Hormonal and genetic control of germ cell apoptosis in the testis

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Programmed cell death is an evolutionarily conserved cell death process that plays a major role during normal development and homeostasis. In many cases, the ordered execution of this internal death programme leads to typical morphological and biochemical changes that have been termed apoptosis. The crucial role of this mode of cell death in the pathogenesis of diverse human diseases including cancer, acquired immunodeficiency syndrome, neurodegeneratives disorders, atherosclerosis and cardiomyopathy is now supported by a wealth of data. In adult mammals, including humans, germ cell death is conspicuous during normal spermatogenesis and plays a pivotal role in sperm output. Withdrawal of gonadotrophins and testosterone further enhances the degeneration of germ cells in the testis. The availability of a quantitative method for analysing the testicular DNA fragmentation and in situ methods to localize specific germ cells undergoing apoptosis, either spontaneously or in response to a variety of death triggering signals, opens new avenues in the understanding of the significance of germ cell apoptosis during normal and abnormal states of spermatogenesis. A growing body of evidence demonstrates that both spontaneous (during normal spermatogenesis) and accelerated germ cell death triggered by deprivation of the gonadotrophic support or moderately increased scrotal temperature in adult rats occur almost exclusively via apoptosis. Although there has been spectacular progress in the understanding of the molecular mechanisms of apoptosis in various systems other than spermatogenesis, elucidation of the biochemical and molecular mechanisms by which germ cell apoptosis is regulated has only just begun. It is likely that germ cell apoptosis is controlled in a cell-type specific fashion, but the basic elements of the death machinery may be universal. In addition, there is increasing evidence that homozygous disruption of a number of genes in mice results in infertility through accelerated germ cell apoptosis. Manipulation of spermatogenesis by survival factor(s) deprivation or increases in extrinsic death signals in loss-of-function or gain-of-function mouse models provides a basis for further attempts to define the intrinsic regulation of various death-related genes by external death signals. Such information is crucial for effective management of male factor infertility as well as more targeted approaches to male contraception.

The process of programmed cell death has become one of the most intensively studied topics in biological sciences in recent years. Much progress has been made in understanding the regulation of apoptosis in various extragonadal cell systems (reviewed in Majno and Joris, 1995; White, 1996; Jacobson et al., 1997; Nagata, 1997) and in the ovary (Hsueh et al., 1994, 1996) with little emphasis on programmed germ cell death in the testis (Hsueh et al., 1996).

The term ‘programmed cell death’ was originally used to describe the coordinated series of events leading to cell demise during development (Lockshin and Williams, 1964). The term ‘apoptosis’, coined by Kerr and colleagues (1972) to refer to a morphologically distinct form of cell death, is a form of cell death that plays a major role during the normal development and homeostasis of multicellular organisms. This mode of cell death is a tightly regulated series of energy-dependent molecular and biochemical events orchestrated by a genetic programme. Apoptosis is either developmentally regulated (launched in response to specific stimuli, such as deprivation of survival factors, exposure to ionizing radiation and chemotherapeutic drugs or activation by various death factors and their ligands) or induced in response to cell injury or stress. It is now widely accepted that apoptosis serves as a prominent force in sculpting body parts, deleting unneeded structures, maintaining tissue homeostasis, and as a defence mechanism to remove unwanted and potentially dangerous cells, such as self-reactive lymphocytes, virus-infected cells and tumour cells (Thompson, 1995; Jacobson et al., 1997). Apoptosis is also increasingly being recognized in the pathogenesis of many diverse human diseases including cancer, acquired immune deficiency syndrome, neurodegenerative disorders, atherosclerosis and cardiomyopathy (Thompson, 1995; Hannun, 1997; Olivetti et al., 1997).

Spermatogenesis is a complex process in which stem spermatogonia, through a series of events involving mitosis, meiosis and cellular differentiation, become mature spermatozoa (Russell et al., 1990; Sharpe, 1994). Germ cell death, in addition to its involvement in cellular proliferation and differentiation, is also conspicuous during normal spermatogenesis of various...
mammalian species, including humans, and plays a critical role in determining the quantitative degree of sperm output (Allan et al., 1987; Russell et al., 1990; Sharpe, 1994). In addition to its function as the underlying mechanism of germ cell death during normal spermatogenesis, apoptosis can be triggered by a wide variety of regulatory stimuli. This review will highlight recent developments in the field of apoptosis in general and spermatogenesis in particular, and will address recent advances in the study of the intratesticular regulatory mechanisms that control germ cell apoptosis.

