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2,5-HEXANEDIONE-INDUCED TESTICULAR INJURY

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■ **Abstract** Now in its third decade of mechanistic investigation, testicular injury caused by 2,5-hexanedione (2,5-HD) exposure is a well-studied model with a rich database. The development of this model reflects the larger changes that have moved biology from a branch of chemistry into the molecular age. Critically examined in this review is the proposed mechanism for 2,5-HD-induced testicular injury in which germ cell maturation is disrupted owing to alterations in Sertoli cell microtubule-mediated functions. The goal is to evaluate the technical and conceptual approaches used to assess 2,5-HD-induced testicular injury, to highlight unanswered questions, and to identify fruitful avenues of future research.

INTRODUCTION

This review is divided into sections that roughly parallel the temporal progression of the experiments and the changing nature of the field. The first section, Background, covers the time frame of approximately 1975–1985 and focuses on chemistry, clinical observations, and histopathology. In the second section, The Tubulin Hypothesis, extending from 1985 to approximately 1990, the experimental approach is dominated by biochemical techniques. The sections Microtubule-Dependent Transport is a Target and Apoptosis, Stem Cell Factor/c-Kit, and Irreversible Injury reflect the advances in cell biology techniques in the early 1990s and their use in defining mechanisms of toxicity. In a final part, Future Directions, unanswered research questions and problems are highlighted and new approaches and molecular techniques are proposed to further refine our mechanistic understanding of this model.

BACKGROUND

n-Hexane is a volatile organic solvent widely used in industry and commerce. Low level environmental exposure is ubiquitous because *n*-hexane is a

component of gasoline (1). *n*-Hexane is metabolically converted to the γ-diketone, 2,5-hexanedione (2,5-HD), via sequential cytochrome p450–dependent ω-1 hydroxylation and oxidation (2, 3) (Figure 1). 2,5-HD can combine with primary amines, such as protein lysyl ε-amines, to form substituted 2,5-dimethylpyrroles (4). Pyrroles form and accumulate on tissue proteins during in vivo exposure to 2,5-HD as a required step in the induction of both testicular and nervous system injuries (5–8). Subsequent oxidation and cross-linking of these tissue-bound heterocyclic aromatic compounds result in a complex array of products, including pyrrole dimers (9, 10).

Toxicologically significant human exposure to *n*-hexane has been reported mainly in occupational settings, although chronic intentional inhalation of consumer products containing *n*-hexane ("glue sniffing" or "huffing") has produced disease (11). The clinical manifestations of "hexacarbon" or γ-diketone-induced toxicity are those of a peripheral polyneuropathy [(12); for review see (13)]. A well-studied example of γ-diketone-induced toxicity occurred among workers in an Ohio fabric printing plant in 1973. Shortly after substituting methyl *n*-butyl ketone as a cleaning agent, a sudden outbreak of polyneuropathy developed among the fabric printing workers who were repeatedly exposed to the solvent via inhalation and skin contact. The distribution of nerve involvement, severity of the deficits, and temporal course correlated with the amount of exposure (14). Sensory symptoms and signs predominated in mild cases; however, both motor and sensory deficits occurred in severe cases. Although the distribution was characteristically distal, proximal involvement occurred in severe cases. Onset was gradual without prominent systemic symptoms and neurological involvement was symmetrical. The symptoms progressed for several months after cessation of exposure and recovery was slow.

These clinical characteristics of 2,5-HD-induced neurotoxicity are most compatible with an axonopathy affecting primarily long fibers. The histopathological manifestations of *n*-hexane polyneuropathy include distal axonal swellings filled with neurofilaments proximal to the nodes of Ranvier. This injury is sometimes followed by Wallerian-like degeneration of the axon distal to the swelling, resulting in muscle atrophy. 2,5-HD-induced neurofilament cross-linking has been proposed as the molecular mechanism of this neurotoxicity (15) based on the chemical reactivity of 2,5-HD and histopathological manifestations, although alternative models have been suggested (16–21).

Initial animal studies demonstrated a close correlation between the blood levels of 2,5-HD, the ultimate toxicant, and neurological symptoms (22). In addition, these initial animal studies identified the testis as a target organ of exposure to *n*-hexane and its metabolites. 2,5-HD targets Sertoli cells, resulting in germ cell apoptosis and testicular atrophy (23, 24). The assignment of the Sertoli cell as the target cell for testicular injury is based on the early histopathological alterations in this cell following exposure (described below). To appreciate the testicular injury, one must understand Sertoli cell biology and the dependence of germ cells on Sertoli cells for structural and trophic support.

