (Table 1).

Accurate identification of all physiological cell deaths by these criteria, however, may not be appropriate as other forms of normal nonpathological cell death have been reported that differ from the stereotypical series of events that define apoptosis (Table 1). Furthermore, problems have arisen from the use of ‘apoptosis’ and ‘programmed cell death’ as synonymous or interchangeable terms (Majno and Joris, 1995), when in fact true programmed cell death (driven by genes that specify the timing of cell death, most notably during tissue development; Lockshin and Williams, 1965; Saunders, 1966) can occur by morphological and biochemical events that are distinct from apoptosis (resulting from genes that encode the effector proteins necessary to carry out the cell death command, generally in response to changes in external stimuli; Kerr et al., 1972). Evidence has also been provided for a distinct type of physiological cell death in vertebrates, termed autophagic vacuolization or autophagocytosis (Ericsson, 1969) (Table 1). As its name implies, this process is associated with the formation of large cytoplasmic vacuoles, suspected to be of lysosomal origin, before or in the absence of any nuclear changes. In contrast to apoptosis, comparatively little is known about this form of cell death; however, in many of the instances where autophagocytosis is observed, apoptosis can also be detected. In any case, the occurrence of irreversible tissue injury leading to ‘accidental’ or ‘pathological’ cell death can be discerned from all physiological cell deaths, since the former is restricted to contiguous tracts of adjoining cells that exhibit evidence of an inflammatory reaction (Kerr et al., 1994). Moreover, at the single cell level, accidental or pathological death is characterized morphologically by chromat in clumping without changes in its distribution, swollen mitochondria and focal disruption of the plasma membrane leading to cellular dissolution (Kerr et al., 1994; Majno and Joris, 1995) (Table 1).

From a biochemical perspective, one of the hallmark features attributed to apoptotic cells is the loss of DNA integrity
following endonuclease-mediated fragmentation of the nuclear genomic pool (Williams et al., 1974). A primary indicator of this event in most cases is the generation of 'DNA ladders'. This phenomenon results from the specific internucleosomal cleavage of DNA into 185 basepair (bp) multiples, and can be visualized by agarose gel electrophoresis of DNA extracted from tissues or cells (Wyllie, 1980; Tilly, 1994) (Fig. 1). Of cautionary note, the generation of DNA ladders is not a prerequisite step in apoptosis, as many instances of this form of cell death have been observed to occur without detectable oligonucleosome formation (Cohen et al., 1992; Collins et al., 1992). Until the recent use of pulsed-field gel electrophoresis (PFGE) for analysis of cell death, this finding confounded efforts to resolve the discrepancy between nuclear pyknosis and the apparent absence of DNA cleavage. The application of PFGE, which permits visualization of the high molecular weight (MW) DNA fraction that cannot be assessed by conventional agarose gel electrophoresis, to the study of DNA integrity confirmed the occurrence of some degree of high MW genomic DNA fragmentation in essentially all instances of physiological cell death (Walker and Sikorska, 1994). These observations have now paved the way for an in-depth analysis of the identity and regulation of the nucleases responsible for DNA cleavage, an event that is believed to be the irreversible point in the cell death cascade.

**Table 1.** Reported morphological and biochemical criteria for identifying physiological (apoptosis, autophagocytosis) versus accidental (pathological) cell death

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Phenotype</th>
<th>Time-frame</th>
<th>Type of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td>Condensation, margination, redistribution along nuclear membrane</td>
<td>Early</td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td>No change?</td>
<td>Early-to-late</td>
<td>Autophagocytosis</td>
</tr>
<tr>
<td>DNA integrity</td>
<td>Condensation, no redistribution</td>
<td>Mid-to-late</td>
<td>Accidental</td>
</tr>
<tr>
<td></td>
<td>Fragmentation, high molecular weight cleavage, DNA ladders (not always)</td>
<td>Early</td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td>High molecular weight cleavage?</td>
<td>Late</td>
<td>Autophagocytosis</td>
</tr>
<tr>
<td></td>
<td>Random cleavage (sometimes DNA ladders)</td>
<td>Mid-to-late</td>
<td>Accidental</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Reduced volume, protease activation</td>
<td>Early-to-mid</td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td>Vacuolization (increased volume), protease activation</td>
<td>Early</td>
<td>Autophagocytosis</td>
</tr>
<tr>
<td></td>
<td>Increased volume (followed by shrinkage at a later point), organelle disruption, lysosome rupture</td>
<td>Early</td>
<td>Accidental</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Maintained</td>
<td>Early-to-late</td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td>Autodigestion</td>
<td>Mid-to-late</td>
<td>Autophagocytosis</td>
</tr>
<tr>
<td>Cell membrane</td>
<td>Swelling, rupture</td>
<td>Early-to-mid</td>
<td>Accidental</td>
</tr>
<tr>
<td></td>
<td>Shrinkage, budding</td>
<td>Early</td>
<td>Autophagocytosis</td>
</tr>
<tr>
<td>Fate of cella</td>
<td>Enlargement</td>
<td>Early-to-mid</td>
<td>Accidental</td>
</tr>
<tr>
<td></td>
<td>Focal disruption, ion pump failure, swelling, rupture</td>
<td>Early</td>
<td>Autophagocytosis</td>
</tr>
<tr>
<td></td>
<td>Heterophagocytosis of apoptotic bodies, absence of typical inflammatory reaction</td>
<td></td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td>Autophagocytosis then heterophagocytosis, absence of typical inflammatory reaction</td>
<td></td>
<td>Autophagocytosis</td>
</tr>
<tr>
<td></td>
<td>Inflammatory reaction, massive immune cell infiltration for 'clean-up' of cell remnants</td>
<td></td>
<td>Accidental</td>
</tr>
</tbody>
</table>

