MINIREVIEW

Role of Sertoli Cells in Injury-Associated Testicular Germ Cell Apoptosis (44558)

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Abstract. **This review examines experimental models of Sertoli cell injury resulting in germ cell apoptosis. Since germ cells exist in an environment created by Sertoli cells, paracrine signaling between these intimately associated cells must regulate the process of germ cell death. Germ cell apoptosis may be signaled by a decrease in Sertoli cell pro-survival factors, an increase in Sertoli cell pro-apoptotic factors, or both. The different models of Sertoli cell injury indicate that spermatogenesis is susceptible to disruption, and that targeting critical Sertoli cell functions can lead to rapid and massive germ cell death.** [P.S.E.B.M. 2000, Vol 225:105–115]

The overall purpose of this presentation is to examine the role of paracrine interactions between Sertoli cells and testicular germ cells in the induction and regulation of germ cell apoptosis associated with testicular injury. Because of this stated purpose, the model systems used to study injury-associated apoptosis and the molecular events that control apoptosis will be discussed in detail. However, before proceeding, it is worth examining the more general questions of the nature and purpose of Sertoli–germ cell interactions.

The concept of the Sertoli cell as a sustentacular cell that performs a supportive role in spermatogenesis has engaged the imaginations and experimental designs of reproductive morphologists, biochemists, cell biologists, and molecular biologists for decades. Perhaps the power of this concept is derived from a primal satisfaction associated with the recreation by cells within the testis of the types of mutually interdependent relationships we experience in our daily lives. Carrying this analogy a bit too far, it takes some adjustment in our thinking to accept that the nurse Sertoli cell is also delivering death signals to a dependent germ cell population; or, conversely, that germ cells are simply parasitic cells using the Sertoli cell for scaffolding and a food source.

The morphological hallmarks of Sertoli-germ cell interdependence are obvious and include the embedding of germ cells within Sertoli cell crypts and an assortment of specialized junctions. The uniqueness of these structures speaks to the special nature of this interaction. However, beyond this unique morphology, little is understood at the biochemical, cellular, and molecular levels about what makes this special Sertoli–germ cell interaction work. We have knowledge at the level of individual pathways, such as the pro-survival c-*kit*/stem cell factor system and the pro-death Fas system. These systems serve as reductionist-type paradigms of those detailed molecular investigations that form the body of this presentation. However, we lack an overall understanding of how these systems integrate. This lack of understanding is painfully demonstrated by the repeated failure of *in vitro* models, such as Sertoli-germ cell co-cultures, to mimic normal spermatogenesis.

This presentation begins with a brief background section about apoptosis and the testis followed by a short conceptual discussion of Sertoli-germ cell interactions designed

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to provide a theoretical framework for understanding injury-associated germ cell apoptosis. We then ask the question: How does Sertoli cell injury lead to germ cell apoptosis? To address this question, we explore three models of testicular injury in which Sertoli cell dysfunction is associated with germ cell death and assess the ability of these models to address mechanisms. The goal is to highlight experimental approaches with the potential for deepening our understanding of this complex system. In a Summary section, we return to the question of systems integration by discussing future research directions that hold promise for revealing a more holistic appreciation of testicular intercellular interactions.

Background

The two fundamentally different forms of cell death, apoptosis and necrosis, involve a sequence of biochemical and morphological changes (1, 2). Apoptosis, or programmed cell death, plays a critical role during mammalian morphogenesis by allowing an organism to eliminate unwanted or defective cells through an orderly process of cellular disintegration without inducing an inflammatory response. Apoptosis is an active process that involves the expression of specific genes and requires mRNA and protein synthesis (1, 2). Necrosis, in contrast, is predominantly a passive process resulting in the progressive breakdown of cellular structure and function following irreversible damage, often accompanied by an inflammatory response (1).

Ultrastructurally, apoptosis is characterized by cell volume shrinkage, membrane blebbing, chromatin condensation, cytoplasmic vacuolization, and the breakup of the cell into membrane-bound remnants termed apoptotic bodies (3). The biochemical features of apoptosis include the translocation of phosphatidylserine to the external leaflet of the plasma membrane, the activation of caspase cascades, and DNA cleavage and fragmentation into a 180–200 base-pair ladder, visualized *in situ* by terminal deoxynucleotide transferase-mediated deoxy-UTP nick end labeling (TUNEL) (4, 5).

Conceptually, it is useful to think of apoptosis as divided into two phases: (i) a signaling phase in which cells are initiated to die by various signals, and (ii) an execution phase in which cells rapidly execute a death program (6). Many different types of signals—growth factor withdrawal, cell cycle perturbations, or DNA damage, to name a few—can initiate apoptosis. Apparently, the cell uses various sensing mechanisms to interpret and integrate the different death-inducing signals and then funnels the initiation message to a common caspase execution phase pathway. Proteins of the *bcl*-2 family, consisting of various antiapoptotic (*bcl*-2, *bcl*-xl, *bcl*-w, and *mcl*-1) and pro-apoptotic (*bax, bak, bik, bad,* and *bcl*-xs) members, are involved in the decision to activate the apoptosis pathway (7). Other proteins important in induction of apoptosis include p53, a tumor suppressor protein, and the Fas receptor (Fas)/Fas ligand (FasL) system, a paracrine signal transduction system that directly activates the caspase cascade (8–11).