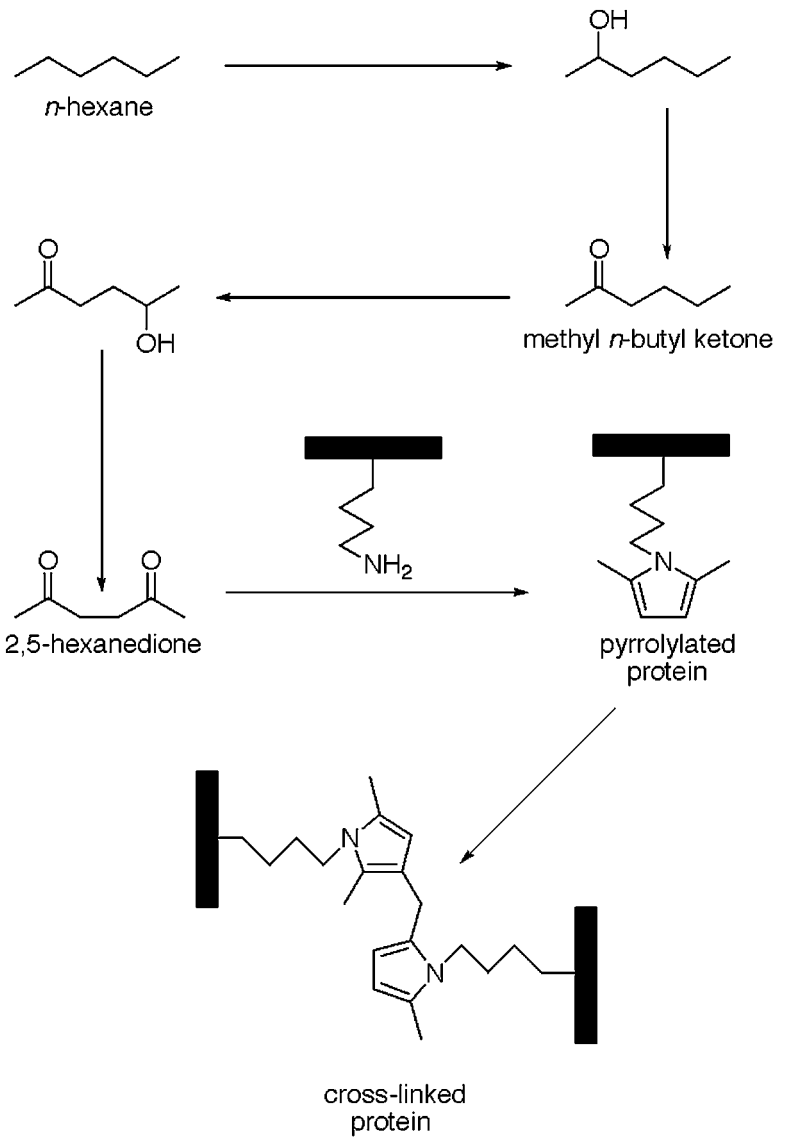

Figure 1 The common solvents *n*-hexane and methyl *n*-butyl methane are converted by ω-1 hydroxylation and oxidation to the ultimate toxicant, 2,5-hexanedione (2,5-HD). 2,5-HD reacts with lysyl ε-amines of proteins (*black rectangle*) to form pyrrolylated proteins, which undergo intra- and intermolecular cross-linking reactions, including dimer formation.

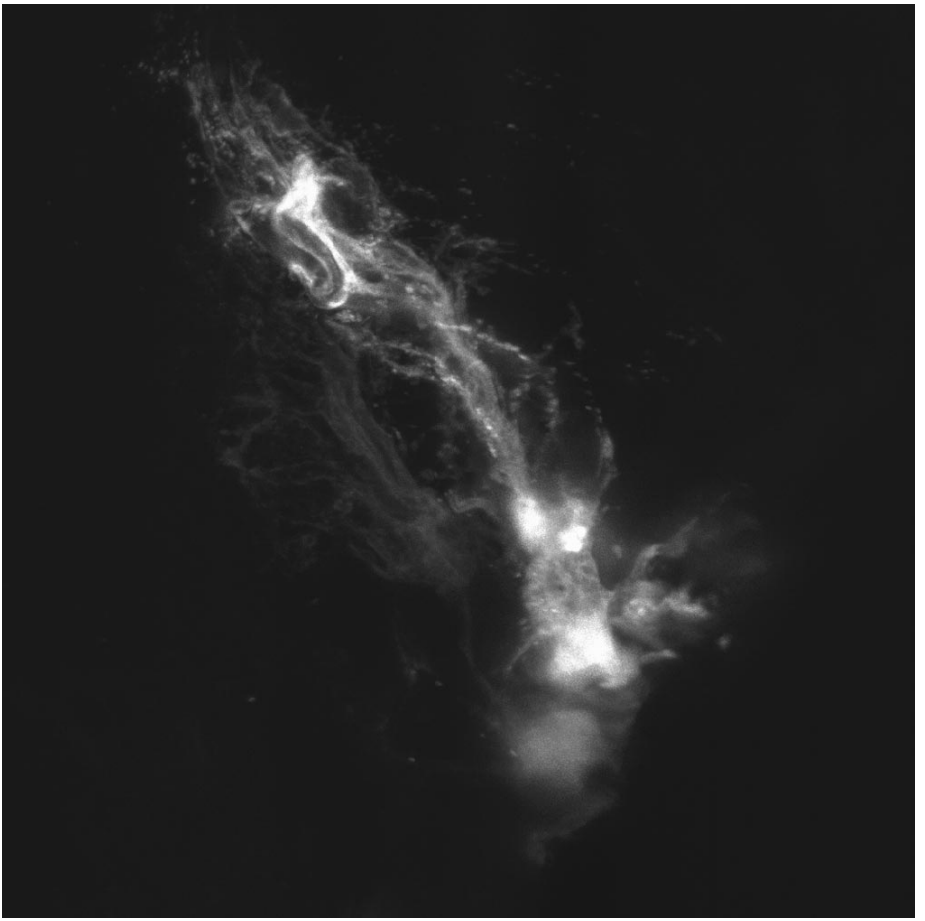

Figure 2 The Sertoli cell extends from the base of the seminiferous tubule (*bottom right*) to the lumen (*upper left*) and has numerous cytoplasmic projections that surround and support germ cells. In this maximum intensity projection of 13 series confocal images, a single Sertoli cell in an intact seminiferous tubule is visible because of green fluorescent protein expression following adenovirus infection. The Sertoli cell nucleus is visible in the basal cytoplasm surrounded by bright fluorescence. The overall length of this Sertoli is approximately 100 μm.

The Sertoli cell, a highly elongated cell type, is found in seminiferous tubules with its base adhering to the basal lamina and its apex extending to the lumen (Figure 2; see also Figure 4). These cells form numerous lateral cytoplasmic processes that surround developing germ cells. Sertoli cells contain a well-developed cytoskeletal network composed of actin filaments, intermediate filaments, and microtubules. Adjacent Sertoli cells are bound together by tight junctions, forming the blood-testis barrier. This barrier, which separates the seminiferous tubule into

a basal and an adluminal compartment, is important in protecting the advanced germ cells from blood-born products, including immune cells. The blood-testis barrier allows Sertoli cells to create the necessary microenvironment for germ cell development by secreting a seminiferous tubule fluid that contains nutrients and proteins, including transport and binding proteins, proteases, antiproteases, and growth factors that regulate proliferation and differentiation. Sertoli cells have many fundamental roles in spermatogenesis; thus, their dysfunction would result in disruption of the normal process of spermatogenesis.

