Diethylstilbestrol Induces Rat Spermatogenic Cell Apoptosis in Vivo through Increased Expression of Spermatogenic Cell Fas/FasL System*

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The significant role that estrogens play in spermatogenesis has opened up an exciting area of research in male reproductive biology. The realization that estrogens are essential for proper maintenance of spermatogenesis, as well as growing evidence pointing to the deleterious effects of estrogen-like chemicals on male reproductive health, has made it imperative to dissect the role estrogens play in the male. Using a model estrogen, diethylstilbestrol (DES), to induce spermatogenic cell apoptosis in vivo in the male rat, we provide a new insight into an estrogen-dependent regulation of the Fas-FasL system specifically in spermatogenic cells. We show a distinct increase in Fas-FasL expression in spermatogenic cells upon exposure to diethylstilbestrol. This increase is confined to the spermatid population, which correlates with increased apoptosis seen in the haploid cells. Testosterone supplementation is able to prevent DES-induced Fas-FasL up-regulation and apoptosis in the spermatogenic cells. DES-induced germ cell apoptosis does not occur in Fas-deficient lpr mice. One other important finding is that spermatogenic cells are type II cells, as the increase in Fas-FasL expression in the spermatogenic cells is followed by the cleavage of caspase-8 to its active form, following which Bax translocates to the mitochondria and precipitates the release of cytochrome c that is accompanied by a drop in mitochondrial potential. Subsequent to this, activation of caspase-9 occurs that in turn activates caspase-3 leading to the cleavage of poly(ADP-ribose) polymerase. Taken together, the data indicate that estrogen-like chemicals can precipitate apoptotic death in spermatogenic cells by increasing the expression of spermatogenic cell Fas-FasL, thus initiating apoptosis in the same lineage of cells through the activation of the apoptotic pathway chosen by type II cells.

The study of the role of estrogens in spermatogenesis has attracted significant interest because of the increasing awareness that estrogen is essential for spermatogenesis (1, 2), spermatogenic cells express estrogen receptors (ER)† 1(1–3), and estrogen-like chemicals present in the environment adversely affect male reproductive health (4). Such chemicals have the ability to affect gene expression and cellular function by binding to the hormone receptors (5) and have been implicated in the declining trend of male fertility and an increase in testicular cancers (4). Effects of endocrine disruptors (ED), a term used to describe agents that mimic hormones have been particularly well documented in wildlife populations (6). In the male animal, one of the most susceptible pathways that can be disrupted by EDs is the hypothalamic-pituitary-gonadal axis. This axis regulates spermatogenesis by controlling the circulating levels of luteinizing hormone and follicle-stimulating hormone through the feedback regulation of steroid hormones (4), and this feedback loop can be interrupted by EDs binding to ERs in the pituitary. Direct interference with spermatogenic cells is also possible because these cells are known to express ERs (7). Diethylstilbestrol (DES) is a stilbene estrogen that can bind to ERs in the pituitary and has been widely used as a model estrogen to study changes in male reproductive function in response to estrogens (8, 9). DES can mimic estrogen action by interfering with the functioning of the pituitary-gonadal axis, leading to the suppression of testosterone levels that result in increased spermatogenic cell apoptosis (4, 10). Therefore, DES-induced spermatogenic cell death is a suitable model to study the pathways involved in estrogen-induced spermatogenic cell apoptosis.

Regular apoptosis of spermatogenic cells is required to maintain proper testicular homeostasis, although increased cell death can result in defective spermatogenesis leading to infertility (11). As in other cells, male spermatogenic cells respond to external signals and to their internal milieu by activating intracellular signaling pathways that ultimately determine their fate. Signals that induce programmed cell death are known to initiate apoptotic pathways in spermatogenic cells involving members of the Bcl-2 family and also the Fas-FasL system (12). In FasL-induced spermatogenic cell death, it is generally accepted that FasL from Sertoli cells kill the spermatogenic cells by engaging the Fas receptors present on them. The Fas system of regulation in the testis is operative under various conditions of stress like ethanol injury (13), heat exposure (14), and cryptorchidism (15), where increases in Sertoli cell FasL expression have been described but no spermatogenic cell FasL expression was reported. However, recently, it has been shown that FasL is expressed in spermatogenic cells (16).

*This work was supported by grants to the National Institute of Immunology from the Department of Biotechnology, Government of India. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: ER, estrogen receptor; ED, endocrine disruptor; DES, diethylstilbestrol; Δψm, mitochondrial membrane potential; JC-1, 5,5′,6,6′-tetrachloro1′,1′,3,3′-tetrachlorobenzimidazolylcarbocyanide iodide; PI, propidium iodide; TdT, terminal deoxynucleotidyltransferase; TUNEL, terminal deoxynucleotidyltransferase enzyme-mediated dUTP nick end labeling; FITC, fluorescein isothiocyanate; GSH, reduced glutathione; GSSG, oxidized glutathione; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; PARP, poly(ADP-ribose) polymerase; DTT, dithiothreitol; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RT, reverse transcription; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)1-propanesulfonic acid.
To our knowledge, no study has so far shown the up-regulation of the Fas-FasL system specifically in the spermatogenic cells as a means of initiating cell death in response to any stimuli. Although the Fas-FasL system is reportedly important for spermatogenic cell apoptosis, a functional Bax protein is also known to be crucial, as expression of this protein helps initiate massive spermatogenic cell apoptosis at a critical time during testicular maturation (17).