Spermatogenesis is a dynamic and highly synchronized process that takes place in the seminiferous tubules of the testis. This process begins with the clonal expansion of stem spermatogonia and involves mitosis, meiosis, and cellular differentiation. As in many tissues throughout the body, the number of cells in the seminiferous tubules of the testis is determined by a dynamic balance between cell proliferation and apoptotic cell death (12). In the testis, spontaneous apoptosis of type A_2, A_3, and A_4 spermatogonia reduces the number of germ cells produced to 25% of that expected if all A_1 spermatogonial progeny were to survive (13).

Germ cell apoptosis during testicular development in the mouse has two peaks corresponding to the time of migration of primordial germ cells into the gonads and the beginning of the first round of spermatogenesis (14). The apoptosis associated with the first round of spermatogenesis ($\approx$ 10–13 days after birth) occurs as a single wave affecting spermatocytes, and is critical to the normal development and function of the adult testis (15, 16). In mature adult rat testis, apoptosis is limited to certain germ cells and stages of spermatogenesis; a large proportion of type A_2, A_3, and A_4 spermatogonia degenerate *via* apoptosis with the highest levels occurring in stages I and XII-XIV (17, 18). Since the somatic Sertoli cells, to which the germ cells are attached, can only support a limited number of germ cells, *de facto* the number of germ cells is regulated to match the supportive capacity of the Sertoli cells (19). Thus, the ratio of the different types of germ cells to Sertoli cells remains relatively constant in mammalian spermatogenesis, and control of this ratio is an important requirement during differentiation (15).

Various types of testicular injuries, including hormonal perturbations (20), heat exposure (21), Sertoli cell toxicants such as 2,5-hexanedione (22) and mono-(2-ethylhexyl) phthalate (23), and germ cell toxicants like x-irradiation (24), all result in germ cell apoptosis. Together, these observations indicate that the seminiferous epithelium responds to most adverse environmental conditions by eliminating germ cells through programmed cell death. And since Sertoli cells are responsible for establishing the environment within the seminiferous epithelium, this implies that Sertoli cells have a way to initiate and control germ cell apoptosis.

Conceptual Framework

To construct our conceptual framework of the Sertoli-germ cell interaction during testicular injury, we will first review the characteristics of a testicular paracrine pro-survival system, the c-*kit*/stem cell factor system, and a testicular paracrine pro-apoptotic system, the Fas/FasL system.

Stem cell factor (SCF, also known as Steel factor) is a Sertoli cell–produced ligand that binds to the c-*kit* receptor on differentiating spermatogonia (25). Our detailed molecu-

lar understanding of this system has resulted, in large part, from the study of mice spontaneously mutant in the *steel (Sl)* locus (that produces SCF) and the *dominant white spotting (W)* locus (that produces the c-*kit* receptor). These mutant mice exhibit abnormalities in hematopoiesis, melanogenesis, and spermatogenesis (26). In a recent investigation of these mouse models, spermatogonial transplantation of germ cells from infertile SCF mutant mice to infertile c-*kit* mutant mice restored fertility in the recipients (27). This experiment demonstrates the specificity and reciprocal nature of this ligand-receptor interaction as well as the reversibility of infertility once an appropriate signaling milieu is restored. The importance of this pro-survival system to testicular homeostasis is underscored by the induction of testicular atrophy in normal mice infused with an antibody that blocks SCF stimulation of the c-*kit* receptor (28, 29).

With SCF binding, the c-*kit* receptor, a tyrosine kinase, undergoes dimerization and autophosphorylation. Subsequent downstream signaling initiated by c-*kit* involves multiple pathways, including phosphatidylinositol-3-kinase, Src family members, the JAK/STAT pathway, and the Ras-Raf-MAP kinase cascade (30). To date, detailed investigations of these signal transduction pathways have been largely limited to the hematopoietic system with little information available about testicular germ cells.

The Fas system is an apoptosis-initiating ligand-receptor system that has been studied extensively because of its importance in immune regulation. Murine mutants with a dysfunctional Fas system (*gld*, the FasL mutant, and *lpr*, the Fas mutant) develop an autoimmune-like syndrome because of an abnormal proliferation of lymphocytes. The FasL-Fas interaction triggers the death of cells expressing Fas, a process best studied in cytotoxic T lymphocyte–mediated cytotoxicity (31). The interaction between FasL and Fas can be autocrine, paracrine, or juxtacrine and is mediated by trimerization of both ligands and receptors (31, 32). The binding of FasL to the Fas receptor, a type I transmembrane receptor protein, directly triggers a caspase cascade that leads to the death of the cell (11).

In searching for the mechanism by which Sertoli cells might modulate germ cell apoptosis, we studied the Fas system in the testis. Using immunohistochemistry, we localized FasL, which is highly expressed in rodent testis (33), to Sertoli cells and Fas to germ cells (9). The functionality of the Fas system in testis was assessed *in vitro* by prolonging germ cell survival with an antisense oligonucleotide that inactivated FasL and increasing germ cell death by activating Fas directly with the Jo-2 antibody (9).

Comparing the expression patterns of FasL and Fas mRNA after toxicant-induced testicular injury, those agents that damaged Sertoli cells led to an upregulation of FasL followed by an upregulation of Fas; conversely, toxic agents that damaged germ cells directly failed to upregulate FasL while upregulating Fas mRNA expression (10). Interestingly, *gld* mutant mice (with an abnormal FasL) have a modest baseline germ cell hyperplasia (34), indicating that the Fas system modulates, at least in part, the level of ongoing spermatogenesis. Furthermore, *gld* mice exposed to an agent that damages Sertoli cells had markedly deficient germ cell apoptosis when compared with exposed wild type mice (34).