*The criteria provided are a summary of observations from several independent studies and do not always hold true for all paradigms of the specific cell death indicated. For more information, the reader is referred to Lockshin and Williams (1965); Saunders (1966); Ericsson (1969); Quatacker (1971); Kerr et al. (1972, 1994); Majno and Joris (1995). bThe point at which a given criterion is observed once a cell has received a death signal. cNote that in some cases resident macrophages play a role in the 'clean-up' of physiological cell deaths; however, these deaths typically do not attract other lineages of white blood cells (lymphocytes, neutrophils) that are involved in normal inflammatory responses resulting from traumatic tissue injury.
Identification of apoptosis during atresia: a new twist on an old story

The first reported observation of apoptosis in the ovary, which is also one of the earliest descriptions of physiological cell death, was made in 1885 after morphological analysis of granulosa cells during degeneration of antral follicles in the rabbit ovary (Flemming, 1885). A process referred to as ‘chromatolysis’ was proposed as a mechanism by which granulosa cell loss was mediated, and in retrospect these observations closely matched all of the morphological criteria now known to be the hallmarks of apoptosis (Kerr et al., 1972, 1994). Since this initial observation, many studies on the morphological changes that occur in granulosa cells and theca-interstitial cells of follicles progressing through atresia have documented that apoptosis is, in all likelihood, the primary mechanism by which cell loss is mediated during follicle degeneration (Tsai and Braw, 1984; Hirshfield, 1991; Tilly and Ratts, 1996). The first biochemical evidence that endonucleases responsible for the generation of DNA oligonucleosomes could be detected in nuclei isolated from rat granulosa and luteal cells was published in 1989 (Zeleznik et al., 1989; recent data suggest that this endonuclease activity shares many properties with deoxyribonuclease I: Boone et al., 1995). Despite these data, the role of apoptosis in either atresia or luteolysis at that time continued to be an area of research that attracted very little attention. However, in 1991 two simultaneous reports documented the occurrence in vivo of internucleosomal DNA fragmentation in granulosa cells isolated from atretic follicles of both avian and mammalian species (Hughes and Gorospe, 1991; Tilly et al., 1991) (Fig. 1), and these served as a catalyst for a tremendous increase in research efforts to understand the roles and regulation of apoptosis in the ovary.

Germ cell attrition, follicular atresia and luteolysis: cell death as an underlying theme

A review of the literature indicates that apoptosis or some form of physiological cell death plays a fundamental role in three discrete processes related to ovarian development or cyclicity: (1) perinatal oogonium and oocyte attrition, (2) follicular atresia and (3) luteolysis. Although the vast majority of available information pertains to the loss of granulosa cells during follicle demise, an increasing number of studies have implicated apoptosis in the degeneration of germ cells and luteal cells. Each of these processes will be discussed separately.

Embryonic and early neonatal germ cell loss

In most vertebrate species, mitotically active primordial germ cells migrate to and colonize the developing genital ridge. In the newly formed embryonic ovary, these dividing germ cells (oogonia) then begin to leave the mitotic cycle, initiate meiotic division and, as oocytes, become arrested in the first prophase (Peters, 1970; Hirshfield, 1991). During both mitosis and meiosis, large numbers of germ cells are culled from the ovary for as yet unknown reasons, resulting in less than one-third of the total number of potential germ cells being endowed in the ovary within primordial follicles shortly after birth. Detailed analyses of germ cell depletion in rodents suggest that there are several discrete peaks of degeneration of both oogonia and oocytes in the embryonic ovary (Beaumont and Mandl, 1961; Borum, 1961). The largest peak of germ cell loss occurs within the dividing oogonial population during the later stages of fetal development, although cellular degeneration continues into the first few days after birth and is observed in both mitotic and post-mitotic germ cells. In the human fetal ovary, a similar scenario is believed to take place, beginning at about week 20 of gestation when the peak germ cell population has been reached (Baker, 1963). Morphometric and cytological studies revealed that at least three discrete waves of germ cell loss probably occur in the human fetal ovary: (1) attrition of dividing oogonia, (2) degeneration of pachytene stage oocytes, and (3) loss of diplotene stage oocytes (Baker, 1963). The effect of germ cell attrition on endowment of the stockpile of follicles available for future ovulation becomes apparent as the total number of germ cells falls from nearly \(7 \times 10^6\) at week 20 of gestation to a range of \(1.3 \times 10^3\) to \(3.85 \times 10^5\) per ovary at birth (Baker, 1963; Forabosco et al., 1991).