Even though it is well established that estrogens induce spermatogenic cell apoptosis, the dissection of complete pathways leading to apoptotic death because of exposure to estrogens have not been worked out. With increasing data accumulating on the role of estrogens in spermatogenesis and the detrimental effects of environmental estrogens on the same process (4), it has become essential to investigate the mechanism of estrogen-induced apoptosis to understand spermatogenic disorders. Most of the studies addressing cellular apoptosis in the testis using different model systems have used total testicular tissue and not isolated spermatogenic cells to dissect pathways leading to cell death (13–15). Failure to use isolated spermatogenic cells might provide a completely different view, as the testis is a heterogeneous organ composed of multiple cell types. To circumvent the possibility of introducing variables contributed by other cell types, we have used a purified spermatogenic cell population that is devoid of mature testicular sperm. This study projects a new possibility that under conditions of estrogen exposure in vivo, increased expression of the spermatogenic cell Fas-FasL system may be responsible for initiating apoptotic death in the same lineage of cells by inducing translocation of Bax from the cytosol to the mitochondria, followed by the release of cytochrome c accompanied by a loss of mitochondrial membrane potential (Δψm), leading to the activation of caspase-9 and -3.

EXPERIMENTAL PROCEDURES

Materials—The Apoptosis Detection System was procured from Pro-mega (Madison, WI). Bicinchonin protein assay reagents A & B were purchased from Pierce. Antibodies and secondary antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) or PharMingen (San Diego, CA) and Jackson Immunoresearch (West Grove, PA). Caspase substrate kit was from Bio-Rad. For color development, VIP peroxidase substrate kit for Western blots and dianaminobenzidine kit for immunohistochemical staining were procured from Vector Laboratories Inc. (Burlingame, CA). Proteinase K and AmpliTaq Gold™ reverse transcriptase were from Roche Molecular Biochemicals GmbH (Mannheim, Germany). 5,6,6′-Tetrachloro1,3,3′-tetraethylbenzimidazolocarbocyanide iodide (JC-1), MitoTracker™ Red AM, and ATP determination kit were obtained from Molecular Probes (Eugene, OR). TRIzol reagent was from Invitrogen. All other chemicals were of the highest reagent grade and were purchased from Sigma.

Animals and Treatments—Adult male Wistar rats (Rattus rattus) were obtained from the Small Animal Facility of the National Institute of Immunology (New Delhi, India). Wild type C57BL/6 (B6) and B6.MRL.lpr (B6-lpr,lpr) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained at the Small Animal Facility of the National Institute of Immunology (New Delhi, India). Rats were injected with different doses of DES (0.01, 0.1, and 1 mg of DES/kg of body weight) every alternate day for varying periods of time with the first day of injection being considered as day 0. After completion of each treatment schedule, rats were killed by CO2 asphyxiation and testes were collected for various studies. Similarly, adult 8-week-old lpr mice were treated with DES at 1 mg/kg body weight for 7 days and were killed as above. For testosterone supplementation studies, testosterone was administered at the dose of 30 µg/day for appropriate time periods.

Preparation of Mitochondria and Cytosol—Spermatogenic cells were prepared according to Meistrich et al. (18) with slight modifications as described previously (19). Briefly, decapitated rat testes were finely chopped in spermatogenic cell medium (Ham’s F-12/Dulbecco’s modified Eagle’s medium, supplemented with 14.2 mM sodium bicarbonate, 10 mM sodium pyruvate, 2 mM l-glutamine, 1 mM sodium pyruvate, 10−7 M testosterone, and 1% BSA) and the cells were filtered through Nitex mesh (1000 and 20 µm), mira cloth, and glass wool sequentially. After centrifugation, flow cytometry was used to check the purity of the cellular preparation as described earlier (19). FLOWJO software (Tree Star, Inc., Stanford, CA) was used for DNA analysis.

Cell viability was checked by propidium iodide (PI) staining at a final concentration of 5 µg/ml. PI-stained cell suspensions were analyzed immediately on Coulter EPICS® ELITE ESP Flow Cytometer (Coulter Corp., Miami, FL). DNA analysis of spermatogenic cells was performed according to the procedure of Blanchard et al. (20). Briefly, cells from treated and control animals were resuspended in 50 µl PBS and chilled ethanol in a 1:1 ratio and incubated on ice for 10 min. After washes, RNase treatment was given at 37 °C for 30 min and PI (50 µg/ml) was added to the cells, which were analyzed in a flow cytometer as described above.