The most commonly used animal model for studying 2,5-HD-induced testicular injury has involved exposure of rats to 2,5-HD as a 1% drinking water solution for three to five weeks (24, 25). Beginning two weeks after exposure, assembly of purified testis tubulin was altered followed by a decrease in seminiferous tubule fluid formation by three weeks (26, 27). Large, basally located Sertoli cell vacuoles in stages I, XII, XIII, and XIV were the first histopathological signs of cellular injury at all doses, followed by sloughing and loss of germ cells by four to five weeks (24, 28). The cycle of the seminiferous epithelium was altered with a decreased prevalence of certain stages and a concomitant increase in other stages (24, 29). The seminiferous tubules were devoid of differentiating germ cells by 8–12 weeks after a 3–5 week 2,5-HD exposure but contained proliferating spermatogonia (28). This "irreversible" atrophic state continued for greater than 70 weeks after exposure in the rat (30).

In the rat, testicular atrophy associated with 2,5-HD exposure can occur at cumulative exposure levels below those that produce clinical neurotoxicity. High-level exposure for relatively brief periods produced testicular injury without clinical evidence of distal polyneuropathy (28), whereas chronic low-level exposure produced clinical evidence of distal polyneuropathy without testicular injury (31). In fact, the testicular injury was dose-rate sensitive, whereas the extent of nervous system toxicity was related to the total dose over a range of dose-rates (22, 32). These tissue-selective pharmacokinetic effects may, in part, explain the predominance of neurotoxicity in human exposures to 2,5-HD precursors. In addition, the clinical manifestations of neurotoxicity are obvious, whereas those of testicular injury are subtle.

THE TUBULIN HYPOTHESIS

Because of the selective injury to the axon by γ-diketones and the known importance of microtubules to axonal structure and function, this cytoskeletal element was considered as a molecular target for γ-diketone neurotoxicity. Because the Sertoli cell has axon-like characteristics (33), tubulin was considered an attractive molecular target in testicular injury as well.

The core proteins of the microtubule are α- and β-tubulin, which combine to form a 100-kDa heterodimer. Tubulin heterodimers join end-to-end to form protofilament chains that, through lateral interactions, wrap into a tube-like structure—the microtubule. Because tubulin dimers are oriented within protofilaments,

microtubules are polar structures with fast-growing (+) ends and slow-growing (−) ends. The assembly of microtubules from soluble tubulin heterodimers consists of three main phases: (*a*) a slow nucleation phase in which tubulin subunits are organized into seeds for microtubule growth, (*b*) a fast elongation phase during which tubulin subunits are readily added to the growing protofilament chains, and (*c*) a steady state phase during which the addition and removal of tubulin subunits is in equilibrium and the aggregate microtubule length remains constant [reviewed in (34–37)] (Figure 3). Many factors are known to influence the kinetics of assembly and the maintenance of microtubules, including temperature, solvent conditions, GTP concentration, and microtubule-associated proteins (MAPs). MAPs serve many functions, including nucleation of assembly and stabilization of the

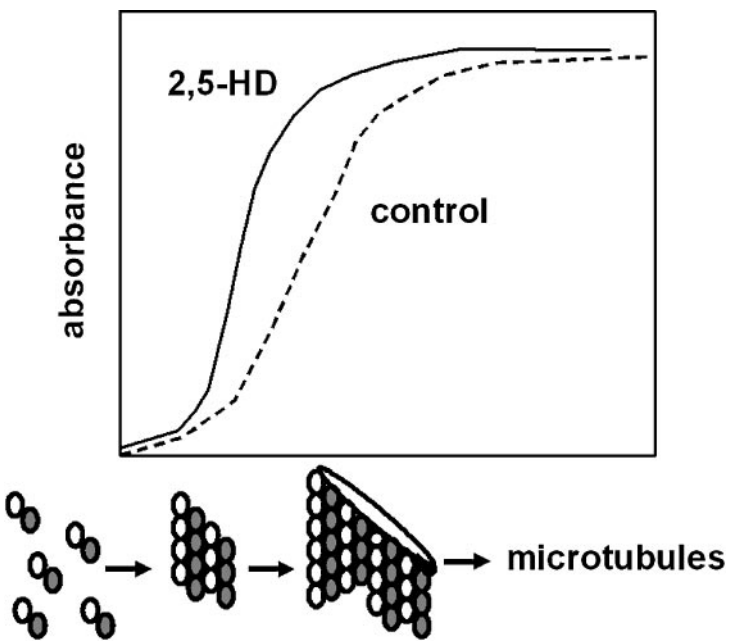

Figure 3 A typical microtubule assembly reaction is initiated by warming a solution of ice-cold tubulin dimers to 37°C in the presence of GTP. Tubulin dimers (*adjacent white and gray circles*) slowly form nucleating seeds (*heptameric tubulin aggregate*), which catalyze a rapid phase of microtubule elongation (*growing microtubule*) enroute to a steady state condition of microtubule formation and destruction. The assembly reaction is monitored by measuring the change in absorbance at 350 nm. In vitro incubation of microtubules with 2,5-HD or in vivo exposure of animals to 2,5-HD followed by tubulin purification yields pyrrolylated tubulin with altered assembly behavior. 2,5-HD-modified tubulin quickly forms numerous seeds, resulting in more rapid assembly into greater numbers of shorter microtubules compared to the control.

microtubule. Presumably, these modifying factors provide a means by which a cell can coordinate microtubule-mediated events in response to different environmental cues and stimuli.

2,5-HD did not alter the proportion of tubulin that polymerized into microtubules when chronically administered to rats (38) or when added to in vitro assembly assays without preincubation (39). However, 2,5-HD exposure did have a dramatic effect on the kinetics of microtubule assembly (25). A detailed evaluation of the 2,5-HD-induced alterations in microtubule assembly behavior followed this initial observation. Brain and testis tubulin isolated from 2,5-HD-exposed rats had a shortened nucleation phase, a more rapid rate of elongation, and contained high-molecular-weight bands on denaturing polyacrylamide gel electrophoresis (SDS-PAGE) that consisted of cross-linked tubulin (25). Tubulin isolated from exposed rats displayed a high level of pyrrole adducts as compared to controls (26). This altered tubulin had remarkable "pro-assembly" characteristics, readily forming microtubules in the presence of calcium and at low temperatures. When rats were exposed to the same total dose of 2,5-HD in the drinking water at dose-rates varying from two to six mmol/kg/day, the increased dose-rate was associated with progressively more severe histopathological alterations in the testis. In concert with these progressive histopathological alterations, the kinetics of microtubules assembled from testicular tubulin purified from the exposed animals were altered. These data positively correlated the extent of histopathological injury with the extent of microtubule assembly abnormality.