Unequivocal evidence that apoptosis is the underlying mechanism responsible for the loss of germ cells in the developing mouse ovary comes from both morphological and biochemical evaluations. Use of fluorescence-activated cell sorting as a measure of DNA content, combined with subsequent evaluations of cellular and nuclear morphology by microscopy, demonstrated that the degeneration of fetal mouse oogonia and oocytes in vivo occurs via apoptosis (Coucouvanis et al., 1993). Additional experiments using light and electron microscopy, as well as biochemical analyses, of mouse primordial germ cells cultured in vitro revealed that germ cells deprived of trophic factor support degenerate with many of the morphological and biochemical features characteristic of apoptotic cell death, including cellular and nuclear condensation, internucleosomal DNA cleavage and increased expression of tissue transglutaminase (Pesce et al., 1993; Pesce and De Felici, 1994).

Two somatic cell-derived growth factors have emerged as primary regulators of germ cell survival: stem cell growth factor (SCF or Steel factor) and leukaemia inhibitory factor (LIF). The first evidence that SCF is required for normal oogenesis was derived from analysis of ovaries of mice deficient in expression of the gene encoding SCF or its receptor, c-kit (Mintz and Russell, 1957). A loss of functional SCF, either through a mutation in the SCF gene or the SCF receptor gene, results in gonadal dysgenesis in both males and females. These findings have been reinforced by reports that provision of SCF, as well as LIF, to rodent primordial germ cells or fetal ovaries cultured in vitro represses spontaneous apoptosis that occurs in hormone-deprived germ cells (Pesce et al., 1993; Martinibeau et al., 1996). Although the pathways activated by SCF and LIF in germ cells that lead to a repression of apoptosis are unknown, recent studies demonstrated that ligand activation of the retinoic acid receptor promotes germ cell survival and proliferation in vitro (Koshimizu et al., 1995).

Follicular atresia

Once the primordial follicle pool is established, depletion of most of the remaining oocytes occurs indirectly as a result of atretic degeneration of follicles not selected for ovulation (Tsai and Braw, 1984; Hirshfield, 1991). Although still controversial, it appears that in most mammals the majority of follicles undergo atresia during the late preantral to the early
Fig. 1. Increased expression of the \textit{bax} death gene heralds granulosa cell apoptosis \textit{in vivo} and \textit{in vitro}. (a) Electrophoretic analysis of DNA prepared from immature rat ovaries before (-eCG) and after (+eCG) priming with equine chorionic gonadotrophin (eCG) \textit{in vivo}. Internucleosomal DNA cleavage (DNA ladder pattern), a hallmark feature of apoptosis, is detectable only in ovaries of unstimulated rats containing many atretic antral follicles. By comparison, priming with eCG \textit{in vivo} prevents granulosa cell apoptosis and follicular atresia as indicated by the lack of DNA oligonucleosomes in ovarian homogenates. (b) Autoradiogram showing expression of the \textit{bax} gene, as assessed by northern blot hybridization analysis, in RNA samples prepared from ovaries of unstimulated (-eCG, widespread atresia) and gonadotrophin-primed (+eCG, no atresia) immature rats. Note the abundant amount of mRNA encoding bax in ovaries containing apoptotic granulosa cells, and the abrupt decrease in \textit{bax} expression after gonadotrophin-promoted follicular survival \textit{in vivo}. (c) Quantitative analysis of changes in mRNA encoding \textit{bax} before and after gonadotrophin treatment \textit{in vivo} following normalization of data against amounts of mRNA encoding \(\beta\)-actin as a product of a ‘house-keeping’ gene. (d) Electrophoretic analysis of DNA integrity in isolated antral follicles, obtained from gonadotrophin-primed immature rat ovaries (see +eCG in (a)), before (0 h, healthy) and after (24 h, atretic) serum-free culture for 24 h. Consistent with morphological evidence of apoptosis and atresia in cultured follicles (see Flaws \textit{et al.}, 1995a, b), the formation of DNA oligonucleosomes is detectable only in follicles induced to undergo atresia \textit{in vitro}. (e) Northern blot and (f) quantitative analysis showing increased expression of \textit{bax} in follicles, again demarcating granulosa cell apoptosis and atresia, in this case initiated \textit{in vitro} by serum-free culture (reproduced with permission from Tilly \textit{et al.}, 1995a).
antral stage when continued growth is dependent upon gonadotrophin. This observation is based on several pieces of evidence, although the most convincing data are derived from analysis of apoptosis via in situ end-labeling of DNA fragments in fixed ovarian sections. In all mammals studied thus far, specific labeling of apoptotic cells by this approach (which has also been confirmed by morphological analyses) is apparently confined to granulosa cells of follicles undergoing maturational transition to the antral stage of development or to large subordinate antral follicles not selected for ovulation (Tilly, 1993; Tilly et al., 1995a, 1996). These histochemical data are supported by qualitative assessments of DNA oligonucleosomes in ovarian extracts throughout postnatal development, which confirm the presence of apoptotic DNA fragments in oocytes only after the first wave of developing antral follicles has formed (Ratts et al., 1995). It is possible that degeneration of immature (primordial, primary, small preantral) follicles does occur, but that the process remains inconspicuous owing to the efficient and rapid removal of the cellular debris (apoptotic bodies) by resident macrophages. Although studies of cells in nonreproductive organs indicate that ‘clean-up’ efficiency may hinder qualitative or quantitative analysis of apoptosis in certain tissues (Bursch et al., 1990), data to support this concept in the ovary are not yet available. In addition, since the ultimate fate of any follicle that exhibits evidence of DNA strand breaks in situ cannot be retrospectively evaluated, it is possible that not all follicles identified by this approach are destined for atresia (Jolly et al., 1994).