Given the above descriptions of the function of the pro-survival SCF/c-*kit* system and the pro-apoptotic FasL/Fas system in spermatogenesis, we should feel some satisfaction in having gained a relatively mature understanding of how these systems work in isolation. On the other hand, our understanding of interactions between these systems is primitive at best. Many additional paracrine interactions must exist between Sertoli cells and germ cells to regulate germ cell apoptosis. Obviously missing are those signals that inform the Sertoli cell of the germ cell state. Also missing, and a real "black box," is any real understanding of the machinery used by the germ cell to integrate the various survival and death signals into a life-or-death decision-making process.

Accepting these limitations, we can develop a simple conceptual framework for the signaling phase by focusing on the paracrine Sertoli-germ cell interaction itself and using what we know about the function of the SCF/c-*kit* and FasL/Fas systems in spermatogenesis (Fig. 1). Using this approach, germ cell apoptosis can be triggered by a deficiency of a pro-survival factor (Fig. 1, top pathway), by an excess of a pro-apoptotic factor (Fig. 1, middle pathway), or by synergistic alterations in both pathways (Fig. 1, bottom pathway). This is a simple model that is likely to be at least

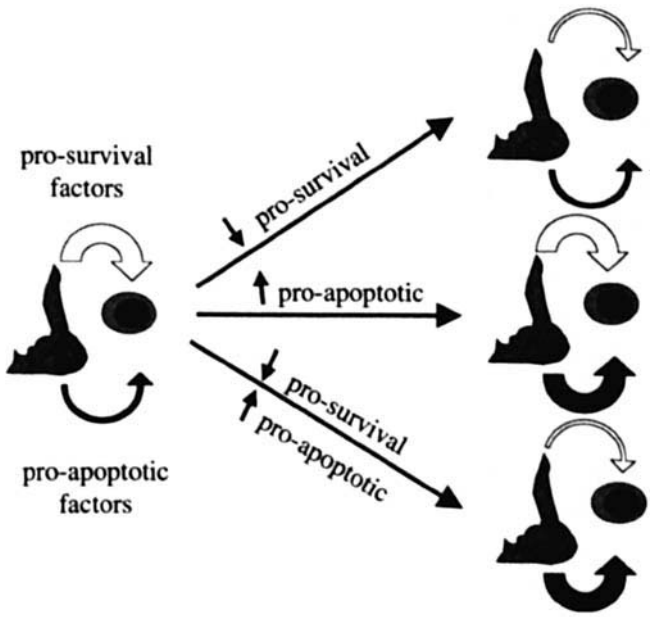

Figure 1. Sertoli cells (irregular in shape) provide pro-survival factors (open arrows) and pro-apoptotic factors (filled arrows) to germ cells (round in shape) to regulate spermatogenesis. Sertoli cell injury can result in excessive germ cell apoptosis because of decreased pro-survival support (top pathway, as may occur with hormone deprivation), increased pro-apoptotic signaling (middle pathway, as in phthalate exposure), or both (lower pathway, as in 2,5-hexanedione exposure).

partially correct, given what we know about the SCF/c-*kit* and FasL/Fas systems, as described above.

Despite its simplicity, the implications of this model are profound. This model says that spermatogenesis is poised at an interface between opposing pro-survival and pro-apoptotic forces and that optimal spermatogenesis requires an appropriate balance of these forces. This leads to a series of questions about the nature of this balance point: Is the interface between pro-survival and pro-apoptotic forces a broad plateau or a knife-edge? Is spermatogenesis finely or coarsely tuned? Tightly or loosely controlled? Easily disrupted or resistant to disruption? To gain some insight into these questions, we turn to three models of testicular injury that appear to operate in different ways.

How Does Sertoli Cell Injury Lead to Germ Cell Apoptosis?

Understanding the mechanism of injury-associated germ cell apoptosis in testis, a highly complex tissue with multiple cell types in various stages of differentiation, is a challenging problem. Not only is it necessary to know the molecular target; in addition, those cells and processes that critically depend upon that molecular target must be determined. In practice, this means that specificity of action is defined by identifying those cells and processes that are first dysregulated in the injury process.

For each of the three models discussed below, the Sertoli cell is the primary target cell of testicular injury resulting in germ cell apoptosis. The reader should approach these models keeping the conceptual framework established in the last section in mind. The model of androgen deprivation, discussed first, is an example of the consequences for germ cells of removing a trophic factor that causes Sertoli cells to be pro-survival (similar to Fig. 1, top pathway). The model of phthalate exposure, discussed second, has features which suggest that germ cell apoptosis occurs primarily because of active pro-apoptotic signaling by an acutely altered Sertoli cell (similar to Fig. 1, middle pathway). The final model, 2,5-hexanedione exposure, has features which suggest that germ cell apoptosis occurs because of both a failure of Sertoli cell pro-survival support and pro-apoptotic signaling (similar to Fig. 1, bottom pathway). After each model is reviewed, we discuss the utility of the model and directions for further research.