These initial observations of 2,5-HD-induced alterations in microtubule assembly led to the articulation of a tubulin-based hypothesis for 2,5-HD-induced testicular injury as follows: "[1] intoxication with 2,5-HD alters microtubule assembly kinetics, [2] altered assembly produces changes in the number and length of Sertoli cell microtubules which compromises Sertoli cell function, and [3] malfunctioning, nonsupportive Sertoli cells disrupt germ cell maturation resulting in testicular atrophy" (25).

The results using tubulin purified from treated animals were confirmed and extended with microtubules treated in vitro with 2,5-HD (40). In vitro incubation with high concentrations of 2,5-HD generated a markedly altered tubulin that could assemble in the absence of added GTP, could readily nucleate the assembly of control tubulin, and was resistant to cold-induced disassembly. The induction of these 2,5-HD-induced assembly alterations required that the incubation take place with assembled microtubules. Negative-stain electron microscopy showed that 2,5-HD incubation followed by assembly led to shorter microtubules than control assemblies, a result explained by the treatment-related induction of numerous nucleating seeds.

Sioussat & Boekelheide (41) explored the biochemical nature of the 2,5-HD effect on tubulin in greater detail. Isolated bovine brain tubulin was treated with 100 mM 2,5-HD for 16 h at 37°C and subjected to three cycles of microtubule assembly with the assembly temperature of each cycle progressively lower, making microtubule assembly progressively restrictive. This procedure effectively concentrated a component of 2,5-HD-treated tubulin with strong nucleating features.

This highly nucleating tubulin preparation had (*a*) a lowered rate of tubulin dissociation from the microtubule polymer, (*b*) a 19-fold decrease in the critical concentration for assembly (the concentration of tubulin required for nucleation to occur), and (*c*) the ability to copolymerize with and seed untreated tubulin assembly at concentrations below that typical for spontaneous nucleation in vitro (39).

The reaction between 2,5-HD and assembled tubulin produced numerous derivatized and cross-linked products visualized as monomers, cross-linked dimers, and higher multimers on SDS-PAGE. The progressively stringent cycling that concentrated the nucleating elements did not change the pattern of bands by SDS-PAGE, indicating that dimers and high multimers were not the nucleating factor. By using limited proteolysis and immunoblotting, the native conformation of the tubulin subunits was probed. Concentration of the 2,5-HD-induced nucleating element by stringent cycling resulted in an altered α-tubulin with a more open structure susceptible to selective tryptic and chymotryptic digestion. With these findings, a biochemical explanation for the assembly alterations was formed; namely, that 2,5-HD treatment of assembled microtubules fixes the tubulin heterodimer into a pro-assembly conformation through a specific intramolecular modification of the α-subunit (41).

The ability of 2,5-HD-modified tubulin generated in vitro to alter microtubule function in an in vivo model system was verified using sea urchin zygotes. Microinjection of 2,5-HD-treated tubulin into normal sea urchin zygotes before the first mitotic cycle caused obvious abnormalities, including small spindles, abnormal chromosomal movement at anaphase, and poor cytokinesis. Depending on the protocol used, mitosis was either grossly disrupted or simply slowed (42).

Having identified microtubule function as an in vivo molecular alteration induced by 2,5-HD, the next phase of the investigation examined unique characteristics of microtubule-dependent activity in Sertoli cells, the testicular target for toxic injury, which were susceptible to disruption.

MICROTUBULE-DEPENDENT TRANSPORT IS A TARGET

In order to maintain the proper microenvironment within the seminiferous tubule, Sertoli cells actively secrete a seminiferous tubule fluid. The elongated structure of Sertoli cells and their ability to target products to different cohorts of germ cells imply a delivery system that is both dynamic and provides exquisite specificity.

Microtubule networks promote targeted secretion in a number of polarized cell types, including MDCK cells (43, 44) and Caco-2 cells (43). Sertoli cell microtubules are oriented parallel to the long axis of the cell and, like those of many polarized cells, arise from nucleation centers located in the apical aspect of the cell (45, 46), not from the basally located centrosome (47).

MAPs include families of motor proteins—kinesins and cytoplasmic dyneins—that use microtubules as a scaffold for transport [reviewed in (48)]. Kinesins and cytoplasmic dyneins convert energy derived from ATP cleavage into movement along the length of the microtubule, mediating the transport of molecules and

vesicles bound to them. Originally, kinesins and cytoplasmic dyneins were believed responsible for transport of cargo to opposite poles of the microtubule, with kinesins directing transport to the (+) end and cytoplasmic dyneins directing transport toward the (−) end; more recent information has made the picture more complex (48).

As predicted by its "minus-end-up" microtubule orientation and active secretion, Sertoli cells express the minus-end microtubule-dependent motor cytoplasmic dynein at a high level. Cytoplasmic dynein consists of a large complex of at least 10 proteins (33, 49). The dynein heavy chain isolated from testis shares features with other cytoplasmic dyneins: It binds microtubules tightly in the absence of ATP, releases from microtubules in the presence of ATP, exhibits microtubule-dependent ATPase activity, is sensitive to inhibitors in a similar manner to that of other dynein species, and is sensitive to vanadate-mediated photocleavage (33). Localization of testicular cytoplasmic dynein to Sertoli cells has been demonstrated by immunofluorescence. Throughout development, and in all stages of spermatogenesis, dynein is observed in a diffuse, granular pattern throughout the Sertoli cell cytoplasm, consistent with its proposed role as a cytoplasmic transport molecule (50, 51). During stages IX–XIV, a more intense Sertoli cell pattern is observed, as well as an intense pattern associated with ectoplasmic specialization regions of step 9 and 10 spermatids (50). Sertoli cell ectoplasmic specializations have been proposed to have a number of roles. They bind microtubule networks and are thought to be involved in spermatid head shaping (50, 52) and in positioning and translocating spermatids in the seminiferous epithelium (46). Ectoplasmic specializations are also thought to be the site at which some Sertoli cell secretory products accumulate (50). This notion was supported by the observation that microtubule-associated cisternae of endoplasmic reticulum coassociate with the Sertoli cell membrane at this point via a thick, hexagonal array of actin filaments (52). Recent work has verified the association of microtubule motors with ectoplasmic specializations in support of the hypothesis that these motors are responsible for the elongate spermatid movements that occur during germ cell maturation (46, 53–55).