Preparation of Mitochondria and Cytosol—Spermatogenic cell mitochondria were prepared according to Graham and Higgins (21). Briefly, 10⁶ spermatogenic cells were suspended in mitochondrial isolation buffer (150 mM sucrose, 10 mM succinate, 5 mM potassium phosphate, 10 mM HEPES-KOH, pH 7.4, 0.1% BSA) and lysed by nitrogen cavitation (450 p.s.i. for 30 min at 4 °C with constant stirring). The lysate was centrifuged sequentially at 500 and 2,500 × g for 10 min at 4 °C to remove unbroken cells and nuclei, respectively. The resulting supernatant was centrifuged at 20,000 × g for 20 min at 4 °C to retrieve the pellet enriched in mitochondria and stored in mitochondrial resuspension buffer.
sion buffer (200 mM mannitol, 50 mM sucrose, 10 mM succinate, 5 mM potassium phosphate, 10 mM HEPES, pH 7.4, 0.1% BSA) for a few hours without significant loss of the mitochondrial membrane potential ($\Delta \psi_m$). The supernatant obtained from the mitochondrial pellet was centrifuged at 100,000 $\times$ g for 1 h at 4°C in an Optima™ XL-100K ultracentrifuge (Beckman, Palo Alto, CA) to obtain the cell cytosol.

Tissue Processing, Terminal Deoxynucleotidyltransferase (TdT) Enzyme-mediated dUTP Nick End Labeling (TUNEL) Assay, and Immunostaining—Testes from rats were fixed in Bouin’s fixative (saturated picric acid:formaldehyde:acetic acid in the ratio of 15:5:1) for assessing morphological changes and in 4% formaldehyde (4% formaldehyde in 50 mM PBS) for TUNEL assays. Tissues were processed by standard procedures. Briefly, post-fixation wash and dehydration in graded ethanol (50–100%) was followed by paraffin embedding. Four-$\mu$m sections were cut with a Reichert Jung Microtome 1640 (Reichert, Germany) and transferred to albumin-coated slides. For assessment of the morphological integrity of the seminiferous tubules, tissue sections were stained with hematoxylin (0.5%) and eosin (0.01%) after deparaffinization and hydration. Enumeration of seminiferous epithelium stages I–IV, V–VI, VII–VIII, IX–XI, and XII–XIV was carried out using a Nikon E600W upright microscope with a 40× objective. For each rat, at least 10 tubules per stage group were analyzed. These stages were identified according to the criteria proposed by Russell et al. (22) for paraffin sections. The rate of spermatogenic cell apoptosis was expressed as the number of apoptotic spermatogenic cells per tubule.

Detection of DNA fragmentation by TUNEL staining was carried out as described previously (19, 23) using a TUNEL assay kit according to instructions from the manufacturer. Briefly, deparaffinized tissue sections were treated with equilibration buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, pH 8.0, 0.2 mM DTT, 0.25 mg/ml BSA, 2.5 mM cobalt chloride) for 10 min at room temperature followed by incubation with TdT buffer containing nucleotide mix (50 $\mu$M dUTP-biotin, 100 $\mu$M dATP, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 7.6) for 1 h at 37°C. The incorporation was visualized with avidin conjugated to horseradish peroxidase (1:500) using a diaminobenzidine substrate kit, and sections were counterstained with hematoxylin (0.5%) and visualized under a Nikon E600W microscope. At least 100 seminiferous tubules per rat from four independent experiments were counted for TUNEL-positive nuclei.

For Fas-FasL immunostaining, fixed sections were deparaffinized and blocked for endogenous peroxidase activity with 0.3% H2O2 in PBS for 15 min and for nonspecific binding with 10% normal goat serum for 30 min. Fas and FasL antibody (1:100 dilution in 10% normal goat serum) incubation was carried out for 1 h at room temperature. After washing, color was developed with a diaminobenzidine kit and sections were counterstained in hematoxylin (0.5%) for 30 s, dehydrated, and mounted in DPX mountant. For co-localization of Fas-FasL, isolated spermatogenic cells fixed in 2% paraformaldehyde were blocked for nonspecific sites with 10% normal

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**Fig. 2. Effect of DES on testicular cell types and seminiferous tubule diameter.** A, sections from testis of rats treated with DES at different time points showing changes in seminiferous tubular cell populations. a, control; b–d, sections from testis collected from 0.1-mg DES-treated rats on days 7, 14, and 31 after treatment. Note the loss of spermatogenic cells in some tubules, whereas others show intact seminiferous epithelium. e–g, sections from testes collected from 1-mg DES-treated rats on days 7, 14, and 22. Note the severe depletion of spermatogenic cells from the seminiferous tubules in g. Bar represents 100 $\mu$m. B, seminiferous tubular diameter determined from testis sections of rats treated with 0.1 mg of DES (a) and 1 mg of DES (b), n = 6.
goat serum and permeabilized with 0.1% saponin prior to staining. The cells were incubated with monoclonal antibodies against FasL (1:50) and Fas (1:50) tagged to phycoerythrin and fluorescein isothiocyanate (FITC), respectively. The dual staining was visualized with a confocal microscope (Zeiss LSM 510, Zeiss Inc., Thornwood, NY) by illuminating with a 488-nm argon ion laser at 150% power and images recorded through a band-pass filter at 505–550 nm and a long pass filter at 560 nm. For staining of Bax and cytochrome c, isolated spermatogenic cells were fixed in 2% paraformaldehyde and stained for the respective antigens with anti-Bax and anti-cytochrome c antibodies (1:50) for 1 h at room temperature. Secondary antibody tagged to FITC was used at 1:200 dilution. For Bax-stained cells labeled with FITC, to identify the at room temperature. Secondary antibody tagged to FITC was used at 1:200 dilution. For Bax-stained cells labeled with FITC, to identify the site of staining, cells were incubated with MitoTracker® Red AM, a mitochondria-specific dye, prior to observation with a confocal system using same settings as above. Stainings for MitoTracker® Red and Bax (green) were overlapped and source of staining with anti-Bax antibody was identified. Cytochrome c-FITC distribution in spermatogenic cells was visualized at 540 nm using an E600W Nikon microscope.