Androgen Deprivation: A Model of Decreased Pro-Survival Support

Spermatogenesis is dependent upon both pituitary gonadotrophins and testicular androgens. In response to hypothalamic gonadotrophin releasing hormone (GnRH), the pituitary releases luteinizing hormone (LH) and follicle stimulating hormone (FSH). The interstitial Leydig cells, under LH stimulation, produce androgens, including testosterone. The Sertoli cells, which contain intracellular androgen receptors as well as plasma membrane FSH receptors, modulate these trophic inputs and support spermatogenesis through the production of a number of paracrine-acting factors (35, 36).

In the classic model of hormone deprivation, hypophysectomy, the stimulatory input of FSH and LH is removed, resulting in the degeneration of spermatogenic cells (37–39). Hypophysectomy-associated cell loss in the testis is the result of germ cell apoptosis (40). A time-dependent decrease in testis weight, amounting to an 80% drop after 6 days correlated with an increase in apoptotic internucleosomal DNA fragmentation (40). Hormonal therapy with testosterone, FSH, and hCG, but not GnRH antagonist, immediately following hypophysectomy, resulted in a reduction of DNA fragmentation (40). Morphology and TUNEL labeling indicate that apoptosis is limited to germ cells (18, 41–44) and does not involve Leydig or Sertoli cells. Because both androgen and FSH receptors are present on Sertoli cells, gonadotrophins and testosterone act as survival factors preventing the death of germ cells with Sertoli cells acting as mediators of this process.

A decline in androgen production by Leydig cells is a trigger for germ cell apoptosis. One model for examining the acute effects of testosterone deprivation on germ cells *in vivo* has been through the use of ethane dimethane sulfonate (EDS), a toxicant that selectively kills Leydig cells, resulting in a suppression of intratesticular and serum testosterone. Although EDS is a primary Leydig cell toxicant, it also provides a useful model to study the effects of androgen ablation independent of a suppression of LH and FSH, features not afforded by models of hypophysectomy and GnRH analog therapy.

The effects of EDS on the Leydig cell population have been well characterized. Within 6 hr of a single intraperitoneal injection of EDS (75 mg/kg), serum, interstitial, and seminiferous fluid testosterone levels fall dramatically (45). Leydig cells show signs of disintegration and fragmentation by 24 hr, and at Days 3–7 following EDS injection, the interstitial space is void of histologically identifiable Leydig cells. Testosterone levels decline further at 3 days, and remain at undetectable levels for 2 weeks postdose. The decline in testosterone to castrate levels at Day 3 is accompanied by a 50% reduction in testis weight between Days 3 and 14 postdose (45). From Days 3–7 after EDS treatment, the first morphologic signs of testosterone withdrawal are apparent within seminiferous tubules from stages VII to IX. Pyknotic changes are noted in pachytene spermatocytes and step 7 and 9 spermatids. In addition, step 19 spermatids are retained by Sertoli cells (46). By Days 5–8 after testosterone deprivation, degenerating step 10 and 11 spermatids at stages X and XI are also observed.

The degeneration of germ cells is associated with stage-dependent alterations in the morphology of Sertoli cells, which accumulate numerous valuoles within their basal cytoplasm. Not all stages of spermatogenesis are equally responsive to testosterone, as testosterone-mediated suppression of EDS-induced germ cell loss is limited to stage VII tubules (46). These observations are consistent with the

known androgen dependence of stages VII and VIII, and with a maximal content of androgen receptors in Sertoli cells at stages VII-VIII (47, 48).

As apoptosis gained recognition as a fundamental developmental process and appropriate techniques became widely available, the germ cell degenerative changes observed following EDS exposure were characterized as apoptotic. Initial studies implicating testosterone in programmed cell death reported DNA fragmentation and hypodiploid DNA content in testis following EDS treatment, with these signs of apoptosis particularly evident in haploid cells (49). Later, TUNEL analysis of seminiferous tubule squash preparations from EDS-treated rats also reflected highest levels of apoptosis in stage VIIab and VIIcd, with apoptotis frequently occurring in preleptotene spermatocytes. In addition, increases in apoptotic cells were observed in stages II–XI. The effects were suppressed by testosterone supplementation, except in stages II–III and VII cd. An opposite effect was observed in stage XII, where the number of apoptotic cells decreased 1, 3, and 7 days after EDS treatment and returned to control levels in animals supplemented with testosterone (50).

Although ample evidence exists for a role of testosterone in the suppression of germ cell apoptosis, little is known regarding the signaling events that allow androgen binding in the Sertoli cell to suppress germ cell apoptosis. Molecular details of steroid hormone receptor activation have been well characterized. Testosterone diffuses into the cell and binds to the androgen receptor inducing conformational changes that favor the displacement of heat shock proteins and DNA binding. The testosterone/androgen receptor complex then binds as a homodimer to specific steroid response elements, resulting in the recruitment of basal transcription factors, coactivators, and other transcription factors that induce and/or repress the transcription of target genes (51).

However, a real gap exists in our knowledge of postreceptor signaling and how androgen receptor binding is able to suppress germ cell apoptosis. One way to examine indirectly the molecular mechanism of apoptotic cell death involved in androgen withdrawal has been to follow the expression of a number of apoptosis-related genes following EDS treatment, including the *bcl*-2 family members and the Fas system. Following treatment with EDS, time-dependent increases in the expression of the pro-apoptotic protein *bax* correlate with androgen loss and the onset of germ cell apoptosis (52). However, androgen ablation did not result in changes in the expression of clusterin, *bcl*-xl, *bak*, or *bad*. *Bcl*-2 levels also rise, but not until 8 days after androgen suppression, suggesting that upregulation of *bcl*-2 by remaining germ cells serves as a survival mechanism to ensure that the atrophied tissue will respond to re-stimulation by androgen (52).