Kinesin participates in vesicle transport in a number of cell types, including fast axonal transport in neurons (56). In Sertoli cells, kinesin has been observed to localize to the *trans* Golgi network, a location suggesting involvement in membrane trafficking within the cell (57). In addition, kinesin is localized to ectoplasmic specializations where it may be involved in the movement and positioning of elongate spermatids within the seminiferous epithelium (55).

Alterations in the distribution of microtubule-associated motor proteins occurred progressively with 2,5-HD exposure. The pattern of Sertoli cell cytoplasmic dynein staining changed from an intense signal associated with ectoplasmic specializations of elongate spermatids to a diffuse cytoplasmic signal (58). 2,5-HD treatment had similar effects on Sertoli cell kinesin distribution, which was tightly associated with the highly organized Golgi network and became more diffuse with treatment-induced Golgi disruption (58). Microtubules treated in vitro with 2,5-HD exhibited functional differences in their ability to support microtubule-dependent transport. Microtubules treated with either 2,5-HD or glutaraldehyde

(a generalized protein cross-linker) exhibited diminished rates of kinesin-based transport in vitro compared with untreated microtubules. In comparison, tubulin treated with acetyl 2,5-HD, a noncross-linking pyrrole-forming agent, supported rates of kinesin-based transport similar to those of untreated microtubules (59). This suggests that 2,5-HD treatment itself is capable of altering microtubule-based transport and that it is the cross-linking activity of the 2,5-HD that promotes those changes (59).

Synthesizing the observed effects on microtubule assembly in vivo and microtubule-dependent transport in vitro led to the hypothesis that disruption of microtubule-dependent vesicle transport from the endoplasmic reticulum to the plasma membrane underlies the seminiferous tubule fluid alterations observed following 2,5-HD exposure (60) (Figure 4). Microtubule-dependent vesicle transport involves the movement of secretory vesicles from the endoplasmic reticulum to the plasma membrane, and these vesicles have been demonstrated to associate with microtubules in polarized cells (61, 62). However, direct assessment of the effect of microtubule disruption on vesicle movement and seminiferous tubule fluid formation is difficult. Measurement of seminiferous tubule fluid production in isolated tubules, however, has revealed that the classic microtubule disrupter, colchicine, as well as the inhibitor of intracellular membrane transport, brefeldin A, cause reduced rates of fluid production (27). Similarly, seminiferous tubules isolated from rats exposed to 2,5-HD for three or four weeks exhibited reduced rates of seminiferous tubule fluid formation compared to those isolated from untreated rats (27). Importantly, the deficit in seminiferous tubule fluid formation preceded the histopathological alterations in germ cells, indicating an etiologic relationship. The mechanism by which this deficit is translated into germ cell loss is considered in the next section.

APOPTOSIS, STEM CELL FACTOR/C-KIT, AND IRREVERSIBLE INJURY

Although the Sertoli cell is the target of 2,5-HD injury, the end result is a depletion of germ cells. This raises several questions: How does Sertoli cell dysfunction translate into germ cell loss? Is the atrophy the result of the loss of a supportive factor normally produced by the Sertoli cell or is it the consequence of a death signal produced by the Sertoli cell? Is the depletion due to a decrease in stem cell commitment to the differentiating germ cell pool or increased germ cell death? In this section, we consider germ cell apoptosis, growth factors [specifically stem cell factor (SCF)], and the "irreversible" nature of the germ cell loss after 2,5-HD exposure.

Apoptosis

Apoptosis, a controlled and highly ordered cell death, occurs during spermatogenesis to regulate germ cell production (63). In the rat, an important control point

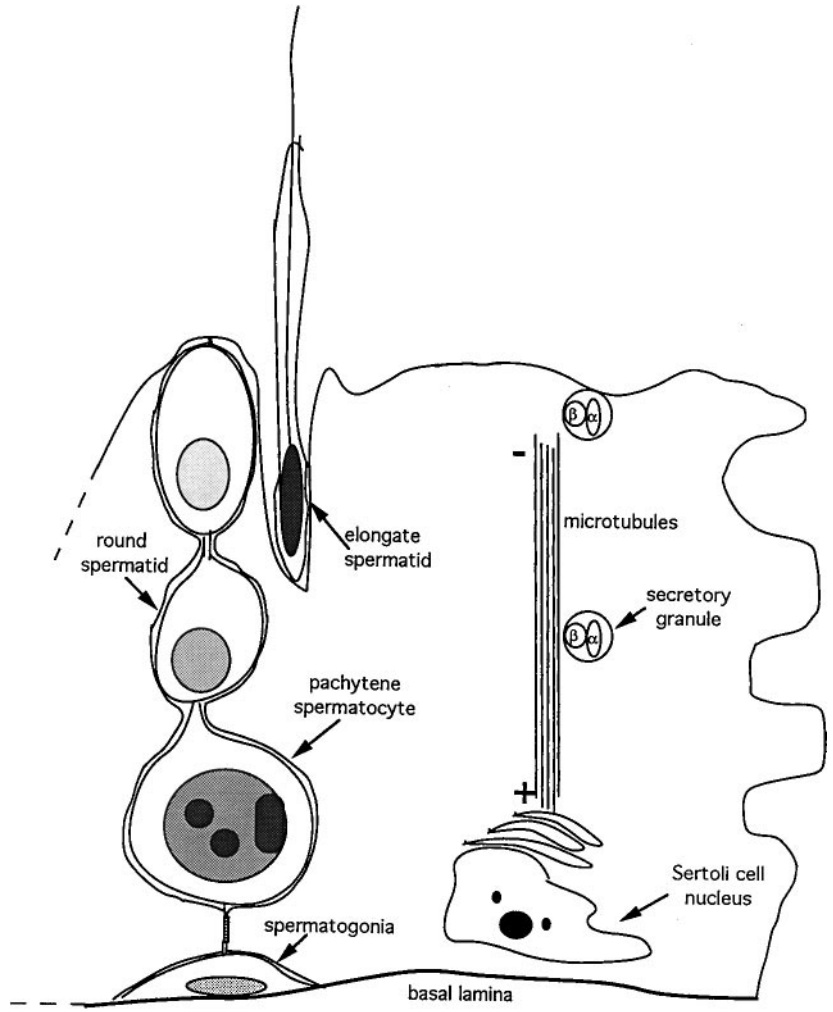

Figure 4 The Sertoli cell provides trophic factors to various germ cells (spermatogonia, pachytene spermatocyte, round spermatid, and elongate spermatid) by secreting a seminiferous tubule fluid. Microtubule-dependent transport facilitates this supportive role, using microtubule motors to move vesicles along the abundant, radially oriented, Sertoli cell cytoplasmic microtubules. In this model, a secretory granule is moving toward the microtubule (−) end, translocating from the perinuclear Golgi apparatus to the seminiferous tubule lumen. Cytoplasmic dynein, an abundant Sertoli cell protein (33), is the presumptive motor to catalyze this basal-to-lumenal microtubule-dependent transport.