Cellular and molecular characteristics of apoptosis

The cellular and molecular characteristics of apoptosis have been well documented (see Majno and Joris, 1995; White, 1996; Jacobson, et al., 1997). Rather than covering this ground again, this review will provide a brief description of the identifying features of apoptotic cells and how these features differ from that of necrosis or accidental cell death. The process of apoptosis is associated with well-defined morphological and biochemical changes, including a reduction in cell volume, blebbing of the cell membrane, chromatin condensation and margination, and formation of apoptotic bodies. In contrast to physiological cell death or apoptosis, necrosis (sometimes called oncosis) is a passive process that does not require energy expenditure by the cell and occurs in response to a wide variety of noxious agents. Necrosis does not occur in a developmental context, usually affects a group of contiguous cells, and is characterized by swelling of the cell and its organelles (as a result of ion pump failure) and results ultimately in membrane rupture and cell lysis.

A unique biochemical event in apoptosis is the activation of calcium–magnesium-dependent endonuclease activity, which specifically cleaves cellular DNA between regularly spaced nucleosomal units (approximately 200 base pairs). Such oligonucleosomal fragments, when separated by agarose gel electrophoresis, form a distinctive DNA ‘ladder’ pattern. This characteristic DNA ladder is considered the hallmark of apoptosis. In necrosis, as opposed to apoptosis, the genomic DNA is degraded randomly by a host of cytosolic and lysosomal endonucleases, producing a continuous spectrum of sizes (a DNA smear) when analysed by agarose gel electrophoresis (Batistatou and Greene, 1993; Tilly and Ratts, 1996).

Another important distinguishing feature of apoptosis is the rapid clearance of dead cells by ‘professional’ phagocytes (such as macrophages or neighbouring cells) before they can lyse, spill their noxious contents and cause an inflammatory reaction. This clearance mechanism is so efficient and rapid that the contribution of apoptosis to overall cell death in tissues has often been grossly underestimated. In contrast, during the pathological or accidental cell death that results from overwhelming cellular injury, cells swell and lyse, releasing noxious contents that often trigger an inflammatory response. An additional change associated with cells during the early phases of apoptosis is the alteration of plasma membrane phosphatidylserine asymmetry (Kroemer et al., 1995). In normal cells, the phosphatidylserine is located on the cytoplasmic side or on the inner leaflet of the plasma membrane. Early in apoptosis, phosphatidylserine is translocated from the inner to the outer surface of the plasma membrane and, consequently, is exposed to the external cellular environment. Surface exposure of phosphatidylserine occurs along with chromatin condensation, precedes the increase in membrane permeability and constitutes one of the principal targets of phagocyte recognition.

A disruption in the mitochondrial transmembrane potential occurring before nuclear changes has been observed in many cells undergoing apoptosis (Kroemer et al., 1997). This permeability transition (PT) involves the opening of a large channel in the inner membrane of the mitochondrion that leads to the release from mitochondria to the cytosol of apoptotic-inducing factors (AIF). In addition, PT causes the mitochondrial generation of reactive oxygen species (ROS), and rapid expression of phosphatidylserine residues in the outer plasma membrane leaflet.

Moreover, during apoptosis, mitochondrial inner membrane proteins, such as cytochrome c, leak out into the cytosol. At least two other cytosolic proteins, apoptotic protease activating factor 1 (Apaf-1) and Apaf-3, have been identified that collaborate with cytochrome c (also known as Apaf-2) to induce proteolytic processing and caspase activation and, in turn, kill cells by apoptosis (Reed, 1997).