Semiquantitative RT-PCR—Total RNA was isolated from whole testis and spermatogenic cells using TRIzol reagent. First strand complementary DNA was made using 1 μg of total RNA in the presence of AmpliTaq Gold™ reverse transcriptase and random primer. After the RT reaction, 1 μl of incubation mixture was used as a template for the subsequent PCR reaction. Primer sets used as a template to obtain PCR products of FasL were 5'-AGCCCGTGAATTACCCATGTC-3' and 5'-TGCTGGGGTTGCTATTTTGCT-3' and for β-actin were 5'-AGCCATCCTGACCCCTGAAGTAC-3' and 5'-CTTTTCAATGGAATGCCTGTCAG-3'. For semiquantitative analysis, β-actin was used as an internal control, and was coamplified with FasL messenger RNA by using β-actin primers and FasL primer (1 μl) in the same mixture. All PCR reactions were performed for 30 cycles with an annealing temperature of 55–65 °C in 1.5 mM MgCl₂.

Western Blot and DNA Analysis—Electrophoresis on 12% polyacrylamide gels, Western blots, and DNA extractions were carried out as described previously (19, 23).

Measurement of Mitochondrial Membrane Potential (Δψm) and ATP Levels—Δψm was estimated using JC-1 as a probe according to the method of Dey and Moraes (24) with slight modifications. JC-1 is a cationic mitochondrial vital dye that is lipophilic and becomes concentrated in mitochondria in proportion to their Δψm; more dye accumulates in mitochondria with greater Δψm and ATP-generating capacity. The dye exists as a monomer at low concentrations (emission, 530 nm; green fluorescence) but at higher concentrations forms J aggregates (emission, 590 nm; red fluorescence). JC-1 was chosen because of its reliability for analyzing Δψm in intact cells, whereas other probes capable of binding mitochondria show a lower sensitivity or a noncoherent behavior because of a high sensitivity to changes in plasma membrane potential (25). Briefly, cells after different treatments were collected and incubated for 10 min with 5 μM JC-1 at 37 °C, washed, and resuspended in media, and Δψm was measured at 590 nm for J-aggregates and at 530 nm for J-monomer. The ratio of 530/590 nm was considered as the relative Δψm value.

ATP was measured by a bioluminescence assay (26) using an ATP determination kit. The assay is based on the requirement of luciferase for ATP in producing light (emission maximum ~ 560 nm at pH 7.8). Briefly, cells (10⁶) after different treatments were resuspended in reaction buffer containing 1 mM DTT, 0.5 mM luciferin, and 12.5 μg/ml luciferase and gently mixed, following which readings were taken in a luminometer (Lumincount, Packard, CT). ATP standard curves were run in all experiments with different concentrations of ATP, and calculations were made against the curve and cellular ATP levels were expressed as n mol/10⁶ cells.

Glutathione Levels—Quantification of oxidized glutathione (GSSG) and reduced glutathione (GSH) in the samples was done using the fluorescent probe α-phthalaldehyde (23, 27).

Assay of Caspase Activity—Cell lysates (200 μg of protein) were incubated with caspase buffer (50 mM HEPES, pH 7.4, 100 mM sodium chloride, 10% sucrose, 1 mM EDTA, 0.1% CHAPS, and 100 mM DTT) containing 100 mM fluorogenic peptide substrate, acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) at 37 °C. Apopain from caspase-3 assay kit was used as a positive control. 7-Amino-4-trifluoromethyl coumarin release was measured with a PerkinElmer Life Sciences LS-50B luminescence spectrometer at excitation wavelength of 400 nm and emission wavelength of 550 nm.

Testosterone Assay—Plasma levels of testosterone were measured by radioimmunoassay according to Suß et al. (28). The sensitivity of radioimmunoassay was 2.7 pg/assay tube. The intra-assay and interassay variations were 10 and 7%, respectively.

Protein Estimation—Bioinorganic acid assay was performed in microtiter plates as described previously (23).

Statistical Analysis—An unpaired two-tailed Student's t test using T-EASE software (version 2.0; Institute for Scientific Information, Philadelphia, PA) and analysis of variance were used for statistical analyses. Data are reported as mean ± S.E. from at least three separate experimental groups unless specifically mentioned. Each experimental group consisted of 6 rats. Data sets were said to be significantly different for p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***)..

