Survival factors regulating ovarian apoptosis – dependence on follicle differentiation

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Only a minute fraction of the ovarian follicles present in a fetal ovary will complete the path to ovulation. Most of the follicles will undergo atresia, a hormonally controlled apoptotic process. Apoptosis occurs at each stage of follicular development and there is a marked reduction in the number of follicles present at birth. Stage-dependent mechanisms of follicle survival can be postulated to achieve co-ordinated development, leading to ovulation of a small fraction of follicles. Indeed, hormone and growth factor regulation of follicular atresia is stage-specific. This short review considers the factors that promote survival of ovarian follicles throughout development, including endocrine, locally produced and intracellular mediators, as exemplified mainly by follicular development in rodents. In primordial follicles, oocyte apoptosis is considered to be the cause of subsequent follicle degeneration. In slow-growing preantral follicles, FSH is not a survival factor, but some locally produced growth factors are. Progression to the antral follicle stage is probably the most critical stage of follicle development *in vivo*, and FSH is a major survival factor at this stage. In addition, insulin-like growth factor I and interleukin 1 β are potent survival factors for cultured rat follicles at the antral stage. Preovulatory follicles express receptors for LH, and both of the gonadotrophins are survival factors at this stage. Relatively little is known about the period between the LH surge and ovulation; however, it has been suggested that at this stage progesterone acts as a survival factor.

The human fetal ovaries contain millions of germ cells: the maximum number is observed at month 5 of pregnancy (6.8×10^6 germ cells). Degeneration of germ cells occurs before formation of the ovarian follicles and thus regulates the number of primordial follicles present within the ovaries. The number of germ cells decreases markedly up to birth, at which stage the number of germ cells in the primordial follicles is less than 20% of the maximum number (Baker, 1963).

More than 99.9% of the ovarian follicles present at birth never reach ovulation. The most common fate of follicles is to undergo atresia, which is mediated by a highly organized type of cell death called apoptosis or programmed cell death (Hsueh *et al.*, 1994; Billig *et al.*, 1996; Kaipia and Hsueh, 1997). The reduction of the large number of growing follicles to a single ovulatory follicle is achieved primarily by cell death of granulosa cells (Hsueh *et al.*, 1994; Reynaud and Driancourt, 2000).

Individual mammalian follicles consist of a central oocyte surrounded by somatic granulosa cell layers and outer layers of somatic theca cells. The maturation of ovarian follicles involves several sequential stages: initiation of growth of

primordial follicles in the resting pool, growth of the follicles, selection of a dominant follicle, ovulation and, finally, luteinization. The initiation of growth of resting primordial follicles, designated initial recruitment (McGee and Hsueh, 2000), is followed by growth, differentiation and atresia. When the follicle acquires an antral cavity, it becomes dependent on hormonal support from FSH. During each reproductive cycle, increasing FSH concentrations recruit growing antral follicles. The term 'cyclic recruitment' has been proposed to describe this rescue from degeneration (McGee and Hsueh, 2000). At the last stage in follicular development, the endogenous LH surge induces ovulation and formation of the corpus luteum. During the growth and development of follicles, atresia occurs at every stage of follicular development (Gougeon, 1986), except the final stage. At the final stage, the number of corpora lutea is equal to the number of follicles that respond to the LH surge, implying decreased sensitivity to apoptosis.

Follicular atresia is regulated by endocrine factors, such as FSH and LH, and by paracrine factors, including insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), activin and interleukin 1 β (IL-1 β). Atretogenic factors include tumour necrosis factor α (TNF- α), GnRH, androgens, IL-6 and free radicals (Kaipia and Hsueh, 1997). Granulosa cell apoptosis

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is an active cellular event that is dependent on transcription and protein synthesis, as incubation in the presence of the transcription inhibitor actinomycin D or the translation inhibitor cyclohexamide decreases the rate of spontaneous apoptosis in a dose-dependent manner (Svanberg and Billig, 1999).