The hormonal regulation of apoptosis in granulosa cells during follicular atresia appears very complex, and probably involves a classic mesenchymal (theca–interstitial)–epithelial (granulosa) cell interaction (Chun et al., 1994; Flaws et al., 1995a; Tilly et al., 1995b) (Fig. 2). Much of our current knowledge of the endocrine, paracrine and autocrine regulation of apoptosis has been derived from studies of rat antral follicles incubated in vitro under defined conditions (Tilly et al., 1992a). Importantly, the temporal series of morphological and biochemical events associated with atresia in vivo (Tsafriri and Braw, 1984; Tilly et al., 1992b) appear to be recapitated faithfully using the follicle culture system (Flaws et al., 1995a; Hirshfield et al., 1995; J. L. Tilly, J. A. Flaws, A. M. DeSanti, K. I. Tilly, D. V. Maravei, A. M. Trbovich and A. N. Hirshfield, unpublished) (Fig. 1). Data derived from studies of rat follicles incubated in vitro have revealed that, as anticipated from in vivo observations (Tsafriri and Braw, 1984; Hirshfield, 1991), gonadotrophins are effective inhibitors of apoptosis that occurs in granulosa cells of follicles deprived of trophic support (Chun et al., 1994; Tilly and Tilly, 1995). The anti-apoptotic actions of FSH and LH can also be mimicked by treatment of follicles with neuropeptides, such as vasoactive intestinal peptide (VIP), and protein kinase A activators (Flaws et al., 1995a).

It has been proposed that intrafollicular-derived insulin-like growth factor I (IGF-I), and paracrine factors produced by theca–interstitial cells, such as transforming growth factor-β, keratino-cyte growth factor and hepatocyte growth factor, which bind specific receptors expressed by the adjacent granulosa cells, mediate the suppression of apoptosis by gonadotrophins and other endocrine factors in granulosa cells of antral follicles (Tilly et al., 1992a, 1995b; Chun et al., 1994; Eisenhauer et al., 1995; Flaws et al., 1995a) (Fig. 2). To add another level of complexity to these findings, the actions of growth factors on apoptosis in rat granulosa cells may in turn involve progesterone as a mediator (Luciano et al., 1994). Lastly, it should be pointed out that pathways distinct from those activated by the binding of hormones to their receptors, such as extracellular matrix protein interactions (Peluso et al., 1996) and gap junction maintenance (Weisen and Midgley, 1993), may play key roles in determining the fate of granulosa cells. Despite the fact that a large volume of useful information has been generated by these investigations, additional studies are clearly needed to determine the general applicability of these findings to other species including humans. Investigations of the regulatory events surrounding survival of less mature follicles within the ovary, and possible differences in the apoptotic potential of cells within follicles at various developmental stages, will also be of great interest.

**Luteolysis**

Relative to atresia, little is known of the requirement for apoptosis in the process of luteal regression. The majority of studies published thus far have identified the presence of apoptotic cells at the time of structural degeneration of the corpus luteum (Orlicky et al., 1992; Juengel et al., 1993; Rueda et al., 1995a, b), although one report links the timing of apoptosis to functional luteolysis in rabbits (Dharmarajan et al., 1994). Other confounding issues are the identity of the cells that undergo degeneration, and the exact nature of the cell death that occurs in the corpus luteum, as the process of luteolysis may also involve several nonapoptotic forms of physiological cellular deletion (Quatacker, 1971; Fraser et al., 1995). At this time, there are also very few published reports on the regulation of cell death within the corpus luteum. Prostaglandin F2α (PGF2α), a known luteolysin in many species, is one factor that has been implicated in the process of apoptosis in the corpus luteum (Orlicky et al., 1992). One action of PGF2α in rat luteal cells appears to involve increased generation of reactive oxygen species (Sawada and Carlson, 1991), an event that has been linked to both a loss of progesterone biosynthesis (Musicki et al., 1994) and the induction of apoptosis (Buttke and Sandstrom, 1994; Tilly and Tilly, 1995). By comparison, the luteotrophic factor, hCG, enhances expression of anti-oxidant factors, such as superoxide dismutase, in the rat corpus luteum (Laloraya et al., 1988) and prevents apoptosis in rabbit luteal tissue (Dharmarajan et al., 1994). The ability of hCG to protect luteal cells from reactive oxygen species may also involve paracrine mediators, such as progesterone, which directly inhibit superoxide radical generation by mononuclear phagocytes in the rat corpus luteum (Sugino et al., 1996). These observations provide a clear impetus for continuing studies on the interactions between luteolysins and luteotrophins in regulating luteal cell death, and for defining the relationships between oxidative free radical damage, apoptosis and the induction of both functional and structural luteolysis.