Molecular mechanisms of apoptosis

The most important clue to the molecular nature of the death programme comes from genetic studies of the nematode Caenorhabditis elegans, in which, of the 1090 somatic cells formed during development of an adult hermaphrodite, 131 die by apoptosis. Three genes, ced 3, ced 4 and ced 9 (ced stands for cell death defective) are known to be directly involved in controlling the execution of apoptosis during development in C. elegans (Ellis et al., 1991). Both ced 3 and ced 4 are killer genes, required for the cell to die, whereas ced 9 is a survival gene that inhibits apoptosis in C. elegans (Ellis et al., 1991; Hengartner and Horvitz, 1994). It appears that ced 4 codes for an adaptor protein linking ced 4 and ced 9 and is essential for the processing of pro-ced 3 into the catalytically active death enzymes. These genes have mammalian homologues that also function in the molecular dance of death (reviewed in White, 1996; Jacobson et al., 1997; Nagata, 1997; Salvesen and Dixit, 1997; Vaux, 1997). In mammals, ced 3 homologues code for a family of at least a dozen cysteine proteases or caspases (formerly designated as the ICE or interleukin-1-converting enzyme family), whereas ced 9 corresponds in mammals to the Bcl-2 gene family. Mammalian counterparts of ced 4 such as Apaf 1 and Apaf 3 have been identified (Vaux, 1997), and these homologues are sufficient to activate pro-caspase 3 into the catalytically active death enzyme. The conservation in both sequence and function between nematode and mammalian cell death genes indicates that the apoptotic programme has been conserved throughout much of animal evolution.

There has been spectacular progress in the understanding of the molecular mechanisms of apoptosis (reviewed in White, 1996; Levine, 1997; Nagata, 1997; Reed, 1997; Salvesen and Dixit, 1997). One of the best studied survival genes is Bcl-2, the first member identified of a growing family of genes that regulates cell death in either a positive or a negative fashion. The Bcl-2 family of proteins, which contains both pro-apoptotic (Bak, Bcl-xL, Bad) and anti-apoptotic (Bcl-2, Bcl-xL, Mcl, A1) proteins, constitutes a critical, intracellular checkpoint within a common cell-death pathway that determines the susceptibility
It is generally believed that the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family proteins is the critical determinant of cell fate, with an excess of Bcl-2 resulting in cell survival but an excess of Bax resulting in cell death. Although these molecules compete through dimerization, extensive mutational studies have not established firmly whether anti-apoptotic or pro-apoptotic members are dominant in determining the key survival-promoting decision point. Paradoxically, a given family member may perform either function, depending on the cell systems used. In addition, the activity of some of these proteins can be regulated by phosphorylation (Gajewski and Thompson, 1996). For example, there is evidence for a negative role of Bcl-2 phosphorylation on a serine residue that lowers the apoptotic threshold and promotes cell death despite the presence of Bcl-2.

Bad, a proapoptotic member of the Bcl-2 family that promotes apoptosis by binding to and blocking the activity of Bcl-xL (a cell survival factor), dissociates from Bcl-xL upon phosphorylation. Instead, the phosphorylated form of Bad associates with 14-3-3, a phosphoserine binding protein that interacts with several signalling enzymes, including Raf-1 and Akt kinase, and is translocated into the cytosol. This sequesters Bad away from Bcl-xL, thus freeing Bcl-xL to exert its anti-apoptotic effect.

Loss of function of the tumour suppressor protein p53, in addition to its known role in cell cycle regulation, plays an important role in the regulation of apoptosis (Levine, 1997; Polyak et al., 1997). The molecular mechanisms of p53-induced apoptosis are only partially understood. Recent studies suggest that p53-induced apoptosis involves: (1) activation of redox-related genes also known as PIGs (p53-induced genes); (2) generation of reactive oxygen species; and (3) oxidative degradation of mitochondrial components permitting the release of apoptosis-inducing factors, including AIF, cytochrome c, Apaf 1, Apaf 3 into the cytosol to activate the caspases. p53-induced cell death could be influenced by Bcl-2, Fas/apo-1 and insulin-like growth factor binding protein 3.