Models for ovarian apoptosis

A range of stimuli can affect whether a cell will undergo atresia. In the ovary, there are several specific regulators of apoptosis, including hormones, growth factors and cytokines (Fig. 1). Investigation of ovarian apoptosis is problematic as the process concludes with phagocytosis of the apoptotic cell by surrounding cells, leaving no detectable traces for investigation. Furthermore, in the ovary, different factors regulate apoptosis in follicle populations of different developmental stages, necessitating models in which homogeneous cohorts of follicles can be isolated at specific stages.

Many models for studying follicular atresia have taken advantage of the fact that isolated follicles or granulosa cells undergo spontaneous apoptosis when cultured in the absence of serum (Tilly *et al.*, 1992). Addition of survival or pro-apoptotic factors to the incubation medium can affect the rate of spontaneous apoptosis. Several rodent models have also been developed to isolate follicles at specific stages of development. For instance, administration of diethylstilboestrol implants to hypophysectomized rats provides an opportunity to study preantral and early antral follicles (Billig *et al.*, 1993). Further differentiation can be achieved by sequential stimulation of the FSH and LH receptors of immature rats. In humans, the source of material is most often periovulatory granulosa cells collected from women undergoing oocyte aspiration before *in vitro* fertilization.

Mechanisms of apoptosis

Once the decision to die has been made, the execution of the apoptotic death requires co-ordinated activation and propagation of several sub-programmes (Hengartner, 2000). Two important families of regulators of the apoptotic process are the caspase family and the Bcl-2 family (Fig. 1). Caspases are considered the executioners of the apoptotic pathway (Hengartner, 2000) and function as proteases, cleaving after an aspartic acid residue. There are at least three mechanisms for caspase activation: (i) processing by an upstream caspase; (ii) association with co-factors; and (iii) cleavage induced by a high local concentration of caspases associated with activated death receptors. More than 100 substrates are known to be cleaved by caspases, including lamins, required for nuclear shrinking and budding, and cytoskeletal proteins, such as fodrin and gelsolin, causing loss of overall cell shape (Hengartner, 2000). An important function of the caspases is to activate caspase-activated DNase (CAD), the endonuclease

responsible for internucleosomal DNA fragmentation, one of the most frequently used hallmarks of apoptosis. CAD and its inhibitory subunit, inhibitor of caspase-activated DNase (ICAD), are constantly expressed in the cells. Caspase-mediated cleavage of the inhibitory subunit results in release and activation of the endonuclease.

The Bcl-2 family of apoptotic regulators comprises both anti-apoptotic members, such as Bcl-2 and Bcl-X_L, and pro-apoptotic members, including Bax, Bid, Bik, BOD and Bcl-X_S. Members of the Bcl-2 family can both homodimerize and heterodimerize with several other family members, thereby regulating activity of one another. The essential function of the Bcl-2 family appears to be to regulate release of pro-apoptotic factors, in particular cytochrome *c*, from the mitochondrion into the cytosol (Antonsson and Martinou, 2000). Many members of the Bcl-2 family have been isolated in the ovary, including BAD, Mcl-1 and Bok (Hsu and Hsueh, 2000).

The mitochondrion is a crucial structure in the apoptotic machinery, harbouring several pro-apoptotic factors that are released into the cytoplasm upon a specific signal (Fig. 1). One of these factors is cytochrome *c*, which is required for caspase 9 activation in the cytosol. How cytochrome *c* crosses the outer mitochondrial membrane remains to be elucidated, but the members of the Bcl-2 family are involved closely in the regulation of this process. Release of cytochrome *c* is an almost universal event during apoptotic cell death. However, in some cases it is a very late event, such as in apoptosis induced by death receptors, in which the mitochondrial pathway is often bypassed and cytochrome *c* is released as a result of caspase activation (Scaffidi *et al.*, 1998). In addition to cytochrome *c*, other pro-apoptotic factors are stored in the mitochondria and are released upon induction of apoptosis. Examples include apoptosis-inducing factor (AIF) and several pro-caspases.

After apoptotic death of a cell, the cellular remains are removed. Exposure of phosphatidylserine on the outer cell membrane is one signalling mechanism that occurs in apoptotic cells (Fig. 1). In the ovary, scavenger receptor class B type I (SR-BI) on theca cells mediates recognition and binding of apoptotic granulosa cells expressing phosphatidylserine on the cell surface (Svensson *et al.*, 1999).