The increase in germ cell apoptosis following androgen withdrawal by EDS has also been temporally correlated with changes in Fas receptor protein expression by germ cells. Fas protein expression increases after androgen suppression, before the onset of germ cell apoptosis. However, if EDS and testosterone are administered simultaneously, apoptosis is suppressed, and testis Fas levels remain unchanged (20). A role of Fas in germ cell apoptosis following androgen ablation remains an open question, since declines in both Fas and FasL protein expression following EDS treatment have also been reported (52).

Androgen receptor is localized to Sertoli, Leydig, and peritubular cells (47, 48, 53), and possibly germ cells (48). In addition, evidence is gaining that FSH deprivation independently contributes to germ cell apoptosis, in a stage-dependent manner distinct from testosterone (54), indicating that FSH and testosterone act together to suppress germ cell apoptosis. Thus, current dogma suggests (with little direct evidence) that Sertoli cells, which express androgen receptors in a stage-specific manner corresponding with germ cell sensitivity to androgen withdrawal and exclusively express FSH receptor, suppress germ cell apoptosis by the production of viability-maintaining factors when stimulated by FSH and testosterone. Whether paracrine-acting pro-apoptotic factors participate in signaling germ cell apoptosis following hormone withdrawal remains to be determined.

Constant Sertoli cell support is required to prevent germ cells from dying by apoptosis; therefore, hypothetically, a decrease in Sertoli cell–derived pro-survival factors alone, in this case triggered by testosterone or FSH deprivation, may be sufficient to initiate a process leading to germ cell death.

Phthalate Exposure: A Model of Pro-Apoptotic Activation

Phthalates are in widespread use as plasticizers in packaging materials, leading to significant exposure through contamination of foods and biomedical products. Because of their commercial importance and long-recognized effects on the male reproductive system, phthalates are the most studied class of testicular toxicants. Many features of phthalate-induced male reproductive toxicity are well understood, including the ability to injure the male reproductive system at all life stages from early development to adulthood and the greater susceptibility of certain stages of the cycle of the seminiferous epithelium.

The Sertoli cell is the primary cellular target of this toxicity, with rapid onset of vacuolation, alteration of intercellular contacts, and retraction of processes after exposure (55–58). Produced as aliphatic diesters of phthalic acid, the metabolite responsible for testicular injury is the monoester resulting from hydrolysis by intestinal esterases and pancreatic lipases (59). For mechanistic investigations, mono-(2-ethylhexyl) phthalate is one of the most potent and widely studied of the phthalates, producing extensive testicular germ cell apoptosis 3–6 hr after high-dose exposure.

Over the past 30 years, a number of distinct research themes related to proposed mechanisms or exposure paradigms have been pursued in the study of phthalate-induced

male reproductive injury, including: (i) compromise of zinc-dependent enzymatic activities; (ii) changes in hormonal status; (iii) altered metabolic function; (iv) disruption of Sertoli-germ cell interactions; (v) alterations in membrane-dependent signaling events; and (vi) the consequences of *in utero* and perinatal exposure to phthalates. Despite the extent of this past and ongoing investigation, the molecular target for phthalate-induced male reproductive toxicity remains unknown. In this review, we focus on the recent literature regarding phthalate-induced alterations in Sertoli cell signal transduction, the Sertoli-germ cell interaction, and germ cell apoptosis.

The observation that phthalate-induced testicular injury of adult rats is limited to stages XI-XIV, I, and II (57), those stages with the highest FSH responsiveness (60), prompted investigation of the FSH receptor system as a molecular target. A further attraction of the FSH receptor system is its unique expression by Sertoli cells, the cellular target of phthalate-induced testicular injury in postnatal animals.

Using cultured Sertoli cells, mono-(2-ethylhexyl) phthalate specifically inhibited FSH-stimulated accumulation of the second messenger, cAMP (61–63). The FSH-stimulated inhibition of cAMP accumulation was partial, time-dependent, dose-dependent, independent of cAMP breakdown or adenylate cyclase inhibitory pathways, and FSH receptor signal transduction system–dependent. FSH binding to Sertoli cell membranes was inhibited only after preincubation with mono-(2-ethylhexyl)phthalate and was a consequence of altered affinity rather than a reduction in receptor number (64). These results suggested that mono-(2-ethylhexyl)phthalate acted at the level of the GTP-binding protein (Gs) that couples the FSH receptor to the adenylate cyclase catalytic subunit (64), and has focused attention on signal transduction events as a potential molecular target. The detailed *in vitro* investigations of phthalate-induced inhibition of FSH-stimulated cAMP accumulation by Sertoli cells have been very useful in identifying a relatively sensitive end point of toxicant action. The suggested modulation of the FSH-stimulated signal by an action at the level of the GTP-binding protein, Gs, opens up the possibility of a broad range of physiological effects arising from this type of membrane-associated target. However, the noncritical role played by FSH itself in adult testicular function argues against the FSH signal transduction system *per se* as an important target of toxicity.