involves type A_{2-4} spermatogonia; a large portion of these germ cells normally undergo apoptosis (64, 65). Germ cell apoptosis also can be upregulated massively in response to hormonal or cytokine deprivation or in response to injuries, such as heat, radiation, or toxicant exposure (66–69). Initiators of apoptosis include intracellular "stress sensors" or external signals, which trigger apoptosis via ligand binding to cell surface receptors. The Fas system is a paracrine pro-apoptotic signaling pathway; in the testis, Fas ligand is expressed by Sertoli cells and induces apoptosis in germ cells expressing Fas receptor (70–73). p53 is a transcription factor with an important pro-apoptotic role in the testis that modulates the expression of bcl-2 family members, the Fas receptor, and other elements of the apoptotic machinery (74, 75).

The apoptotic signal, whether intracellular or extracellular in origin, results in activation of a caspase cascade that disassembles the cell (76–78). This controlled destruction includes the cleavage of cytoskeletal proteins, changes in the lipid membrane, and DNA fragmentation evident as a DNA ladder by gel electrophoresis. Blanchard et al. (79) established that the mechanism of germ cell death in response to 2,5-HD exposure is indeed apoptosis. Using gel electrophoresis to visualize DNA fragmentation, ladders were most prominent five weeks into the toxicant exposure, and in situ DNA end-labeling showed an increase in germ cell apoptosis as early as two weeks into treatment.

How the apoptotic system is activated depends on the mechanism of injury and how the cells sense the injury. Because the Sertoli cell is the target of 2,5-HD-induced injury, germ cell death is secondary to Sertoli cell dysfunction and could be the result of a paracrine death signal initiated by the Sertoli cell or the lack of a survival or proliferation factor produced by the Sertoli cell. The possibility that the Sertoli cell actively signals germ cell death is supported by the concomitant increases in Fas ligand and Fas mRNA expression with the onset of germ cell apoptosis (71, 80). Mutant mice lacking functional Fas ligand have less germ cell apoptosis than wild-type controls following exposure to the model Sertoli cell toxicant, mono-(2-ethylhexyl)phthalate (81). An intellectually satisfying model is one in which Sertoli cells send a death signal to the population of dependent germ cells they can no longer support, thereby explaining the increased Fas system mRNA.

Stem Cell Factor/c-Kit

Whatever the signals are that trigger germ cell apoptosis, the initiating event could be a deficiency of a survival factor due to failure of the normal Sertoli cell microtubule-dependent formation of seminiferous tubule fluid. One important survival factor made by Sertoli cells and required by germ cells is stem cell factor (SCF) (82).

SCF is a ligand that is expressed on Sertoli cells. A proteolytic cleavage site within exon 6 results in two isoforms of SCF, a soluble isoform (sSCF) and a membrane-bound isoform (mSCF). c-Kit, the SCF receptor, is a 145-kD transmembrane tyrosine kinase expressed on germ cells and Leydig cells, the

testosterone-producing cells in the testicular interstitium. Structurally, c-kit belongs to the platelet-derived growth factor receptor superfamily (83, 84).

Two well-studied mutants of SCF and c-kit are Steel factor (Sl) and dominant white spotting (W) mice, respectively. Sl and W mice are deficient in melanogenesis, erythropoiesis, and gametogenesis (85). During embryogenesis, SCF and c-kit are essential for maintenance and proliferation of primordial germ cells (86, 87). Fertility is restored upon transplantation of W mice with Sl or wild-type germ cells, but not by transplantation of W germ cells into Sl mice (88). In another genetic complementation experiment, injection of mSCF-expressing replication-deficient adenovirus into the seminiferous tubule lumens of Sl mice resulted in Sertoli cell infection and restoration of spermatogenesis (89). Immunolocalization with an anti-c-kit antibody showed expression on type A, In, and B spermatogonia along with Leydig cells and preleptotene spermatocytes. Use of an anti-c-kit antibody to block SCF binding in vivo led to a loss of type $A_{2–4}$ spermatogonia (90). These data implicate binding of Sertoli cell-derived SCF to germ cell c-kit as an important regulatory mechanism controlling survival of spermatogonia.

The seminiferous tubule atrophy induced by 2,5-HD treatment persists long after the exposure has ended (30). Spermatogonia were present in reduced (91), albeit relatively constant, numbers following toxicant-induced injury (28). Interestingly, the remaining spermatogonia were actively proliferative but failed to repopulate the atrophic testis (92). Spermatogonial modeling in 2,5-HD-treated rats with irreversible injury has shown a large increase in apoptosis of type A_3 and A_4 spermatogonia (92). As discussed above, this block in spermatogenesis at type $A_{3–4}$ spermatogonia correlates with the germ cell developmental stage requiring SCF/c-kit activity for survival.

Because 2,5-HD is a Sertoli cell toxicant and SCF is expressed by Sertoli cells, alterations in its function after toxicant exposure could contribute to the persistence of testicular atrophy. Interestingly, SCF was present in testis after exposure; however, the ratio of sSCF to mSCF was altered (91). Normally in adult rats, twofold more mSCF is expressed than sSCF. Exposure to 2,5-HD led to a preferential expression of sSCF, which correlated temporally with the onset of atrophy (91). Reversal of the atrophic state with GnRH agonist therapy (see below) resulted in a significant increase in the proportion of mSCF that was expressed, returning the expression pattern toward normal (93). In a pharmacologic test, exogenous SCF was administered to the atrophic testes of 2,5-HD-treated rats. This therapy was associated with an increase in the percentage of seminiferous tubules with large clones of proliferating germ cells (91), suggesting that manipulations of the in vivo level of SCF could affect proliferation and survival of type $A_{2–4}$ spermatogonia in 2,5-HD-induced irreversible injury.