Caspases are cysteine proteases that promote apoptosis in mammals (reviewed in Salvesen and Dixit, 1997). About ten different caspases have been identified in mammals (Table 1). Evidence for the role of caspases in cell death is based on findings that their inhibition can prevent apoptosis, whereas their over-expression and activation cause apoptosis. Caspases mediate apoptosis by cleaving selected intracellular proteins, including poly (ADP) ribose polymerase (PARP), lamin and actin, and cause morphological changes to the cell and nuclei. The programmed cell death cascade can be divided into at least three phases: signal activation, control and execution, and structural alterations (Fig. 2). Multiple signalling pathways lead from death-triggering extrinsic signals to a central control and execution stage. During this stage, the activation of caspases occurs, which is controlled by a number of genes, including Bcl-2s, p53, AIF, cytochrome c, Apaf 1, Apaf 2 and many death receptors and their ligands. Downstream, caspase activation leads to DNA fragmentation and morphological changes of the cells and nuclei that are typical for apoptosis. Mechanisms regulating programmed cell death are perhaps best-exemplified in cytokine-induced apoptosis (Gura, 1997; Kastan, 1997; Nagata, 1997). Thus far, within this family, three death factors, such as tumour necrosis factor (TNF), Fas ligand, and TNF-related apoptosis inducing ligand (TRAIL) and seven genuine death receptors, including Fas (also known as APO-1 or CD95), TRAIL-R1, TRAIL-R2, cytopathic avian leukemia-sarcoma virus receptor (CAR-1), and DR5 (also known as KILLER) have been identified. All these receptors contain a region of 60–80 amino acids within their cytoplasmic regions that is responsible for transducing the death signal, and is termed a death domain.
A number of signalling molecules also carry a DD that binds directly to these receptors. These adaptor molecules include Fas-associating protein with death domain (FADD or MORT 1), TNFR1-associated death domain protein (TRADD), receptor interacting protein (RIP), RIP-associated Ich/ced 3 homologous protein with a death domain (RAIDD). Upon activation by ligand (FasL), Fas recruits FADD directly whereas TNFR 1 and DR 3 bind FADD indirectly through TRADD. FADD, in turn, activates caspase 8 (also known as FADD-like interleukin-1β converting enzyme or FLICE), initiating a series of caspase-dependent events leading to cell death. TRADD, in an additional pathway, recruits RIP and transduces the death signals. The involvement of RAIDD in the TNF-induced apoptotic pathway is not known. However, there is evidence that RAIDD binds RIP through its death domain and recruits caspase-2 to RIP. One of the most significant breakthroughs in apoptosis research relates to the discovery of a novel death-signalling TRAIL pathway that specifically kills tumour cells but not normal cells. Like FasL–Fas, the TRAIL–DR4 or DR5 ligand–receptor complex engages the caspase cascade but does so in a FADD-independent manner. However, normal tissues are resistant to such killing, whereas most tumour cells are sensitive to it. A potential explanation for the tumour cell selectivity was provided when a related protein, TRID (TRAIL receptor without an intracellular death domain also known as DcR1), was found to be expressed selectively in normal tissues but at substantially lower amounts in most tumour cells (Pan et al., 1997). It is possible that this protein acts as a ‘decoy’ that can block TRAIL-induced apoptosis.

**Characterization of germ cell apoptosis**

The presence of internucleosomal chromatin degradation in the majority of observations of apoptotic cells has resulted in the use of DNA fragmentation as a reliable diagnostic tool for the occurrence of apoptosis. DNA 3’-end labelling is being used routinely to detect germ cell apoptosis in a large number of studies (Tapanainen et al., 1993; Billig et al., 1995; Sinha Hikim et al., 1995, 1997a). In brief, testicular DNA is extracted from tissues and labelled at 3’-ends with 32P-labelled dideoxy-ATP.
using terminal deoxynucleotidyl transferase (TdT), and then size fractionation in agarose gel by electrophoresis. A ladder-like DNA fragmentation can be seen in cells undergoing apoptosis. However, a drawback with this method is that it does not allow characterization of individual cell types undergoing apoptosis. Neither is this method appropriate for detecting spontaneous germ cell apoptosis during normal spermatogenesis, as the proportion of germ cells undergoing apoptosis is much lower than the proportion of non-apoptotic cells. Therefore, it is not surprising that assessment of apoptotic DNA fragmentation in individual germ cells has proven to be of considerable use for many investigators in this field. TdT-mediated dUTP nick-end labelling (TUNEL) is being used routinely to detect apoptotic cells in various tissues. The technique uses TdT to catalyse template-independent addition of digoxigenin-dUTP and dATP to 3'-OH ends of fragmented DNA generated by internucleosomal cleavage. The incorporated nucleotides form a random heteropolymer of digoxigenin dUTP and dATP. An anti-digoxigenin antibody conjugated to peroxidase is then added, which generates an intense signal for chromogenic substrates.