Hallmarks of phthalate-induced disruption of the Sertoli–germ cell interaction are germ cell sloughing *in vivo* (55) and germ cell detachment *in vitro* (65). In an attempt to explain these phenomena, the Sertoli cell cytoskeleton has been examined as a potential target (23). Following *in vivo* exposure, mono-(2-ethylhexyl) phthalate altered the distribution of vimentin within the Sertoli cell cytoplasm (23). Within 3 hr of exposure, Sertoli cell vimentin filaments collapsed around the nucleus instead of projecting radially toward the seminiferous tubule lumen. At this early time point, no changes were observed in Sertoli cell microtubules or actin networks. Based on their location, vimentin filaments may be involved in positioning Sertoli cell nuclei, in Sertoli-germ cell attachment, and in signal transduction between the Sertoli cell plasma membrane and the nucleus (66, 67). However, Sertoli cell vimentin filaments *per se* are not candidates for the initial target of phthalate-induced injury, since vimentin-null mice develop and reproduce without an obvious phenotype (68).

Interestingly, the collapse in vimentin filaments seen at 3 hr was accompanied by a decrease in the baseline rate of germ cell apoptosis; at later time points, supranormal rates of germ cell apoptosis were observed as the germ cells died following phthalate-induced injury. A model, involving membrane-dependent alterations in Fas signaling, couples the observed cytoskeletal alterations to this pattern of germ cell apoptosis (69). This model assumes that the Fas system performs a critical role in effecting germ cell apoptosis in phthalate-induced testicular injury, an assumption supported by the well-documented upregulation of Sertoli cell FasL and germ cell Fas receptor expression after exposure (9, 10, 69) and the inhibition of the usual germ cell apoptosis seen after phthalate exposure in FasL-deficient *gld* mice (34). According to this model (69), the collapse of Sertoli cell vimentin filaments reflects germ cell detachment from the Sertoli cell membrane. The detachment of germ cells has two effects: first, it prevents the interaction of Sertoli cell membrane-bound FasL with Fas receptor on germ cells, explaining the observed decrease in germ cell apoptosis at early times after exposure; second, it signals Sertoli cells to increase the expression of soluble FasL (sFasL) through metalloproteinase-mediated cleavage of membrane-bound FasL, causing the massive germ cell apoptosis that ultimately ensues. This internally consistent and satisfying model is supported by Western blots quantitating the temporal pattern of sFasL and Fas protein expression in the injured testis, and explains the peculiar suppression of germ cell apoptosis immediately following exposure to mono-(2-ethylhexyl) phthalate.

To summarize, the phthalate model is an excellent example of Sertoli cell targeting leading to paracrine pro-apoptotic signaling of germ cell death. The rapid onset of phthalate-induced testicular injury suggests a relatively simple mechanism of action specific to the Sertoli cell. To account for the range of phthalate-induced metabolic and functional abnormalities and, in particular, the alterations observed in FSH-signaling, the initial molecular target for male reproductive toxicity is likely to occupy a central role in a basic signal-transduction system within the Sertoli cell. Although the nature of this initial molecular target remains unknown, a plausible cascade of events involving vimentin collapse, germ cell detachment, and Fas system activation has been proposed to explain how Sertoli cell dysfunction leads to germ cell apoptosis. The rapid induction of pro-apoptotic signaling after Sertoli cell targeting by phthalates indicates that certain Sertoli cell functions are absolutely and immediately critical to successful spermato-

genesis. Therefore, identification of the phthalate target should provide insight into basic pathways important to testicular homeostasis.

2,5-Hexanedione Exposure: A Complex Model

To first summarize this model briefly, 2,5-hexanedione is the toxic γ-diketone metabolite of *n*-hexane and methyl *n*-butyl ketone. Exposure of rats to 2,5-hexanedione produces a slowly progressive syndrome of selective nervous system and testicular dysfunction. The temporal sequence of biochemical and morphological testicular alterations has been studied in detail in young adult rats exposed for 5 weeks to 1% 2,5-hexanedione in the drinking water. The earliest alteration detected, 2 weeks after initiating exposure, is enhanced polymerization of purified rat testis tubulin, which is predominantly of Sertoli cell origin (70). By 3 weeks after initiating exposure, seminiferous tubule fluid formation is decreased (71), possibly due to decreased microtubule-dependent transport in the Sertoli cell (72). By 4 weeks after initiating exposure, Sertoli cell vacuolization (73) and a profound decrease in seminiferous tubule fluid formation is followed by a rapid and progressive apoptosis of germ cells (22, 74). After exposure and return of the rats to normal drinking water, the testis continues to lose germ cells and, by 12 weeks after initiating exposure, enters a persistent state of irreversible testicular injury (75).

Several points raised in this brief summary require greater attention, namely: the identity of the molecular target, the temporal progression of Sertoli cell histopathological and functional abnormalities, and the nature of the wave of germ cell apoptosis. As a γ-diketone, 2,5-hexanedione reacts ubiquitously with tissue nucleophiles, particularly protein lysyl ε-amines, leading to the formation of pyrrole adducts and cross-linked proteins. The cross-linking of tubulin, the building block of microtubules, is noteworthy because it is associated with altered microtubule assembly kinetics in 2,5-hexanedione–exposed rats (76, 77). Indeed, tubulin extracted from brain or testis and incubated with 2,5-hexanedione *in vitro* exhibited more rapid nucleation and assembly and greater resistance to cold than untreated tubulin. More importantly, testis-derived tubulin extracted from 2,5-hexanedione–exposed animals exhibited similar alterations in its assembly properties.