**Intracellular effectors of ovarian cell death: lessons from nongonadal tissues**

The bcl-2 gene family

The initiation of apoptosis in various ovarian cell lineages probably depends upon cell-specific stimuli received via hormonal signals, the absence or presence of which activates
an intracellular cascade of events that now appears to share many common features regardless of the cell type examined (see above). Much of our recent knowledge of the cytoplasmic and nuclear effectors of the apoptotic pathway in the ovary has come from studies of genes believed to regulate apoptosis in cells of various nonreproductive tissues (Korsmeyer, 1995; Stellar, 1995; Wyllie, 1995) (Table 2) or from genetic mutation studies of invertebrate species such as the nematode, *Caenorhabditis elegans* (Hengartner and Horvitz, 1994). One such gene in vertebrates is *bcl-2*, a proto-oncogene that encodes a membrane-anchored intracellular protein that prevents apoptosis induced by a variety of stimuli, including trophic hormone deprivation, ionizing radiation, hypoxia, hypoxia and receptor-linked death signals, such as those elicited by tumour necrosis factor α (Reed, 1994; Korsmeyer, 1995).

Although the role of BCL-2 in ovarian function remains to be fully elucidated, a growing number of reports have implicated this anti-death factor and other related members of this gene family in the three instances of ovarian cell death discussed in the previous section. For example, expression of the...
the ovarian phenotype observed in SCF- SCF receptor (c-kit)- and BCL-2-deficient mice has raised the possibility that the downstream survival actions of SCF on developing oogonia and oocytes are linked to enhanced expression of the bcl-2 gene (Ratts et al., 1995). Data to prove unequivocally that this association occurs \textit{in vivo} are not yet available. However, development of defined \textit{in vitro} models, such as isolated primordial germ cells or intact fetal ovaries placed in culture (Pesce et al., 1993; Martimbeau et al., 1996), should permit future testing of this hypothesis.

Another member of the bcl-2 gene family which warrants discussion in the context of ovarian function is the death-susceptibility gene, bax. The BAX protein was originally identified via its ability to noncovalently interact with BCL-2 in cells (Oltvai et al., 1993). This interaction is thought to blunt BCL-2 bioactivity and thus may serve as one of its mechanisms of action as a death-inducing factor. However, BAX probably also acts independently of BCL-2 heterodimerization to induce apoptosis (Korsmeyer, 1995; Sedlak et al., 1995), although its precise function in this regard remains to be fully clarified. Recent studies suggest that this protein plays a pivotal role in the life-and-death decision ultimately faced by ovarian cells. For example, expression of the bax gene in the ovary has been reported from studies of both the mRNA (Rueda et al., 1994; Tilly et al., 1995a) and the protein (Krajewski et al., 1994a). In addition, enhanced expression of the bax death-susceptibility gene in rat granulosa cells temporally coincides with the occurrence of apoptosis and the onset of follicular atresia \textit{in vivo} and \textit{in vitro} (Tilly et al., 1995a) (Fig. 1).

Convincing evidence that BAX plays a fundamental role in ovarian somatic cell death has been derived from studies of genetically manipulated mice that do not express functional BAX protein (Knuudson et al., 1995). Disruption of the bax gene leads to the development of grossly misshapen follicles which possess degenerating oocytes indicative of atresia. However, granulosa cells within degenerating follicles of BAX-deficient mice appear to be resistant to the induction of apoptosis (Knuudson et al., 1995), substantiating reports that increased expression of bax coincides with and may herald the onset of follicle demise under normal physiological conditions (Tilly et al., 1995a) (Fig. 1). Further evaluation of the potential effects of bax knock-out on numbers of oocytes and follicles, as well as on alterations in the ovarian follicle response to apoptotic stimuli, should yield new clues to aid in our understanding of the precise role of BAX in the ovary.

In addition to bcl-2 and bax, other members of the bcl-2 gene family have been cloned (Table 2), and several of these factors are expressed in the ovary (Krajewski et al., 1994b; Tilly et al., 1995a; Johnson et al., 1996; S. Martimbeau, K. I. Tilly and J. L. Tilly, unpublished). One of these genes, bcl-x, is unique among the other members of this family as it is alternatively processed to yield both positive (‘short’ isoform, death inducer) and negative (‘long’ isoform, death suppressor) regulators of the cell death pathway (Boise et al., 1993). Data currently available indicate that bcl-x\textsubscript{long} is the primary, if not only, isoform of the message expressed in ovarian granulosa cells of mammalian (Tilly et al., 1995a) and avian (Johnson et al., 1996) species. In agreement with the reported anti-apoptotic actions of BCL-X\textsubscript{long} protein, recent observations have revealed that expression of bcl-x\textsubscript{long} mRNA in the avian ovary is most abundant