Dependence on stage of follicle differentiation

Follicular atresia is a hormonally regulated process, and different factors are affecting the decision to die at different stages of ovarian follicle development. Some of the factors that promote survival during the growth and differentiation of follicles are summarized (Fig. 2). Although a number of locally produced growth factors affect follicle cell survival, the main physiological regulators of ovarian follicle survival are the gonadotrophins. Most, if not all, of the factors reported in published studies as inhibitors of follicle atresia are regulated by FSH and LH. When the growing follicles reach the antral stage, they express receptors for FSH and become dependent on FSH stimulation. Sufficient FSH

Fig. 1. Schematic overview of the apoptotic process in follicular granulosa cells. A range of hormones and locally produced factors regulates the 'decision to die' by way of their receptors. Examples of survival factors include the gonadotrophins FSH and LH, the steroids progesterone (P₄) and oestrogen (E₂), and other factors including growth hormone (GH), insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin. The signalling of these factors is mediated via a range of different types of receptor. Execution of the apoptotic programme converges in the mitochondria, in which pro- and anti-apoptotic members of the Bcl-2 family interact at the surface. Excess pro-apoptotic activity causes release of an array of molecules from the mitochondrial compartment. One of the principal molecules is cytochrome c, which associates with apoptotic protease-activating factor 1 (APAF-1) and pro-caspase 9 to form the apoptosome complex. This complex subsequently activates downstream caspases, such as caspase 3. Downstream of caspase 3, the apoptotic programme branches into a multitude of sub-programmes, among which is exposure of phosphatidylserine on the cell surface. Another effect of caspase activation is cleavage of inhibitor of caspase-activated DNase (ICAD) resulting in release of caspase-activated DNase (CAD), the endonuclease responsible for internucleosomal DNA fragmentation. Two other pathways for induction of apoptosis are also represented. One pathway is DNA damage caused by, for example, irradiation, which initiates the apoptotic process via p53. The second pathway is the death receptor pathway, which has been characterized mainly in immune cells. Binding of ligand to these receptors (for example CD95/Fas, tumour necrosis factor receptor I) causes receptor clustering and formation of a death-inducing signalling complex (DISC). This complex recruits multiple copies of pro-caspase 8, via the adaptor molecule Fas-associated death domain protein (FADD), resulting in activation of caspase 8.

Fig. 2. Examples of factors that regulate stage-dependent survival of ovarian follicles: gonadotrophins (red); locally produced survival factors (blue); and intracellular mediators (green). Jagged ends indicate that studies have not been presented regarding earlier or later developmental stages. Examples of relevant references are provided. bFGF: basic fibroblast growth factor; EGF: epidermal growth factor; GH: growth hormone IGF-I: insulin-like growth factor; IL-1β: interleukin 1β; KGF: keratinocyte growth factor; NO: nitric oxide; P₄: progesterone; PR: progesterone receptor.

concentrations are critical for survival of follicles that have differentiated to the antral stage or beyond. During each reproductive cycle, increasing FSH concentrations rescue developing follicles, that is, there is cyclic recruitment. LH is important for follicles approaching ovulation and expressing the LH receptor. Inhibition of the LH surge results in massive atresia in rats (Braw and Tsafiriri, 1980). In the adult human ovary, the degree of atresia has been estimated to be highest in antral follicles > 5 mm in diameter (Gougeon, 1986).

The differentiation-dependent regulation that occurs throughout follicular development has to be acknowledged to understand the finely tuned balance of follicle growth and atresia.

Primordial follicles

In primordial follicles, it is likely that oocyte apoptosis is responsible for subsequent follicular degeneration. Oocyte apoptosis has been reviewed recently by Morita and Tilly (1999) and Reynaud and Driancourt (2000), who described the importance of, for instance, Kit–Kit ligand interaction and the growth factors EGF and bFGF in rodents. It has been reported that Kit–Kit ligand interaction is important for survival of primordial follicles in fetal as well as postnatal ovaries. Interaction probably prevents degeneration of follicles by rescuing the oocyte (Driancourt *et al.*, 2000). Another oocyte-derived factor that is important for survival of small follicles is growth and differentiation factor 9 (GDF-

9). In mice lacking GDF-9, follicles do not develop beyond the primary or early secondary stage (Dong *et al.*, 1996).