Tubulin is of interest as a potential molecular target for 2,5-hexanedione because of the role of microtubules in microtubule-dependent transport. Evidence that microtubule-based transport is impaired by 2,5-hexanedione has been obtained primarily from *in vitro* experiments. Compared with untreated microtubules, kinesin-based transport along 2,5-hexanedione–treated microtubules is slowed in a dose-dependent fashion (72). In studies of the nervous system injury, axonal microtubule–dependent transport is inhibited following 2,5-hexanedione exposure (78).

Seminiferous tubule fluid, formed by Sertoli cells by a microtubule-dependent transport process, contains growth factors and nutrients that are essential for the support of differentiating germ cells. Seminiferous tubule fluid formation is easily quantitated using an *in vitro* system that monitors oil droplet movement in the lumen of intact seminiferous tubules (71). Using this approach, colchicine, a specific and irreversible microtubule disassembly agent, rapidly stopped oil droplet movement. Brefeldin A, a reversible inhibitor of membrane flow in the cellular secretory apparatus, acted as a reversible inhibitor of oil droplet movement. Most importantly, seminiferous tubules isolated from rats exposed to 2,5-hexanedione for 3 weeks had decreased seminiferous tubule fluid formation in the absence of histopathological alterations in the seminiferous epithelium (71).

Only limited information is available concerning the behavior of specific Sertoli cell–derived growth factors during the time course of 2,5-hexanedione–induced testicular injury. By Northern analysis, stem cell factor mRNA levels rise after germ cell loss due to the relative enrichment for Sertoli cells in the atrophic testis (79). The ratio of expression of stem cell factor as either the soluble or transmembrane transcript has been examined throughout the time course of 2,5-hexanedione; coincident with the onset of germ cell loss, there is an increase in the soluble-to-transmembrane stem cell factor transcript ratio (80). This ratio is of interest because it varies during development with high soluble transcript levels early in life during primordial germ cell migration and high transmembrane transcript levels once spermatogenesis is established (81). In addition, mutant mice that produce only the soluble stem cell factor transcript, known as steel Dickie mice, have an aborted first wave of spermatogenesis followed by atrophy (82). An attempt to reverse 2,5-hexanedione–induced germ cell loss by the intratesticular infusion of stem cell factor met with limited success; rats receiving stem cell factor had an increase in the size of their germ cell cohorts (79).

The earliest identifiable histopathological lesions in testis appear at 4 weeks of 2,5-hexanedione exposure, after the observed alterations in tubulin assembly and seminiferous tubule fluid formation. At this time, characteristic signs of Sertoli cell dysfunction appear in the form of large vacuoles in the basal cytoplasm (70, 73). Ultrastructurally, these vacuoles have been identified as swollen components of the endoplasmic reticulum. Soon after the appearance of Sertoli cell vacuolization, germ cells begin to die.

Although it was initially believed that germ cell death was due to necrosis, it is now clear that apoptosis is the mechanism by which germ cells are eliminated after 2,5-hexanedione exposure. The appearance of DNA ladders, a hallmark of apoptosis, coincided with the appearance of dying germ cells (22). Five weeks after initiating 2,5-hexanedione exposure, the frequency of TUNEL positivity increased markedly from a baseline of<10% to>60% of seminiferous tubules containing ≥3 TUNEL positive germ cells per seminiferous tubule cross section (22).

Weeks before the wave of germ cell apoptosis induced by 2,5-hexanedione exposure, FasL mRNA expression is

upregulated as assessed by semiquantitative RT-PCR (10). On the other hand, Fas mRNA expression is increased only later at the time of germ cell apoptosis. This temporal pattern of Fas/FasL system expression suggests that early in the time course of 2,5-hexanedione exposure, Sertoli cell dysfunction is sensed and translated into a pro-apoptotic signal.

Future research on the 2,5-hexanedione model of testicular injury should focus on a better understanding of the molecular target and how the deficiency of pro-survival factors and upregulation of pro-apoptotic signals trigger germ cell apoptosis. Assessing the dependence of germ cells upon Sertoli cell microtubule-dependent functions in the intact animal has been difficult. *In vivo* exposures to colchicine or taxol, a microtubule-stabilizing compound, invariably produce unwanted effects on germ cells. To address this problem, approaches that deliver molecular probes selectively to Sertoli cells are needed. We have previously demonstrated that adenoviruses, when delivered to the lumen of the seminiferous epithelium by retrograde perfusion of the rete testis, specifically infect Sertoli cells (83). By using an adenovirus engineered to express γ-tubulin, a superstable form of tubulin, we hope to simulate the microtubule assembly alteration induced by 2,5-hexanedione and test the hypothesis that Sertoli cell microtubule dysfunction *per se* can trigger germ cell apoptosis. By this mechanism, it will be possible to assess directly the dependence of germ cells upon intact Sertoli cell microtubule networks.

A more detailed understanding of changes in secretion, expression, and concentration of specific Sertoli cell–derived growth factors during the time course of 2,5-hexanedione–induced testicular injury would be informative. However, these types of studies are inherently difficult because any measurements made on such a complex tissue as the testis do not easily translate into insight at the level of the Sertoli-germ cell interaction.