A large number of studies with rats have used the TUNEL technique for detection of germ cell apoptosis in situ in 4% formalin-fixed (Billig et al., 1995; Henriksen et al., 1995; Sinha Hikim et al., 1995, Blanco-Rodriguez and Martinez-Garcia, 1996) and Carnoy’s-fixed (Brinkworth et al., 1995) tests. However, a disadvantage of these fixations is inadequate structural preservation, which means that accurate identification of a given cell type in a diversely cellular tissue such as testis is difficult. A recent study (Sinha Hikim et al., 1997b) has shown that the use of glutaraldehyde fixation provides improved TUNEL sensitivity and allows the superior structural preservation needed for the quantitative assessment of cell death involving various germ cells at different phases of their development after removal of hormonal support in adult rats (Fig. 3). The same method was used for the detection of germ cell necrosis to examine whether this improved TUNEL technique was also specific for apoptosis. Rats were given a single subcutaneous injection of cadmium chloride (4 mg kg⁻¹ body weight) to induce germ cell necrosis. Early studies in rats indicated that a single injection of cadmium chloride in the dose range of 3.4–10.0 mg kg⁻¹ body weight produced germ cell necrosis involving all the seminiferous tubules (Mason et al., 1964). Glutaraldehyde-fixed, paraffin-wax embedded testicular sections from rats 7 days after a single exposure to cadmium were processed under the same conditions as applied to detect apoptosis of germ cells triggered by hormonal deprivation (Sinha Hikim et al., 1997a,b). Necrotic germ cells were clearly devoid of any specific labelling for low molecular weight DNA fragmentation. However, there was strong background staining, most likely due to the leakage of the degraded DNA after cell lysis. These results do not agree with those of Yan et al. (1997), which show the incidence of apoptosis in the urogenital organs, including testis in male Wistar rats 48 h after cadmium administration (0.03 mmol kg⁻¹). The reason for this discrepancy between our results and those of Yan et al. (1997) remain unclear but may be due to various factors, including strain-susceptibility, treatment duration and the intensity (exposure time or concentration) of the insult (Mason et al., 1964; Leist and Nicotera, 1997). Major advantages of glutaraldehyde fixation are that it permits recognition of apoptotic germ cells with high sensitivity and specificity and that it allows an accurate identification of their stage(s) of occurrence. In addition, the remaining portion of the glutaraldehyde-fixed testis from the same animal can be used for assessment of overall germ cell death by morphological criteria using high-resolution light and electron microscopy.