### Table 2. Reported cell death regulators and their potential role in apoptosis

<table>
<thead>
<tr>
<th>Name</th>
<th>Role in apoptosis</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL-2</td>
<td>Repression</td>
<td>Cellular redox reactions, calcium homeostasis, growth factor signalling</td>
</tr>
<tr>
<td>BCL-X\textsubscript{long}</td>
<td>Repression</td>
<td></td>
</tr>
<tr>
<td>BCL-X\textsubscript{short}</td>
<td>Induction</td>
<td>BCL-2 antagonism</td>
</tr>
<tr>
<td>BAX</td>
<td>Induction</td>
<td>BCL-2/BCL-X\textsubscript{long} antagonism</td>
</tr>
<tr>
<td>MCL-1</td>
<td>Repression</td>
<td>BAX antagonism</td>
</tr>
<tr>
<td>A1</td>
<td>Repression</td>
<td>?</td>
</tr>
<tr>
<td>BAG-1</td>
<td>Repression</td>
<td>BCL-2 potentiation</td>
</tr>
<tr>
<td>BAD</td>
<td>Induction</td>
<td>BAX displacement from BCL-2/BCL-X\textsubscript{long}</td>
</tr>
<tr>
<td>BAK</td>
<td>Induction</td>
<td>BCL-2/BCL-X\textsubscript{long} antagonism</td>
</tr>
<tr>
<td>DAD-1</td>
<td>Repression</td>
<td>?</td>
</tr>
<tr>
<td>PD-1</td>
<td>Induction</td>
<td>?</td>
</tr>
<tr>
<td>p53</td>
<td>Induction</td>
<td>bcl-2/bax gene transcription</td>
</tr>
<tr>
<td>c-myc</td>
<td>Induction</td>
<td>Gene transcription (bax?)</td>
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<tr>
<td>Superoxide dismutase</td>
<td>Repression</td>
<td>Anti-oxidant</td>
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<td>Glutathione peroxidase</td>
<td>Repression</td>
<td>Anti-oxidant</td>
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<td>Catalase</td>
<td>Repression</td>
<td>Anti-oxidant</td>
</tr>
<tr>
<td>ICE</td>
<td>Induction</td>
<td>Protease</td>
</tr>
<tr>
<td>ICH-1\textsubscript{long} (Nedd-2)</td>
<td>Induction</td>
<td>Protease</td>
</tr>
<tr>
<td>ICH-1\textsubscript{short}</td>
<td>Repression</td>
<td>ICH-1\textsubscript{long} antagonism</td>
</tr>
<tr>
<td>CPP32 (Yama, Apopain)</td>
<td>Induction</td>
<td>Protease</td>
</tr>
<tr>
<td>ICE\textsubscript{III} (TX)</td>
<td>Induction</td>
<td>Protease</td>
</tr>
<tr>
<td>MCH-2</td>
<td>Induction</td>
<td>Protease</td>
</tr>
<tr>
<td>Calpain</td>
<td>Induction</td>
<td>Protease</td>
</tr>
<tr>
<td>Ceramide</td>
<td>Induction</td>
<td>Kinase induction</td>
</tr>
<tr>
<td>Clusterin (TRPM-2)</td>
<td>Passive</td>
<td>Membrane changes</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Evidence suggests that these various cell death regulators act in parallel pathways as well as in a cascade fashion. Much of our knowledge concerning the function of these intracellular effectors in cell death is derived from gene-transfer (transgenic mice; cell transfection and over-expression) and gene knock-out experiments. Thus, it should not be assumed that, under physiological conditions, any one factor is sufficient for the induction or repression of apoptosis.

\textsuperscript{b}The mechanisms of action of the cell death regulators listed remain to be fully characterized. The examples provided are taken from available data.

\textit{bcl-2} gene has been detected in the ovary of many species (Johnson et al., 1993; Rodger et al., 1995; Tilly et al., 1995a), and ablation of functional BCL-2 through targeted disruption of the gene in mice (gene ‘knock-out’) leads to significantly fewer oocytes and primordial follicles in the postnatal ovary (Ratts et al., 1995). By comparison, the extent of granulosa cell apoptosis does not appear to be affected by a loss of BCL-2, suggesting that there are cell type-specific differences in the requirement for various genes to carry out or suppress the cell death command appropriately. In any case, the commonality of the ovarian phenotype observed in SCF- SCF receptor (c-kit)- and BCL-2-deficient mice has raised the possibility that the downstream survival actions of SCF on developing oogonia and oocytes are linked to enhanced expression of the bcl-2 gene (Ratts et al., 1995). Data to prove unequivocally that this association occurs \textit{in vivo} are not yet available. However, development of defined \textit{in vitro} models, such as isolated primordial germ cells or intact fetal ovaries placed in culture (Pesce et al., 1993; Martimbeau et al., 1996), should permit future testing of this hypothesis.

Another member of the bcl-2 gene family which warrants discussion in the context of ovarian function is the death-susceptibility gene, bax. The BAX protein was originally identified via its ability to noncovalently interact with BCL-2 in cells (Oltvai et al., 1993). This interaction is thought to blunt BCL-2 bioactivity and thus may serve as one of its mechanisms of action as a death-inducing factor. However, BAX probably also acts independently of BCL-2 heterodimerization to induce apoptosis (Korsmeyer, 1995; Sedlak et al., 1995), although its precise function in this regard remains to be fully clarified. Recent studies suggest that this protein plays a pivotal role in the life-and-death decision ultimately faced by ovarian cells. For example, expression of the bax gene in the ovary has been reported from studies of both the mRNA (Rueda et al., 1994; Tilly et al., 1995a) and the protein (Krajewski et al., 1994a). In addition, enhanced expression of the bax death-susceptibility gene in rat granulosa cells temporally coincides with the occurrence of apoptosis and the onset of follicular atresia \textit{in vivo} and \textit{in vitro} (Tilly et al., 1995a) (Fig. 1).