The aryl hydrocarbon receptor (AhR) may be important in regulating the size of the pool of primordial germ cells. Ovaries collected from fetal mice and cultured *in vitro* show a high rate of oocyte apoptosis. The number of non-apoptotic oocytes after culture of fetal ovaries from AhR-deficient mice was almost three times higher than that in cultured fetal ovaries from wild-type mice (Robles *et al.*, 2000). However, at present, the endogenous ligand for AhR is unknown.

Preantral follicles

Preantral follicles are characterized by slow growth, and there are distinct differences between human and rat follicles at this stage. Human follicles reach the preantral stage several months before ovulation, whereas rat follicles become preantral only a few days before ovulation. Thus, although they are at the same morphological stage, there may be developmental differences between preantral rat and human follicles (McGee, 2000). Relatively little is known about regulation of survival of preantral follicles in rodents and humans compared with understanding of survival at later stages of development.

When isolated preantral follicles from 12–14-day-old rats are cultured in serum-free medium, there is a spontaneous onset of apoptosis in the granulosa cells, similar to that seen in preovulatory follicles. At this early stage of differentiation, FSH and its downstream mediator cAMP have no effect on apoptosis in cultured rat follicles (McGee *et al.*, 1997a). However, this lack of effect is not due to lack of FSH receptors, as FSH has the ability to enhance expression of steroidogenic enzymes in preantral follicles (Dunkel *et al.*, 1994; Rannikki *et al.*, 1995). In contrast, both FSH and LH receptor stimulation are important for development of preantral rat follicles *in vivo* (McGee *et al.*, 1997b). This finding indicates that gonadotrophins do not act directly on preantral follicles, but that the effect is mediated via other gonadotrophin-sensitive cells, such as cells in follicles at a later stage of differentiation.

Locally produced survival factors for preantral follicles include keratinocyte growth factor (KGF). KGF is produced by theca cells in the ovary, and receptors are present in granulosa cells. In cultured preantral rat follicles, KGF suppresses apoptosis and also promotes growth and differentiation (McGee *et al.*, 1999). Similarly, FGF suppresses apoptosis in cultured preantral rat follicles (McGee *et al.*, 1999). Other locally produced survival factors are oestrogens, which cause a decrease in the rate of granulosa cell apoptosis in preantral follicles *in vivo* in hypophysectomized rats. This effect is inhibited by treatment with testosterone (Billig *et al.*, 1993).

Intracellular mediators for survival of preantral follicles include cGMP, but not cAMP. It has been reported that treatment of isolated preantral rat follicles with a cGMP analogue reduces the degree of apoptosis by as much as 75% (McGee *et al.*, 1997a).

Dietary ascorbic acid (vitamin C) also affects fertility in humans. Murray *et al.* (2001) reported that addition of ascorbic acid to mouse preantral follicles cultured *in vitro* decreases the rate of apoptosis and increases the percentage of follicles that maintain basement membrane integrity.

Factors that promote growth of ovarian follicles, for example FSH and bFGF, also inhibit apoptosis, although there are some exceptions. For instance, Müllerian inhibitory substance (MIS) promotes the growth of preantral rat follicles without affecting granulosa cell apoptosis or differentiation (McGee *et al.*, 2001).

Early antral follicles

During the normal reproductive cycle in humans and rodents, the early antral stage is the most critical stage of follicle development. Early antral follicles express receptors for FSH and become dependent on FSH stimulation for survival. Owing to the lack of FSH support, many follicles never progress beyond this point in development (see Hirshfield, 1991).

In isolated early antral follicles from rats, FSH is the most important survival factor. FSH is able to suppress apoptosis by up to 60%. The effect was partially reversed by addition of the IGF binding protein IGFBP-3, indicating a mediatory role of IGF-I. In contrast, stimulation of LH receptors has a limited effect on survival of rat follicles at this stage (Chun *et al.*, 1996).