The spontaneous mouse mutant called Steel-Dickie (Sl^d), which expresses only the soluble form of SCF, provides a molecular model of deficient mSCF expression. In the Sl^d mutant, a 4-kb intragenic deletion of the SCF sequence leads to expression of only sSCF, resulting in anemia, white coat color, and infertility (94, 95). This mutant has normal migration of primordial germ cells to the genital ridge but fails to maintain normal spermatogenesis. In vitro, germ cells exhibit a

diminished capacity to bind Sl^d Sertoli cells, a defect corrected by expression of recombinant mSCF by Sertoli cells (96). Notably, the Sl^d mouse mutant and the 2,5-HD-injured atrophic rat testis share in common a deficiency of mSCF expression and a failure of the seminiferous epithelium to support germ cell development beyond spermatogonia.

The apoptotic machinery involved in cell death following SCF deprivation has not been well characterized. However, germ cell apoptosis in c-kit-deficient mice has been shown to be p53 dependent—that is, germ cell apoptosis in W mice is reduced if functional p53 is missing (97). Additionally, decreases in bax and other pro-apoptotic bcl-2 family members and increases in anti-apoptotic bcl-2 family members have been demonstrated in response to SCF (98).

Irreversible Injury

The reason for the persistence of 2,5-HD-induced testicular atrophy is unknown. Both the accumulation of testicular pyrroles and the alteration in testicular microtubule assembly that occur during treatment with 2,5-HD return to baseline levels soon after exposure ends (26).

Treatment with a gonadotropin releasing hormone (GnRH) agonist can successfully reverse the "irreversible" 2,5-HD-induced testicular atrophy. In rats treated with 2,5-HD for 23 days and given depot GnRH agonist therapy for 10 weeks immediately after toxicant exposure, greater than 90% of seminiferous tubule cross-sections showed signs of germ cell repopulation, with approximately 80% of the seminiferous tubules containing mature spermatids. In contrast, toxicant-treated controls repopulated only 1% of their seminiferous tubules (93). A similar reversal of testicular atrophy has been observed following treatment of testis-irradiated LBNF1 rats with GnRH agonists, GnRH antagonists, or testosterone (99, 100). Testicular atrophy induced by the chemotherapeutic agent procarbazine is also reversed by GnRH agonist therapy (101).

The ability of GnRH agonist therapy to stimulate spermatogenesis in atrophic testes is apparently mediated by a suppression of intratesticular testosterone levels (102). It is widely accepted that spermatogenesis is dependent upon gonadotropin support, mediated by complex feedback through the hypothalamic-pituitary-gonadal axis, and that withdrawal of testosterone and FSH results in a stage-specific loss of germ cells. However, the ability of testosterone-suppressing therapies to stimulate spermatogenesis in the atrophic testis suggests that testosterone may be inhibitory to the differentiation of type A spermatogonial cells in cases of testicular atrophy, perhaps by enhancing apoptosis among spermatogonial cells. Although the evidence supporting the involvement of intratesticular testosterone suppression in the reversal of testicular atrophy is quite compelling (103), ablation of Leydig cells in atrophic testes from 2,5-HD-treated rats failed to stimulate a recovery of spermatogenesis. Acute exposure to ethane dimethane sulphonate (EDS) suppressed intratesticular testosterone levels, yet regardless of whether EDS was administered alone or in combination with GnRH agonist therapy, failed to reverse

2,5-HD-induced testicular atrophy (104). These results suggest that Leydig cell factors, in addition to a lowered intratesticular testosterone level, may be important to the reversal of 2,5-HD-induced testicular atrophy.

FUTURE DIRECTIONS

Considerable effort has been applied to investigating 2,5-HD-induced testicular injury, both to enhance our understanding of pathways susceptible to disruption and to identify those complex mechanisms that govern spermatogenesis. As described in this review, the proposed pathogenic sequence for 2,5-HD-induced testicular injury is as follows: 2,5-HD-induced cross-linking of tubulin leads to altered microtubule assembly, which results in altered microtubule-dependent transport; in Sertoli cells, this altered microtubule-dependent transport is manifested as decreased seminiferous tubule fluid formation and a failure to provide adequate support to germ cells causing them to undergo apoptosis. In developing this mechanistic hypothesis, many unanswered issues have been raised. A brief listing of some of these issues follows in the hope that their articulation will pique the interest that they deserve:

- Additional structural studies could further elucidate the biochemical basis of the 2,5-HD-induced alteration in microtubule assembly, but progress in this area is limited by the poorly understood chemistry of γ-diketone adducts and cross-links.
- The underlying basis for the selective nervous system and testicular injury resulting from 2,5-HD exposure is unknown, but this pattern of toxicity suggests either a similar molecular target or a shared architectural vulnerability in the two tissues.
- Although indirect evidence supports the assertion that 2,5-HD alters Sertoli cell microtubule-dependent transport and inhibits seminiferous tubule fluid formation, the molecular connections between these processes remain to be elucidated.
- Because deficiencies in the p53 and Fas system pathways protect against germ cell loss, effective pharmaceuticals resulting from a better molecular understanding of the apoptotic machinery could ameliorate the consequences of toxicant-induced testicular injury.

In a sense, the very general chemical reactivity of 2,5-HD is both its greatest strength and greatest weakness as an investigative tool. As a blunt instrument, 2,5-HD has been useful for identifying microtubule-dependent transport as a vulnerable, failure-prone pathway within the Sertoli cell, but the lack of specificity in its reactivity has made it difficult to progress from correlation to causation. As described below, future experiments using molecular techniques are being designed to specifically disrupt Sertoli cell microtubule-dependent processes and examine subsequent germ cell effects.

Historically, insight into the role of Sertoli cell microtubules in promoting spermatogenesis has been gleaned from analyzing the actions of known microtubule disrupters in the testis. Both colchicine and taxol lead to defects in spermiation and residual body elimination, activities attributed to Sertoli cells (105–107). Because microtubules are present in all cells, however, these studies cannot rule out potential actions on other cellular targets, particularly on the germ cells themselves.