Convincing evidence that BAX plays a fundamental role in ovarian somatic cell death has been derived from studies of genetically manipulated mice that do not express functional BAX protein (Knuudson et al., 1995). Disruption of the bax gene leads to the development of grossly misshapen follicles which possess degenerating oocytes indicative of atresia. However, granulosa cells within degenerating follicles of BAX-deficient mice appear to be resistant to the induction of apoptosis (Knuudson et al., 1995), substantiating reports that increased expression of bax coincides with and may herald the onset of follicle demise under normal physiological conditions (Tilly et al., 1995a) (Fig. 1). Further evaluation of the potential effects of bax knock-out on numbers of oocytes and follicles, as well as on alterations in the ovarian follicle response to apoptotic stimuli, should yield new clues to aid in our understanding of the precise role of BAX in the ovary.

In addition to bcl-2 and bax, other members of the bcl-2 gene family have been cloned (Table 2), and several of these factors are expressed in the ovary (Krajewski et al., 1994b; Tilly et al., 1995a; Johnson et al., 1996; S. Martimbeau, K. I. Tilly and J. L. Tilly, unpublished). One of these genes, bcl-x, is unique among the other members of this family as it is alternatively processed to yield both positive (‘short’ isoform, death inducer) and negative (‘long’ isoform, death suppressor) regulators of the cell death pathway (Boise et al., 1993). Data currently available indicate that bcl-x\textsubscript{long} is the primary, if not only, isoform of the message expressed in ovarian granulosa cells of mammalian (Tilly et al., 1995a) and avian (Johnson et al., 1996) species. In agreement with the reported anti-apoptotic actions of BCL-X\textsubscript{long} protein, recent observations have revealed that expression of bcl-x\textsubscript{long} mRNA in the avian ovary is most abundant
in granulosa cells of follicles destined for ovulation (Johnson et al., 1996). At this point, however, the precise requirement for either isoform of BCL-X in ovarian cell fate is uncertain. This question may remain unanswered since disruption of the bcl-x gene in genetically manipulated mice results in embryonic lethality between day 12 and day 13 after coitus (Motoyama et al., 1995).

Anti-oxidant pathways

The mechanisms by which BCL-2 and related proteins direct cell fate in any tissue are not well understood. The membrane localization of BCL-2 within mitochondria (Hockenberry et al., 1990) prompted early investigations into the potential interactions of this cell death protein with metabolic function. Use of gene-transfer techniques with cultured cells showed that overexpression of bcl-2 protected cells from oxidative stress-induced death and reduced accumulation of certain reactive oxygen species or their intermediates (Hockenberry et al., 1993; Kane et al., 1993). A follow-up study demonstrated that BCL-2 may cause a slight pro-oxidant state which then serves to trigger oxidative stress defence mechanisms of the cell in the form of increased activity of enzymes such as superoxide dismutase (Steinman, 1995). The link between BCL-2 and oxidative stress in cells of nonreproductive tissues spawned a study on the potential role of oxygen free radicals in the process of granulosa cell apoptosis during atresia (Tilly and Tilly, 1995). Data from these investigations revealed that gonadotrophin-mediated follicular survival coincides with enhanced expression of anti-oxidant factors. Moreover, provision of inhibitors of oxidative stress to follicles cultured in vitro mimics the ability of FSH to prevent apoptosis in granulosa cells (Tilly and Tilly, 1995), which is consistent with data suggesting that uncontrolled free radical damage induces apoptosis in diverse cell types (Buttke and Sandstrom, 1994).

The physiological significance of these findings may be related to the stage of follicular development at which the majority of atresia is thought to occur. The transition of developing follicles to the antral stage is associated with a marked increase in metabolic function of granulosa cells, most notably in the production of steroids via enhanced activity of cytochrome P450 enzymes (Hirshfield, 1991; Richards, 1994). Electron transport associated with steroidogenic output is a primary site of free radical generation, suggesting that differentiation of granulosa cells within the follicle coincides with an increased pro-oxidant status. As a consequence, it has been hypothesized that a potential trigger for atresia in follicles not selected for ovulation is an inadequate amount of protection from oxygen free radicals that accumulate in steroidogenically active granulosa cells (Tilly and Tilly, 1995). Along these lines, a similar role for oxidative stress has been proposed as a basis for both functional (Sawada and Carlson, 1991; Musicki et al., 1994) and structural (Rueda et al., 1995a) luteolysis.