The very complexity of the 2,5-hexanedione exposure model, as portrayed in the discussion above, is both its greatest weakness and its greatest strength. The fact that the protein cross-linking produced by 2,5-hexanedione is ubiquitous means that there are many targets altered by exposure. And yet the biochemical lesioning is subtle, leading to a protracted period of incipient dysfunction before the occurrence of catastrophic germ cell apoptosis. The slow progression of dysfunction and the initial subtlety of the abnormalities suggests that this model can be used to identify important pathways and targets, from those that are less important, in a dynamic *in vivo* setting. Careful study of changes taking place in the production of pro-survival and pro-apoptotic factors early during 2,5-hexanedione exposure, before massive germ cell apoptosis, offers us an opportunity for insight into how these various factors interact and integrate.

To summarize, following 2,5-hexanedione exposure, early abnormalities are observed in Sertoli cell function. The nature of the abnormalities indicates a failure by Sertoli cells to provide adequate germ cell support accompanied by active pro-apoptotic signaling. The protracted time of abnormal Sertoli cell support and pro-apoptotic signaling before the onset of germ cell apoptosis indicates that spermatogenesis is resistant to this type of injury. On the other hand, once the decision-making process decides in favor of death, germ cells undergo rapid and massive apoptosis.

Summary

A great deal of experimentation remains before the factors influencing germ cell apoptosis in the setting of Sertoli cell dysfunction will be understood. Few systems regulating germ cell survival *in vivo* have been studied in sufficient detail to know the molecular nature of both the Sertoli cell and germ cell factors. In addition to identifying the molecular players regulating germ cell survival and apoptosis, large gaps exist in our understanding of the interplay between these factors and the integrating mechanisms that maintain homeostasis.

Despite the obvious limitations, the injury models have provided us with a general understanding of how the paracrine Sertoli-germ cell interaction works in response to injury. To recap, we return to the series of questions asked at the beginning: Is the interface between pro-survival and pro-apoptotic forces a broad plateau or a knife-edge? Is spermatogenesis finely or coarsely tuned? Tightly or loosely controlled? Easily disrupted or resistant to disruption? Based on what we know from the injury models, the answer to these questions is all of the above. In fact, spermatogenesis is both very robust and very sensitive to disruption. The model of injury by acute hormone deprivation suggests that withdrawal of an absolutely critical pro-survival factor results in a gradual failure of spermatogenesis. Phthalate exposure produces rapid and extensive germ cell apoptosis, presumably by targeting a critical Sertoli cell system that activates a pro-apoptotic paracrine pathway. Injury by 2,5-hexanedione has both a gradual onset and an abrupt phase of germ cell loss.

An appropriate analogy for spermatogenesis might be a very complex machine with lots of parallel pathways and redundancy; to be working exactly optimally, every system must be performing well, but removing a single system is unlikely to be disabling. The ongoing spermatogenesis observed in *gld* FasL-deficient mutant mice and p53 knockout mice are examples that support this view. Unstressed, spermatogenesis in these mice is very close to normal; when injured, these knockout mice respond quite abnormally (34, 84).

Even with the built-in flexibility of redundancy, spermatogenesis has critical vulnerabilities; as in any complex machine, the process requires power and a master controller. The model of phthalate injury highlights the critical vulnerability of spermatogenesis and underscores the importance of the Sertoli cell. But is the Sertoli cell the master controller of this very complex machine? Given both our still rudimentary state of knowledge and that interdependency *per se* between Sertoli cells and germ cells is the

hallmark of spermatogenesis, speculation about the nature of the master controller is best left to the future. Indeed, recent cross-species experiments in which rat germ cells were transplanted into recipient mice have provided the surprising result that the timing of the cycle of the seminiferous epithelium is controlled by germ cells, not Sertoli cells (85).

Future progress in this field will be driven by both advances in technology and shifts in the conceptual framework. The rapid and ongoing advances in molecular technology—cell specific gene expression, transgenics, and knockouts—combined with refined analytical tools—green fluorescent protein and laser capture microdissection—allow the observation and analysis of critical biological processes in complex living systems. The power of these techniques and approaches is obvious.

Paradigm shifts in our conceptual framework should be expected and anticipated. As stated above, Figure 1 illustrates a simple model that is likely to be at least partially correct. But the complex machine of spermatogenesis cannot be fully explained by a simple model. Some of the necessary complexity that we can anticipate will be filling in gaps in existing paradigms. But real advances in understanding are more likely to come from new insights into previously unappreciated systems that lead to new paradigms. To illustrate this point, cadherins may represent an important molecular system that governs interactions between Sertoli and germ cells and promotes the survival of germ cells. Cadherins are transmembrane proteins that bind in a homophilic manner to induce adhesion between neighboring cells. It is thought that cadherin-based adhesion generates intracellular signaling cascades that govern cell survival, growth, and differentiation (86, 87). Because Sertoli-germ cell adhesion enhances the survival of germ cells, and cadherins mediate adhesion between Sertoli and germ cells *in vitro* (88–90), cadherins may be crucial regulators of spermatogenesis and modulators of germ cell apoptosis. Supporting this view, a large number and variety of cadherins are expressed in testis, as recently discovered (91).

We are currently in a phase of ever accelerating comprehension of the systems and pathways necessary for successful mammalian spermatogenesis. At some point, hopefully in the not too distant future, our comprehension of spermatogenesis will allow the *in vitro* recreation of the stem germ cell to spermatozoa proliferation and maturation sequence, a breakthrough that will herald a synthetic understanding of this very complex machine.

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