### Table I

<table>
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<th>Testicular weight</th>
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<td>Day 7</td>
<td></td>
<td>Day 14</td>
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<td>Control</td>
<td>2.40 ± 0.59</td>
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<table>
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RESULTS

DES Treatment Results in the Loss of Spermatogenic Cells and Decrease in Serum Testosterone Levels—DES, a potent estrogenic analogue, is known to disrupt spermatogenesis by the suppression of pituitary gonadotropins, which leads to the inhibition of testosterone production (8) leading to insufficient circulating and intratesticular concentration of the hormone. This results in abnormal spermatogenesis, as testosterone is vital for spermatogenic cell survival (4). Even though the importance of estrogen-induced deregulation of spermatogenesis in male infertility has been acknowledged, the mechanism of DES-induced apoptosis in the adult testis has not been worked upon. Therefore, we first examined the kinetics of DES-induced cell death in the adult testis by using two different doses administered for varying periods of time. We observed a loss of testicular weight in treated animals in a dose- and time-dependent manner (Fig. 1A), indicating a decrease in the number of testicular cells. This observation corroborates previous studies where DES treatment in rat (8, 29) and hamsters (10) have been shown to reduce testis weight that was accompanied by cell death. Because DES is known to interfere with testosterone levels (30), we checked serum testosterone and found a significant decrease in circulating testosterone in treated animals in comparison to controls, although a total shut-off was not achieved (Fig. 1B). In addition to the above two doses of 0.1 and 1 mg of DES, we used 0.01 mg of DES to look at similar parameters, but no appreciable change in either testicular weight or testosterone levels were found (data not shown).

Histological examination of sections from the treated and control testis showed that spermatogenic cell loss was more severe with 1-mg DES treatment as compared with the 0.1-mg DES-treated group (Fig. 2A, a–g), even though reduction in testosterone achieved with both the doses was similar. The loss in testicular weight and spermatogenic cells from the testis corresponded to a time- and dose-dependent decrease in mean cross-sectional area of seminiferous tubules (Fig. 2B, a and b). The seminiferous tubular atrophy observed during exposure to DES was accompanied by important modifications in seminiferous epithelium composition and morphology. There was an apparent decrease in total number of spermatogenic cells, with both the number of spermatozoa and spermatid having decreased. The type of spermatogenic cells affected by DES-in-
duced testosterone suppression is different from those affected by gonadotropin-releasing hormone antagonist-induced suppression of testosterone (31). In the regimen of hormone administration that we used, the group treated with 1 mg of DES exhibited a severe depletion of spermatogenic cells by day 22, whereas treatment with 0.1 mg of DES showed a nearly comparable depletion of spermatogenic cells only by day 31, showing that depletion of spermatogenic cells was dependent on dose as well as time of treatment. Testosterone treatment along with DES was able to restore serum testosterone levels to control levels from day 7 onward (Table I). However, as shown in Table I, the cell viability was not restored to control levels until day 14. This was possibly a result of the combined suppressive effect of testosterone and DES on the pituitary at the onset of treatment schedule. As testosterone levels in the serum and the testis increased with subsequent administrations, repopulation of the seminiferous epithelium took place. The approximate time for spermatids to reappear in the seminiferous epithelium would be 12 days according to the rat seminiferous epithelial cycle length (22); therefore, the restoration of cell viability in the testosterone-supplemented groups to control values after 14 days was logical. Taken together, the above data show the ability of DES to reduce serum testosterone levels and induce a loss of testicular spermatogenic cells in a dose- and time-dependent manner that is paralleled by a loss in testicular weight. Because spermatogenic cell loss was more extensive with 1 mg of DES as compared with 0.1 mg of DES, the 1-mg dose regimen was chosen for further study on the mode of DES action on spermatogenic cell death.

**DES-induced Spermatogenic Cell Death Is Apoptotic in Nature**—Based on the above data, we carried out a flow cytometric analysis of spermatogenic cells prepared from testis of treated and control animals to confirm histological observations. A significant cell loss was observed by day 7 in the 1-mg group as compared with the controls (Fig. 3A, a), thus confirming cell loss observed in earlier experiments. Because it is known that testosterone withdrawal results in spermatogenic cell apoptosis (30) and in our model DES was causing a reduction in testosterone levels as well as death of spermatogenic cells, we next investigated whether DES-induced cell death expressed apoptotic or necrotic phenotype. By day 7 of treatment, there was a substantial increase in cells with sub-haploid DNA content as compared with controls (Fig. 3A, b) with a concomitant reduction of haploid cell population, but there was no change in the diploid population, indicating that the haploid spermatid population was primarily affected. To ensure that spermatogenic cell death was indeed apoptotic, we examined other apoptotic phenotypes like DNA fragmentation by labeling the fragmented DNA ends with fluorescent labeled nucleotides (TUNEL assay) (19, 23). TUNEL assay showed a significant number of spermatogenic cells staining positive in the DES-treated groups (Fig. 3B, b–d) as compared with controls (Fig. 3B, a). The number of apoptotic cells per seminiferous tubule on days 7 and 14 after injection were 1.13 ± 0.1 and 6.66 ± 0.8, respectively, as compared with control values of 0.03 ± 0.001, indicating increased apoptosis in the seminiferous tubules (Fig. 3C). This average number, however, is not a true representation of a particular tubule at a given stage of the seminiferous epithelial cycle. Stage VII showed the maximal number of TdT-positive cells (14 ± 2) (n = 25), the numbers in other tubules being lower on day 7 (1.0 ± 0.01) (n = 25). Biochemically, internucleosomal DNA strand breaks, which are detected as a ladder pattern on agarose gel electrophoresis of DNA are regarded as a hallmark of apoptosis (32). Genomic DNA prepared from spermatogenic cells of animals after day 3 of DES treatment showed distinct DNA laddering regarded as a hallmark of apoptosis (Fig. 3D, b) as compared with controls (Fig. 3D, a). Therefore, the above data showing nucleosomal DNA...
laddering, increase in TUNEL-positive cells and changes in the ploidy of spermatogenic cells clearly established the apoptotic nature of spermatogenic cell death, particularly in the haploid cell type as induced by DES.