**Programmed germ cell death during normal spermatogenesis (spontaneous germ cell apoptosis)**

In mammals, germ cell death is conspicuous during spermatogenesis and occurs spontaneously at various phases of germ cell development such that seminiferous epithelium yields fewer spermatooza than might be anticipated from spermatogonial proliferations (Clermont, 1962; Huckins, 1978; Russell et al., 1990; Sharpe, 1994). Extending these earlier observations, recent studies in our laboratory have demonstrated that apoptosis is the underlying mechanism of germ cell death during normal spermatogenesis in various mammals, including rats (Sinha Hikim et al., 1995, 1997a,b), hamsters (Lue et al., 1997), mice (A. P. Sinha Hikim, Y. Lue, T. B. Rajavashisth, C. Wang, W. E. Salameh and R. S. Swerdloff, unpublished) and humans (Sinha Hikim et al., 1998). Spontaneous apoptosis of a few differentiating spermatogonia, in particular type A spermatogonia, and spermatocytes during their meiotic divisions was observed in rats and hamsters. In mice, spontaneous apoptosis was most commonly observed in spermatocytes, including the dividing spermatocytes, and less frequently in spermatogonia and seldom in spermatids. Unlike rat, mouse, or hamster testes, human testes exhibit spontaneous occurrence of germ cell apoptosis involving all three classes of germ cell, including spermatogonia, spermatocytes and spermatids. The incidence of spontaneous germ cell apoptosis (apoptotic index, expressed as the number of apoptotic germ cells per 100 Sertoli cells) in humans varies with ethnic background (Fig. 4). For example, the incidence of spermatogonial and spermatid apoptosis was higher in Chinese men than in Caucasian men. A higher incidence of spermatocyte apoptosis was also noted in Chinese compared with Caucasian men, but the difference was not statistically significant. The triggering factors for spontaneous germ cell apoptosis during normal spermatogenesis are not known and it is uncertain why there are ethnic differences in the inherent susceptibility of germ cells to programmed cell death. However, it should be noted that, in testes, as in many other tissues, the contribution of spontaneous germ cell apoptosis has been grossly underestimated due to the rapid and efficient clearance of apoptotic cells by professional phagocytes (Sertoli cells).

**Apoptosis as the underlying mechanism of germ cell death in response to gonadotrophin and testosterone deprivation**

A growing body of evidence has now demonstrated that the withdrawal of trophic support in adult rats results in the acceleration of germ cell apoptosis at specific stages of the seminiferous epithelial cycle (Brinkworth et al., 1995; Henriksen et al., 1995; Sinha Hikim et al., 1995). The possibility that apoptosis is
Fig. 3. Representative examples of TdT-mediated dUTP nick-end labelling (TUNEL) of apoptotic DNA fragmentation detected in germ cells in the absence (a,c,e) or presence (b,d,f) of a counterstain in paraffin wax sections of rat testes after various fixation protocols ((a,b) 4% (v/v) neutral buffered formalin, (c,d) 4% (v/v) paraformaldehyde, and (e,f) 5% (v/v) glutaraldehyde). Animals were given an injection of GnRH-A (1.25 mg kg⁻¹ body weight) each day for 7 days to induce germ cell apoptosis. Note the improved specificity of TUNEL of fragmented DNA in germ cells and the excellent structural integrity of the testis fixed with glutaraldehyde. Scale bar represents 10 μm.
A. P. Sinha Hikim and R. S. Swerdloff

The sole mechanism of germ cell death was tested in adult rats by examining the temporal and stage-specific changes in the kinetics of germ cell apoptosis after selective withdrawal of gonadotrophins and testosterone for up to 2 weeks by a potent GnRH antagonist (GnRH-A) treatment (Sinha Hikim et al., 1997a). The temporal relationships between the rates of overall germ cell death (characterized by morphological criteria) and apoptosis were then examined to define clearly the contribution of apoptosis to the loss of germ cells in response to hormonal deprivation. A low incidence of germ cell apoptosis (0.06–0.09, expressed as numbers per Sertoli cell) was detectable at stages I, IX–XI and XII–XIV in control rats (Fig. 5). The mean number of apoptotic germ cells specifically at stages VII–VIII increased significantly by day 5, and increased another 2.2-fold (over the 5 day treatment values) on day 7 after GnRH-A treatment when compared with controls, in which no apoptosis was detected. Significantly increased apoptosis at stages IX–XI over controls was also noted by day 7. Within the study paradigm, the largest number of dying cells occurred by day 14, at which time an increased incidence of apoptosis was also noted at stages I, II–IV, V–VI and XII–XIV in control rats (Fig. 5). The mean number of apoptotic germ cells specifically at stages VII–VIII increased significantly by day 5, and increased another 2.2-fold (over the 5 day treatment values) on day 7 after GnRH-A treatment when compared with controls, in which no apoptosis was detected. Significantly increased apoptosis at stages IX–XI over controls was also noted by day 7. Within the study paradigm, the largest number of dying cells occurred by day 14, at which time an increased incidence of apoptosis was also noted at stages I, II–IV, V–VI and XII–XIV in control rats (Fig. 5).