Locally produced factors that are important for survival of isolated early antral rat follicles include IGF-I, EGF, bFGF, activin and the cytokine IL-1 β . Growth hormone (GH) is unable to suppress apoptosis in cultured early antral follicles, despite inducing increased expression of IGF-I mRNA (Chun *et al.*, 1996). However, several of these locally produced factors are more potent as survival factors in later stages of follicle development. For instance, IL-1 β is threefold more effective in preventing apoptosis in preovulatory than in early antral follicles. Since IL-1 β probably mediates its effects via receptors on theca cells, the higher degree of suppression in preovulatory follicles might be the result of the presence of more differentiated theca cells at that developmental stage (Chun *et al.*, 1996). At the early antral stage, there is a differential effectiveness of the hormones and growth factors that regulate survival of cultured rat follicles: FSH > IGF-I, IL-1 β > LH, EGF, bFGF, activin >>> GH (Chun *et al.*, 1996). As in preantral follicles, oestrogens are important for survival of early antral follicles *in vivo* in rats (Billig *et al.*, 1993). However, there are no reports that demonstrate that oestrogens affect survival of cultured follicles or granulosa cells.

Preovulatory follicles

At the preovulatory stage of development ovarian follicles express LH receptors and are able to respond to the impending LH surge. Both FSH and LH suppress the degree of apoptosis in isolated preovulatory rat follicles (Chun *et al.*, 1994). Studies of cultured preovulatory rat follicles have

shown that the suppression of apoptosis observed after treatment with gonadotrophins may be partially mediated by endogenous IGF-I (Chun *et al.*, 1994). At least three lines of evidence support this hypothesis (see Chun *et al.*, 1994): (i) addition of IGFBP-3 results in a dose-dependent decrease in the apoptosis suppressive effect of LH receptor stimulation; (ii) concentrations of IGFBP-4 and -5 are higher in atretic human follicles than in healthy human follicles; and (iii) LH receptor stimulation results in an increase in IGF-I mRNA content in cultured preovulatory rat follicles. However, IGF-I has no effect in isolated granulosa cells from preovulatory follicles (Tilly *et al.*, 1992), indicating interaction with other follicular cells. The cytokine IL-1 β also mediates part of the apoptosis suppressive effects of gonadotrophins in rats. Addition of an IL-1 β receptor antagonist partially decreases the effect of gonadotrophins (Chun *et al.*, 1995). Furthermore, IL-1 β alone suppresses apoptosis in cultured preovulatory follicles, and simultaneous addition of IL-1 β and an IL-1 β antagonist reverses the effect. The effect of IL-1 β is probably mediated via generation of NO and cGMP (Chun *et al.*, 1995).

In rat preovulatory follicles and isolated granulosa cells the locally produced survival factors EGF and bFGF suppress apoptosis as effectively as do gonadotrophins (Tilly *et al.*, 1992). This effect is mediated through the tyrosine kinase pathway, as addition of the tyrosine kinase inhibitor genistein inhibits the effect of EGF and bFGF. The EGF receptor is upregulated by gonadotrophins (Fujinaga *et al.*, 1994), which probably explains why there is no effect or only a small effect on apoptosis in preantral and early antral rat follicles (Tilly *et al.*, 1992; McGee *et al.*, 1997a). Transforming growth factor α (TGF- α) is a structural and functional homologue of EGF that shares the same cell membrane receptor. TGF- α mimics the effects of EGF in isolated preovulatory rat follicles (Tilly *et al.*, 1992). KGF, a member of the FGF family, is also anti-apoptotic when added to cultured preovulatory rat follicles (McGee *et al.*, 1999).

Insulin is another survival factor for cultured preovulatory rat follicles. It has been reported that insulin decreases sensitivity to apoptosis *in vitro* (Chun *et al.*, 1994). This effect is probably mediated via the IGF-I receptor, and IGF-I has a stronger anti-apoptotic effect than does insulin. Insulin has no effect on isolated preovulatory rat granulosa cells (Tilly *et al.*, 1992), indicating the involvement of other ovarian cells. GH is also able to suppress apoptosis in cultured preovulatory rat follicles. As the effect of GH is inhibited by addition of IGFBP-3, it is probably mediated by IGF-I (Eisenbauer *et al.*, 1995). In addition, it has been reported that mice overexpressing bovine GH have an increased number of healthy preovulatory follicles and a smaller percentage of preovulatory follicles undergoing a high degree of apoptosis compared with non-transgenic littermates (Danilovich *et al.*, 2000). This effect is in contrast to the situation at the earlier developmental stages, in which GH has no effect on apoptosis (Chun *et al.*, 1996).