Increased Expression of FasL and Fas Occurs in Spermatogenic Cells Undergoing Apoptosis in DES-treated Rats—Because it was evident that DES-induced cell death occurred via apoptosis, the expression of possible apoptosis-inducing proteins was studied to dissect the apoptotic pathway. Given the existing evidence of the involvement of the Fas-FasL system in the testis, we first looked at the expression of Fas and FasL after DES treatment. Fas, a 45-kDa member of the tumor necrosis factor receptor superfamily (33), binds to its cognate ligand, FasL, and initiates the apoptotic pathway (34). It is generally accepted that, in the testis, FasL is expressed in Sertoli cells, which precipitates cell death by engaging its cognate receptors on spermatogenic cells (4, 9). One recent report suggests that FasL mRNA is expressed in the spermatogenic cells only (16). Our studies revealed a clear up-regulation of FasL in spermatogenic cells within 24 h of DES treatment as determined by Western blots and RT-PCR with FasL specific antibodies and probes, respectively (Fig. 4A, a and b). Similar up-regulation of Fas was also detectable by Western blots of purified spermatogenic cell extracts (Fig. 4A, c). Interestingly, in isolated spermatogenic cells from DES-treated animals on day 1, FasL and Fas showed differential localization. Although Fas was distributed evenly throughout the cell, FasL staining was associated with vesicular bodies (Fig. 4B, a–f). It has been reported that FasL is stored in special secretory vesicles in certain cell types (35); therefore, it is possible that, during the early part of DES exposure, FasL is localized to secretory vesicles in spermatogenic cells. To further clarify the cell types

![Immunohistochemical staining of Fas-FasL in testis sections of DES-treated and untreated rats.](image-url)
expressing these proteins, immunohistochemical studies were carried out with testis sections. Clearly, considerable up-regulation of FasL was visible in spermatogenic cells. A lower intensity staining was observed in spermatocytes and round spermatids, but more intense staining was detectable in the elongated spermatid cytoplasm by day 7 after treatment (Fig. 5). Interestingly, the cytoplasm of the elongated spermatids of stage V and stage VII of the seminiferous epithelial cycle were most reactive, with residual bodies in stage VII that are remnants of spermatid cytoplasm staining intensely as well (Fig. 5, A (c-f) and C (a and b)). This differential staining for FasL in different stages of the seminiferous epithelial cycle is possibly related to variations in cell population and physiological status of different stages of the cycle. It is known that expressions of proteins and hormone binding capabilities vary with the stages of the seminiferous epithelium (36). It is possible that spermatogenic cells of stage V and VII were more susceptible to estrogen-induced changes in terms of FasL induction. Fas staining was visible in all stages of the seminiferous epithelial cycle, being particularly prominent in stages VII and XII in all groups of spermatogenic cells (Fig. 5, B (c-f) and C (c-f)). When sections of testis from animals treated with DES along with testosterone were examined for Fas and FasL immunostaining, it was observed that there was a distinct down-regulation of staining of both Fas and FasL in the testosterone-treated groups by day 14 (Fig. 6) as compared with DES only (Fig. 5). In summary, the above data show that DES treatment selectively increases FasL and Fas expression in spermatogenic cells, indicating an apoptosis modulating capacity of the spermatogenic cells independent of Sertoli cell control, and provides a model for the study of Fas-FasL interaction in the most complex epithelium of the body. In addition, testosterone supplementation clearly down-regulates the Fas-FasL up-regulation induced by DES, showing that testosterone is important for regulation of apoptotic signals to germ cells. The fact that Fas is an absolute necessity in case of DES-induced apoptosis was proven by studies with lpr mice, where DES was unable to induce changes in lpr mice ((testicular weight: lpr mice control, 0.17 ± 0.003 g; lpr mice + 1 mg of DES, 0.18 ± 0.004 g), (no. of TdT-positive cells/100 spermatogenic cells: lpr mice control, 1 ± 0.008; lpr mice + 1 mg of DES, 0.8 ± 0.005)).