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A comparison of mean (± SEM) apoptotic index (number of apoptotic cells per 100 Sertoli cells) of germ cells between Chinese (♀; n = 5) and Caucasian (♂; n = 9) men. SPG, spermatogonia; SPC, spermatocytes; STD, spermatids *Significantly different (P < 0.05). (Reprinted with permission from Sinha Hikim et al., 1998).

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Fig. 5.

Quantitative assessment of mean (± SEM) apoptotic germ cell population (expressed as number per Sertoli cell) after GnRH antagonist (GA) treatment at (a) stages (♀) VII–VIII and (♂) IX–XI and (b) stages (♀) I and (♂) XII–XIV of the seminiferous epithelial cycle. abcMeans with different superscripts differ significantly (P < 0.05). the sole mechanism of germ cell death was tested in adult rats by examining the temporal and stage-specific changes in the kinetics of germ cell apoptosis after selective withdrawal of gonadotrophins and testosterone for up to 2 weeks by a potent GnRH antagonist (GnRH-A) treatment (Sinha Hikim et al., 1997a). The temporal relationships between the rates of overall germ cell death (characterized by morphological criteria) and apoptosis were then examined to define clearly the contribution of apoptosis to the loss of germ cells in response to hormonal deprivation. A low incidence of germ cell apoptosis (0.06–0.09, expressed as numbers per Sertoli cell) was detectable at stages I, IX–XI and XII–XIV in control rats (Fig. 5).

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Linear regression analysis of the data demonstrated a very high degree of correlation (r = 0.94; P < 0.001) between rates of germ cell degeneration and apoptosis. A similar relationship between germ cell apoptosis and degeneration was also noted 7 days after GnRH-A treatment at stages I, IX–XI and XII–XIV (A. P. Sinha Hikim and R. S. Swerdloff, unpublished). Collectively, these results support the concept that germ cell death after removal of hormonal support in the adult rat occurs almost exclusively via apoptosis.

Fig. 6.

Temporal relationship between the rates of germ cell degeneration (●, assessed by morphological criteria) and apoptosis (○; characterized by TdT-mediated dUTP nick-end labelling) measured at stages VII–VIII after GnRH antagonist (GA) treatment. (Reprinted with permission from Sinha Hikim et al., 1997b).

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Temporal relationship between the rates of germ cell degeneration (●, assessed by morphological criteria) and apoptosis (○; characterized by TdT-mediated dUTP nick-end labelling) measured at stages VII–VIII after GnRH antagonist (GA) treatment. (Reprinted with permission from Sinha Hikim et al., 1997b).
Table 2. Partial list of genes the deletion of which, in mice, results in defects in spermatogenesis

<table>
<thead>
<tr>
<th>Gene disrupted</th>
<th>Phenotype</th>
<th>References</th>
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<tr>
<td>Bax</td>
<td>Accumulation of atypical premeiotic germ cells but no mature haploid spermatozoa. Marked increase in germ cell apoptosis. Infertile.</td>
<td>Kundson et al., 1995</td>
</tr>
<tr>
<td>CREM</td>
<td>Late spermatids are completely absent and there is a significant increase in germ cell apoptosis. Sterile.</td>
<td>Nantel et al., 1996, Blendy et al., 1996</td>
</tr>
<tr>
<td>HR6B</td>
<td>Severely impaired spermatogenesis with only small numbers (&lt; 6% of controls) of predominantly abnormal spermatozoa. Marked increase in germ cell apoptosis. Defects in postmeiotic condensation of chromatids.</td>
<td>Roest et al., 1996</td>
</tr>
<tr>
<td>Hsp70-2</td>
<td>Failure of meiosis with a marked increase in spermatocyte apoptosis. Infertile.</td>
<td>Dix et al., 1996</td>
</tr>
<tr>
<td>Atm</td>
<td>Complete arrest at pachytene spermatocyte. Increased germ cell apoptosis. Infertile.</td>
<td>Xu et al., 1996, Barlow et al., 1996, 1997</td>
</tr>
<tr>
<td>MLH-1</td>
<td>Complete arrest at pachytene spermatocyte stage. Accelerated germ cell apoptosis. Infertile.</td>
<td>Edelmann et al., 1996</td>
</tr>
<tr>
<td>A-myb</td>
<td>Arrest at pachytene spermatocyte stage. Complete absence of post-meiotic cells such as spermatids or spermatozoa. Infertile.</td>
<td>Toscani et al., 1997</td>
</tr>
<tr>
<td>Dazla</td>
<td>Complete absence of meiotic (spermatocytes) and post-meiotic (spermatids or spermatozoa) germ cells. Infertile.</td>
<td>Rugglu et al., 1997</td>
</tr>
<tr>
<td>Bclw</td>
<td>Progressive depletion of germ cells through accelerated apoptosis to a Sertoli cell-only phenotype by approximately 6 months of age followed by a loss of Sertoli cells.</td>
<td>Ross et al., 1998</td>
</tr>
<tr>
<td>p53</td>
<td>Increased spermatogonial proliferation, decreased spermatocyte apoptosis and increased sperm output.</td>
<td>A. P. Sinha Hikim, Y. Lue, T. B. Rajavashisth, C. Wang, W. E. Salameh and R.S. Swerdloff, unpublished</td>
</tr>
</tbody>
</table>