The p53 tumour suppressor protein

One of the primary responses to cellular DNA damage, such as that elicited by reactive oxygen species, is the stabilization and nuclear translocation of the anti-oncogenic transcription factor, p53 (Kastan et al., 1991). In agreement with these data and the hypothesis that oxidative stress triggers atresia, accumulation of p53 protein in nuclei of granulosa cells of antral follicles destined for atresia has been reported (Tilly et al., 1995c) (Fig. 3). Although the mechanisms of p53 action in any tissue are not fully understood, recent investigations demonstrated that p53 induces apoptosis in many cell types (Clarke et al., 1993), including granulosa cells (Keren-Tal et al., 1995). Furthermore, this effect may be the result of the ability of p53 to enhance transcriptional activity of the bax death-susceptibility gene (Miyashita and Reed, 1995), while concomitantly suppressing expression of the bcl-2 survival gene (Miyashita et al., 1994). The relationship between nuclear p53 accumulation and altered expression of cell death genes in ovarian cells has not yet been explored.

The ICE gene family

Lastly, recent evidence indicates that a newly emerging family of cell death regulators, belonging to a group of cytoplasmic proteases that demonstrate significant homology to the Caenorhabditis elegans death gene, cel-3 (Hengartner and Horvitz, 1994), may also contribute to the initiation or progression of apoptosis in granulosa cells during atresia (Flaws et al., 1995b). Death proteins encoded by members of this gene family in vertebrates include interleukin-1β-converting enzyme (ICE), ICE-and-ced-3-homologue 1 (ICH-1), cysteine protease P32 (CPP32), ICErelII (TX, ICH-2), ICErelIII and MCH-2 (Martin and Green, 1995). These proteases share a conserved active site sequence consisting of a QACRG pentapeptide motif, and appear to be unique in their ability to cleave proteins at aspartate residues. Members of this family of proteases attack a diverse spectrum of specific homeostatic and structural proteins, including poly(ADP)-ribose polymerase (an enzyme involved in DNA repair), small nuclear ribonucleoproteins (factors responsible for spliceosome assembly and mRNA processing) and structural proteins of the nuclear scaffold (Martin and Green, 1995).

In the rat ovary, expression and gonadotrophin regulation of ICE, CPP32 and ICH-1 have been reported (Flaws et al., 1995b). It appears that ICE per se is not involved in the apoptotic death of granulosa cells as the abundance of ovarian ICE mRNA is extremely low, expression of the ICE gene in the ovary is not gonadotrophin-regulated, and follicles cultured in vitro to induce apoptosis and atresia do not contain detectable ICE activity (Flaws et al., 1995b). This proposal is in agreement with recent gene knock-out data demonstrating that ablation of ICE in mice does not alter apoptosis in two well-defined model systems for cell death, the glucocorticoid-treated thymus gland and the post-lactational mammary gland (Li et al., 1995). In contrast, expression of the death-inducing proteases, CPP32 and ICH-1, in the ovary is inhibited by gonadotrophins (Flaws et al., 1995b), and preliminary data suggest that endonuclease activation (Flaws et al., 1995b) and cleavage of small nuclear ribonucleoproteins (Trbovich and Tilly, 1995) may be among the consequences of enhanced ICE-related protease activity in granulosa cells. Future analysis of the ovaries of genetically manipulated mice deficient in specific members of this protease gene family, complemented by in vivo and in vitro examinations of the expression and actions of these cell death regulators in ovarian cells, should aid in elucidating the role of this new family of genes in ovarian function.
Implications and future considerations

The concept that apoptosis is fundamental to many aspects of ovarian development and cyclic function, and the possibility that there is a universal pathway for the execution of cell death in most tissues, have opened previously unexplored avenues for research on oogenesis, follicular development and corpus luteum function. The application of state-of-the-art molecular biological techniques, combined with classic histological and morphometric analyses, has provided the first hints that the manipulation of apoptosis in discrete ovarian cell populations may lead to the development of new strategies for combating a host of reproductive disorders and for novel contraceptive approaches. For example, the ability to reduce or prevent perinatal oocyte loss may lead to a surplus of follicles that could maintain appropriate ovarian steroid secretory function throughout a woman’s life as a means to delay or prevent the occurrence of the menopause. In addition, these data can be used to develop strategies to protect the oocyte pool from environmental insults known to cause sterility (for example, radio- and chemotherapeutics and industrial toxicants), and to enhance success rates for oocyte retrieval and survival through cryopreservative techniques required for assisted reproductive technology programmes. Lastly, new contraceptive measures may arise from the ability to destroy selectively the quiescent germ cell pool (permanent sterility), the ovulatory cohort of antral follicles (temporary sterility), or the corpus luteum (post-conception pregnancy termination) via cell-specific manipulation of the apoptotic mechanism. The impact of knowledge derived from studies of cell death on advances in these fields and others related to reproductive function and health will be exciting to watch and participate in over the coming years.

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Fig. 3. Immunocytochemical localization of the p53 tumour suppressor protein in the nucleus of a rat ovarian granulosa–cumulus cell destined for apoptosis. Nuclear accumulation of the p53 protein (dark blue immunoreactive staining), an event that probably leads to altered transcription of p53-responsive genes (for example, bax), is detectable only in granulosa cells of follicles undergoing atresia. By comparison, p53 immunoreactivity is completely absent in healthy follicles of rat ovaries primed with exogenous gonadotrophin (Tilly et al., 1995c; reproduced with permission). Scale bar represents 10 μm.
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