Fas-FasL Up-regulation Is Followed by Increased Caspase-8 Breakdown and Activation of Caspase-9 and -3—Taking a lead from the above data, we sought to determine the downstream effectors of the Fas-FasL pathway including aspartate-specific cysteiny1 protease (caspases) family (37, 38). Our studies show that caspase-8 is cleaved to its active form by day 1 after treatment (Fig. 7A). At this point, there were two possible pathways that the spermatogenic cells could enter into; one was to activate caspase-3 directly through the active caspase-8, and the other was to activate caspase-9 by involving the members of the Bcl-2 family of proteins. In our studies, processing of caspase-9 known to activate caspase-3 (39) to its active form of 37 kDa (37) was detected prominently from 8 h onward (Fig. 7B). Caspase-3 cleavage was detectable on Western blots of treated spermatogenic cell extracts from day 1 onward (Fig. 7C). To further confirm the activation of caspase-3, we directly determined caspase-3 activity in DES-treated cell extracts by monitoring the release of 7-amino-4-trifluoromethyl coumarin from Ac-DEVD-AFC, a substrate of caspase-3. Caspase-3 activity was higher on day 7 as compared with day 1 (Fig. 7D). Having established that caspase-3 is active after DES treatment, we next checked for the cleavage of poly(ADP-ribose) polymerase (PARP), a known endogenous substrate for caspase-3. Cleavage into an 85-kDa C-terminal breakdown product was detectable on Western blots, confirming that caspase-3 was acting on cellular substrates (40) (Fig. 7E). Existing studies show that Fas expression on the cell surface of some cell types is p53-dependent (41). Because p53 can be activated by changes in DNA and estrogen is known to induce changes in DNA (42), p53 expression was checked, but no change was detectable (Fig. 7F). Therefore, the above studies showing a temporal relationship between the activation of caspase-8, translocation of Bax to mitochondria, release of cytochrome c, and subsequent activation of caspase-3 provide a clear picture that exposure to potent estrogens could induce spermatogenic cell apoptosis in a caspase-dependent manner.

**Induction of Apoptosis by Caspase-8 Is Amplified through the Mitochondrial Release of Cytochrome c Preceded by Bax Translocation to the Mitochondria**—Because Bax, a multidomain, proapoptotic member of the Bcl-2 family, has been shown to be involved in the first wave of spermatogenesis (17), we first checked for changes in Bax localization, as pro-apoptotic members of the Bcl-2 family translocate from the cytosol to the mitochondria resulting in the release of cytochrome c (43). Fig. 8 (A and B) shows the migration of Bax from cytosol to the
mitochondria of spermatogenic cells after DES exposure, where there was a gradual loss of the monomeric form of Bax from the cytosol and accumulation of oligomeric Bax in the mitochondria. Cytochrome c was released by 8 h into the cytosol from the mitochondria (Fig. 9, A and B). The involvement of mitochondria in the activation of caspase-3 clearly shows that adult spermatogenic cells follow the apoptotic pathway taken by type II cells in response to estrogen treatment.

Early Changes Upstream of Cytochrome c Are Mitochondrial Potential Loss and ATP Depletion—Looking at changes upstream to cytochrome c release, we checked for mitochondria related events like changes in $\Delta \Psi_m$ and ATP generation. Alteration in the $\Delta \Psi_m$ are known to be a major cause of precipitation of apoptosis in many cell types (43). Measurement of mitochondrial membrane potential was carried out using the fluorimetric dye, JC-1 which showed a drop in $\Delta \Psi_m$ within 4 h after the first injection (Fig. 10A). This drop in $\Delta \Psi_m$ did not change significantly until day 7 after treatment with DES (data not shown). Any disruption of mitochondrial function would reflect in the levels of ATP generation, which was confirmed by a
gradual fall in the ATP levels that reduced significantly by day 7 of treatment with DES (Fig. 10B). These data are consistent with the fact that the apoptotic process requires ATP and therefore a minimal level maintained until day 7 might be sufficient for continuing cellular apoptosis (44, 45). Glutathione being a major detoxification molecule within the cell and changes in the level of GSH can contribute to \( \Delta \phi_m \) loss, we investigated GSH and GSSG levels. However, in case of estrogen-induced spermatogenic cell death, there was no change in GSH and GSSG levels (GSH, \( n \) mol/10⁶ cells: control, 0.97 ± 0.08; day 1, 1.09 ± 0.1; day 7, 1.07 ± 0.09), (GSSG, \( n \) mol/10⁶ cells: control, 3.68 ± 0.2, day 1, 3.60 ± 0.4; day 7, 3.92 ± 0.5)), showing that GSH, even though important in other models of germ cell death (19, 23) does not seem to play any role in estrogen-mediated cell death. The above data suggest that the loss of \( \Delta \phi_m \) that occurs by 4 h is accompanied by a loss of cytochrome c and an increase in Bax protein concentration in the mitochondria.