A true test of this model requires selective access to Sertoli cell microtubule networks in vivo, independent of direct effects on germ cells. Such a mechanism precludes toxicant or pharmacologic manipulations of microtubules, which exert effects globally. Cell-type specificity can only be afforded by molecular interventions that are capable of being either delivered or expressed in subsets of cells.

To date, few cell-specific interventions have been devised. Progress has been made with tissue-specific transgenics, which allow for expression of proteins in subsets of cells (108); however, these constructs are difficult to make and validate. Moreover, effects of ectopic gene expression on spermatogenesis in adult animals may not be easily separable from developmental effects, unless expression is tightly controlled.

Recent success has been achieved with the use of adenoviral vectors in the testis (22, 109–111). Adenoviral vectors are capable of expressing high-levels of transgene and may be used both in atrophic testes and in testes with intact spermatogenesis (22, 109–111). They have been demonstrated to be effective therapeutic agents to correct defective Sertoli gene products, resulting in partial restoration of spermatogenesis (22). Their utility in targeting adult Sertoli cells in testes with intact spermatogenesis makes them particularly useful for studying Sertoli–germ cell interactions in adult animals.

To address the question of whether selective disruption of Sertoli cell microtubule networks could impair spermatogenesis, it would be necessary to deliver a gene product that was capable of promoting microtubule polymerization or stabilization to Sertoli cells in intact, adult testes. γ-Tubulin, a microtubule nucleating protein localized to the centrosome of dividing cells, has been observed to disrupt microtubule networks and leads to mitotic arrest when overexpressed in mitotically active cells (112–115). Similar to 2,5-HD or taxol treatment of tubulin, γ-tubulin promotes microtubule assembly by serving as a seed or template for microtubule elongation (116–118). Delivery of an adenoviral vector that overexpressed γ-tubulin would be expected to perturb the Sertoli cell microtubule network similar to treatment with 2,5-HD, without direct effects on germ cells, which do not express adenoviral transgenes (109). Indeed, overexpression of γ-tubulin was observed to alter the distribution of tubulin immunoreactivity in infected Sertoli cells (data not shown) and to inhibit spermatid release and residual body processing (Figure 5), processes presumed to be dependent upon normal microtubule function.

This model also has the potential to address more fundamental questions of Sertoli cell microtubule organization and Sertoli–germ cell interdependence. The mechanism of microtubule nucleation in any polarized epithelial cell is presently

unknown. The apical orientation of the microtubule organizing center of Sertoli cells has been well documented (47); however, no information is available about the underlying structures that determine the distribution of microtubules. That Sertoli cell microtubule networks are important for spermatogenesis is suggested by their highly dynamic nature throughout the seminiferous epithelial cycle and their close apposition to associated germ cells (107, 119). This implies that a precise mechanism of microtubule organization is in place to facilitate restructuring of the seminiferous epithelium by accommodating changes in germ cell position and shape.

Of perhaps greater importance for understanding the nature of the Sertoli–germ cell interaction is determining the degree to which germ cells depend upon Sertoli cell secretory products. Given the elongated structure of the Sertoli cell and the elaborate architecture of its cytoplasmic associations with germ cells, it is likely that microtubule disruption would impair timely delivery of secretory products to their appropriate germ cell destinations. Inhibition of Sertoli cell secretory pathways would provide insight about whether germ cells could undergo normal differentiation in the absence of a functional Sertoli cell secretory system, and would provide insight into the roles that Sertoli cells perform in maintaining spermatogenesis.

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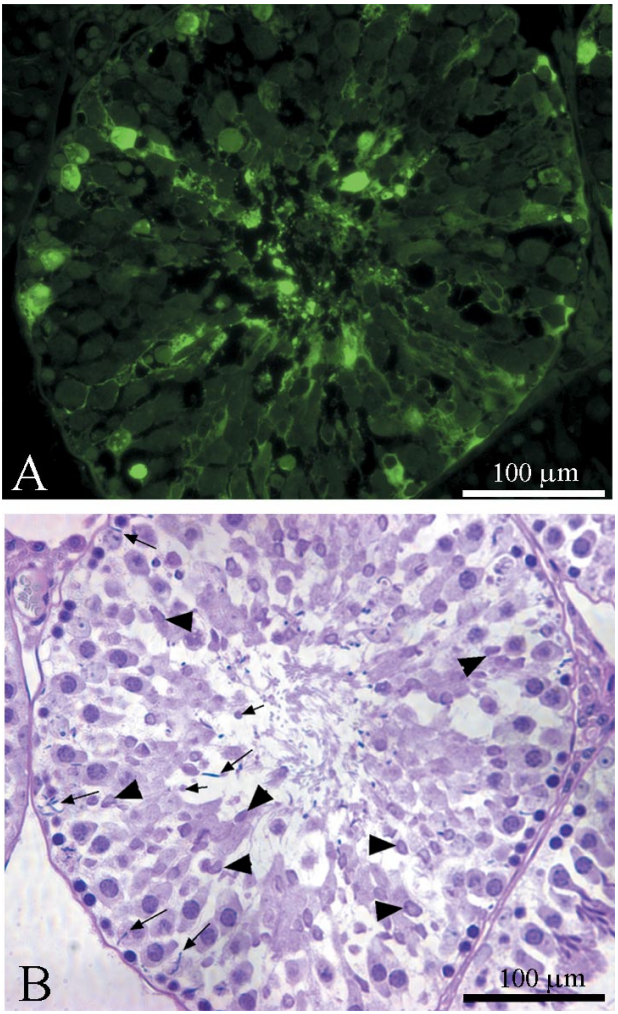

Figure 5 γ-tubulin overexpression in Sertoli cells in vivo leads to disruption of spermatogenesis. An adenovirus co-expressing γ-tubulin and green fluorescent protein was injected into the rete testis and back-perfused into seminiferous tubule lumens. After 72 h, the infected testis was embedded and serially sectioned. Many Sertoli cells in this seminiferous tubule cross section were infected and are expressing green fluorescent protein (*A*). Although structurally intact, the seminiferous epithelium is significantly disrupted with retention of advanced spermatids (*long arrows*) after the next generation of spermatids has begun elongating (*arrow heads*). Sertoli cells in infected tubules also failed to eliminate residual cytoplasm appropriately from elongate spermatids (*short arrows*). Staining (*B*) was performed with periodic acid Schiff's reagent and hematoxylin.