**Genes regulating germ cell apoptosis**

An exciting advance in the understanding of the genetic modulation of apoptosis is the use of genetically altered mice either overexpressing or harbouring null mutations of specific genes. Studies using these mice are making an increasing contribution to the understanding of the role of various genes in regulating germ cell apoptosis (Table 2).

There is clear evidence that homozygous disruption of a number of genes results in infertility through accelerated germ cell apoptosis in mice. These findings give a first glimpse of the regulatory mechanisms involved in the regulation of germ cell apoptosis. Males deficient in bax were infertile and displayed accumulation of premeiotic germ cells but complete loss of advanced spermatids. In striking contrast, bax-deficient mice displayed, as expected, hyperplasia of thymocytes and B cells. Ovaries in these mice also contained unusual atretic follicles with an excess of granulosa cells. In addition, mice misexpressing bcl-2 in spermatogonia displayed an accumulation of spermatogonia before puberty but, during adulthood, exhibited loss of germ cells in the majority of the tubules (Furuchi et al., 1996). Thus, although it appears counterintuitive, both these genetically altered mice exhibited tissue- or lineage-specific effects of these genes on cellular homeostasis. How precisely these mouse mutations model human fertility will only become clear as the molecular basis of human fertility is elucidated. Nonetheless, these observations in mice clearly define important genetic principles that may apply to genes important for human fertility.

**Conclusions and perspectives**

This review has attempted to highlight the recent development in the field of apoptosis in general and the role of this mode of cell death in spermatogenesis, as well as to summarize the intratesticular regulatory mechanisms that control germ cell apoptosis. Essentially, all animal cells have the ability to kill themselves by activating an intrinsic cell suicide programme when they are no longer needed or become seriously damaged. It is now widely accepted that apoptosis is a genetically driven form of cell death that plays a major role during normal development and homeostasis, and in many human diseases. Recent studies using spermatogenesis as a model system for studying the regulation of germ cell death have provided evidence that both spontaneous and induced germ cell death after removal of hormone support in adult rats occur almost exclusively via apoptosis. Although germ cell apoptosis can also be triggered by various non-hormonal regulatory stimuli, including testicular toxins, heat stress and chemotherapeutic agents, the mechanisms by which these hormonal and non-hormonal factors regulate germ cell apoptosis are not well understood. Studies using genetically altered mice either overexpressing or harbouring a null mutation of specific genes indicate that germ cell apoptosis, like that of other cell systems, is regulated by multiple genes that either inhibit or promote cell death. The challenge is now to identify the intracellular regulators of germ cell apoptosis and to elucidate their regulation by a variety of death signals. The mechanisms by which various pro-apoptotic and anti-apoptotic genes control spermatogenesis will provide...
insight into the molecular components of the death machinery within the testis that determine whether germ cells grow, divide or die. This information is likely to be applicable to the assessment and management of male factor infertility as well as to more targeted approaches to male contraception.

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