**DISCUSSION**

Compelling evidence has accumulated on the detrimental effects of estrogen-like chemicals present in the environment on male reproductive function. These chemicals include industrial pollutants like bisphenol A and polychlorinated biphenols, and pesticides like DDT, methoxychlor, or chlorodecone. The extent of exposure to these chemicals on members of a population differs as occupations in agriculture, petrochemicals, and the construction industry entail higher exposure. Because estrogen receptors are present in the pituitary and spermatogenic cells (1–3), estrogen-like chemicals can act as agonists or antagonists for the hormone and interfere with spermatogenesis. The DES-induced spermatogenic cell apoptosis model was ideal to study the mechanism of estrogen-induced spermatogenic cell death, as DES can mimic estrogen action and has also been widely used as a model estrogen to study the effects on the neonatal male rat reproductive tract (8, 9).

The finding that primarily haploid spermatogenic cells were undergoing apoptosis in response to DES is in contrast to what is found in other toxin-induced cell death models, where the diploid population of spermatocytes are most affected (13, 14). Interestingly, immunoneutralization of follicle-stimulating hormone (46) and treatment with gonadotropin antagonist (47), which suppress serum testosterone levels like DES, precipitate apoptosis primarily in spermatogonia, spermatocytes, and, to a lesser extent, spermatids. Clearly, in case of DES treatment, the spermatids are mainly affected, suggesting that estrogen-induced cell death primarily involves apoptosis of the haploid population. This result is not surprising considering the fact that two regulators of the cell death pathway, FasL and Fas, were overexpressed in spermatids after DES exposure. Interestingly, the largest number of apoptotic spermatogenic cells were visible in stage VII of the seminiferous epithelial cycle, a stage were both FasL and Fas expression were most pronounced and not in stage V or XII, where FasL and Fas expression alone were most prominent. Therefore, the failure to express FasL in a stage of the cycle where Fas was expressed or vice versa showed substantially reduced number of cells undergoing apoptosis. In other models of testosterone withdrawal in the male, it has been shown that stage VII of the seminiferous epithelial cycle was most susceptible in terms of apoptosis (48). Our study clearly shows that the increased susceptibility of stage VII to cell death is possibly a contribution of Fas-FasL interaction. The belief that Sertoli cells are the only source of FasL dominated thinking in testicular biology (12); however, in the recent past it has been shown that the
spermatogenic cells have the ability to express both Fas and FasL (16). The presence of Fas-FasL in testicular cells has been linked to the ability of the testis to act as an immune privileged site and maintenance of testicular homeostasis by Sertoli cells eliminating spermatogenic cells. However, the recognition that both the Fas receptor and its cognate ligand FasL can be expressed in spermatogenic cells has led to the speculation that, apart from the above functions, the expression of both these proteins on spermatogenic cells may indicate an as yet unidentified function. Here, we demonstrate an important novel function for the Fas-FasL system in the testis. We show that estrogen exposure induces an increase in the expression of spermatogenic cell Fas-FasL leading to the activation of components downstream of Fas that indicate control of cell death within the same lineage. More importantly, the observation that the number of apoptotic cells is highest in seminiferous tubule stages where both Fas and FasL expression was high reinforced the idea that these two proteins expressed in spermatogenic cells are the main modulators of death. Interestingly, in the female, strong FasL expression occurs in the endometrium in estrogen-dependent late proliferative and secretory phases (49). Additionally, in female rats, expression of FasL protein and mRNA levels in ovarian cell increases upon estrous phases (49). Additionally, in female rats, expression of FasL protein and mRNA levels in ovarian cell increases upon estrous phases (49). Furthermore, in female rats, expression of FasL protein and mRNA levels in ovarian cell increases upon estrous phases (49).

The intrinsic and extrinsic pathways are linked by the pro-apoptotic members of the Bcl-2 family (43). In case of DES treatment, Bax protein translocation to mitochondria occurred after caspase-8 was activated. A role for Bax has been predicted in germ cell apoptosis using different models, but predictions of involvement of Bax have largely been made based on cellular localization (17, 52) or from Western blots of whole testis extracts (53). Using purified spermatogenic cells, our study clearly demonstrates that there is a translocation of Bax from the cytosol to the mitochondria resulting in the release of cytochrome c. In certain cell types, cytochrome c release is accompanied by a fall in mitochondrial potential (54) and our studies show that in spermatogenic cells the \( \Delta \psi_m \) fall is preceded by a transient increase in reactive oxygen species (data not shown) and followed by a fall in ATP levels.

Taken together, the data essentially illustrate the importance of the Fas-FasL system in spermatogenic cell death in the event of estrogen exposure. Furthermore, this study establishes germ cells as type II cells that are able to utilize the extrinsic and intrinsic apoptotic pathways, the link between the two pathways being the proapoptotic protein Bax in the event of estrogen exposure. We also postulate that regular testicular homeostasis may involve the Fas-FasL system to maintain proper spermatogenic cell number, which may be under estrogen regulation.

Acknowledgments—We are grateful to G. S. Neelaram for assistance in immunocytochemistry and to T. Nagarjuna for help in flow cytometry.

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Diethylstilbestrol Induces Rat Spermatogenic Cell Apoptosis in Vivo through Increased Expression of Spermatogenic Cell Fas/FasL System
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doi: 10.1074/jbc.M209319200 originally published online December 10, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209319200

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