Intracellular mediators of preovulatory follicle survival

include cAMP and cGMP. However, stimulation of protein kinase A or C signalling pathways in isolated rat granulosa cells has no effect on apoptosis (Tilly *et al.*, 1992).

Periovarian follicles

Follicles that survive to the periovarian stage are dependent on the endogenous LH surge. Inhibition of the LH surge by hypophysectomy or pentobarbital treatment causes the follicles to degenerate (Talbert *et al.*, 1951; Braw and Tsafiriri, 1980). The decrease in spontaneous apoptosis observed in isolated preovulatory rat follicles treated with hCG to stimulate the LH receptor may be mediated, at least in part, by endogenous production of pituitary adenylate cyclase-activating polypeptide (PACAP). LH-induced suppression of follicular apoptosis is partly inhibited by co-treatment with a PACAP antagonist (Lee *et al.*, 1999).

After the LH surge, the follicles are less susceptible to atresia than are follicles at earlier stages, as shown by decreased sensitivity to apoptosis *in vitro* (Svensson *et al.*, 2000). The mechanism for this reduced sensitivity has not been studied in detail; however, in granulosa cells isolated from periovarian follicles in rats (Svensson *et al.*, 2000) and humans (Makrigiannakis *et al.*, 2000), progesterone functions as a regulator of apoptosis via its nuclear receptor. Effects of progesterone that are not mediated by its nuclear receptor have also been suggested. Progesterone has been attributed an apoptosis-inhibiting effect in immature and preovulatory (but not LH receptor-stimulated) rat granulosa cells via a GABA receptor-like receptor (Peluso and Pappalardo, 1998). However, addition of GABA receptor antagonists (picrotoxin and bicuculline) and the GABA agonist muscimol to isolated granulosa cells from rat periovarian follicles did not influence the degree of apoptosis (Svensson *et al.*, 2000).

Expression of the nuclear progesterone receptor is induced shortly after the LH surge in both rat and human granulosa cells, coinciding with the apoptosis suppressive effects of progesterone. In rat granulosa cells expression of progesterone receptors is transient (Park and Mayo, 1991; Natraj and Richards, 1993), whereas in humans expression of progesterone receptors is prolonged, and the progesterone receptor is also present in the corpus luteum (Press and Greene, 1988; Iwai *et al.*, 1990). Mice lacking both isoforms of the progesterone receptor (A and B) are anovulatory, supporting the contention that progesterone has direct effects in the ovary (Lydon *et al.*, 1995, 1996). Mice lacking only the A isoform of the receptor show decreased ovulation after stimulation (Mulac-Jericevic *et al.*, 2000), whereas mice lacking only the B isoform are fertile and produce normal-sized litters (Mulac-Jericevic *et al.*, 2001). It has also been reported that addition of progestins augments gonadotrophin-stimulated progesterone production by cultured rat granulosa cells (Fanjul *et al.*, 1983). Recently, genes that are regulated by the progesterone receptor have been identified in granulosa cells during the periovarian interval, including PACAP and its receptor

PAC₁ (Ko *et al.*, 1999; Ko and Park-Sarge, 2000) and the proteases ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin-like motifs) and cathepsin L (Robker *et al.*, 2000).

As the progesterone receptor–progesterone complex acts as a transcription factor and inhibition of progesterone increases apoptosis, further studies of progesterone receptor-regulated gene expression will probably identify pathways that are important for follicle cell survival.

Conclusions

Follicular development, characterized by high rates of proliferation and apoptosis, is a tightly regulated process, which is dependent on a balance between local ovarian growth factors and circulating hormones. However, the diminishing number of follicles in each developmental stage indicates that the control of follicular survival or atresia is not the same at each stage. All follicles are not alike: both the susceptibility to apoptosis and the regulators of follicle survival change during the different stages of development (Fig. 2). Increased understanding of the sequential regulatory events of follicle development will aid development of new contraceptives and new methods for increasing the female reproductive lifespan and will lead to improvements in culture methods for oocytes for *in vitro* fertilization.

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