0013-7227/97/$03.00/0
Endocrinology
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Vol. 138, No. 5
Printed in U.S.A.

The Fas System Is a Key Regulator of Germ Cell Apoptosis in the Testis*

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ABSTRACT

Apoptosis occurs in the testis as an important physiological mechanism to limit the number of germ cells in the seminiferous epithelium. Sertoli cells, which tightly regulate germ cell proliferation and differentiation, are implicated in the control of germ cell apoptosis. Fas (APO-1, CD95), a transmembrane receptor protein, transmits an apoptotic signal within cells when bound by Fas ligand (FasL). The Fas system has been implicated in immune regulation, including cytotoxic T cell-mediated cytotoxicity, activation-induced suicide of T cells, and control of immune-privileged sites. Here we propose the Fas system as a key regulator of spermatogenesis. In this model, FasL expressed by Sertoli cells initiates the apoptotic death of germ cells expressing Fas. Using immunohistochemistry, we localized Fas to germ cells and FasL to Sertoli cells. The expression of these genes was dramatically up-regulated after exposure to mono-(2-ethylhexyl) phthalate and 2,5-hexanedione, two widely studied Sertoli cell toxicants known to induce germ cell apoptosis. Mouse germ cells *in vitro* were susceptible to anti-Fas antibody-induced death, and the survival of rat germ cells was increased after disruption of FasL by antisense oligonucleotide treatment. Unlike its expression in other tissues, testicular expression of Fas in the *lpr* mouse, a spontaneous mutant of the Fas gene, is similar to that in the normal mouse, arguing for the importance of the Fas system in maintaining testicular homeostasis. These data implicate the Sertoli cell in the paracrine control of germ cell output during spermatogenesis by a Fas-mediated pathway. (*Endocrinology* **138:** 2081–2088, 1997)

SPERMATOGENESIS is a dynamic process of germ cell proliferation and differentiation from stem spermatogonia to spermatozoa. Sertoli cells and germ cells, the only cell types within the seminiferous epithelium, are in close contact. Sertoli cells, spanning the thickness of the seminiferous epithelium, orchestrate spermatogenesis by providing structural and nutritional support to germ cells.

In the mammalian testis, germ cells clonally expand through many rounds of mitosis before undergoing the differentiation and maturation steps that result in spermatozoa. This clonal expansion is excessive, requiring that a mechanism exist to match the number of germ cells with the supportive capacity of Sertoli cells. Overproliferation of early germ cells is tempered by selective apoptosis of their progeny (1–3). Large numbers of spermatocytes undergo apoptosis in the testis of a 4-week-old rat, and spermatogonia become the main cell type undergoing apoptosis in the adult rat (2, 3). Testicular germ cell apoptosis occurs normally and continuously throughout life (1, 3). In addition, massive testicular germ cell loss is known to result from toxicant exposure (4, 5), depletion of growth factors (6), alterations of hormonal support (testosterone or pituitary hormones including FSH and LH) (7, 8), heat exposure (9), radiation (10), or treatment with chemotherapeutic compounds (10). In many of these situations, germ cells are known to undergo apoptosis (1, 3), indicating that a specific pathway is activated when the testicular environment cannot support spermatogenesis. However, the elements that control this process have not previously been identified.

Fas (APO-1, CD95) is a transmembrane receptor protein that belongs to the tumor necrosis factor/nerve growth factor receptor family (11, 12). It contains a "death domain" and is capable of initiating apoptosis when stimulated by receptor cross-linking or binding to its ligand, FasL (CD95L) (11). FasL is a tumor necrosis factor-related type II transmembrane protein (13). The FasL-Fas interaction triggers the death of cells expressing Fas, a process best studied in lymphoid cells (11). A variety of Fas mediators that are associated with its cytoplasmic domain have been identified by the two-hybrid system (14–16). Fas-associating protein with death domain (FADD)/MORT-1 and receptor interacting protein are positive effectors of Fas-mediated apoptosis (14, 15), and Fas-associated phophatase is suggested to be an inhibitor of Fas-mediated apoptosis (16). A downstream effector protein that binds to FADD is a protease of the interleukin-1β-converting enzyme (ICE) family, named FLICE (FADD-like ICE)/MACH (17). Activation of ICE is required for Fas-mediated apoptotic cell death (18). These data identify a Fas-mediated cell death pathway with FADD linking Fas directly to an ICE-like enzyme.

The biological importance of the FasL-Fas interaction is underscored by abnormalities seen in several strains of mutant mice, in which the Fas system is not functional (11). *lpr* (lymphoproliferation) mice express little or no Fas protein due to insertion of a transposon in the intron 2 region of the Fas gene (19–21). *gld* (generalized lymphoproliferative disease) and lpr^{cg} (*lpr* complementing *gld*) mice have a point mutation in the FasL and Fas genes, respectively, resulting in nonfunctional proteins (11, 22). All of these mice suffer

Received September 30, 1996.
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* This work was supported in part by a grant from the Burroughs Wellcome Fund and in part by a grant from the USPHS NIH (ES05033).
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from dramatic lymphoproliferation and systemic autoimmunity due to altered lymphocyte apoptosis (11, 22).

The Fas system is involved in many areas of immune regulation, including the maintenance of peripheral T and B cell tolerance, cell-mediated cytotoxicity (CTL), and control of immune-privileged sites (11, 22–26). The tissue distribution of Fas messenger RNA (mRNA) is universal and particularly high in thymus and spleen as well as nonlymphoid tissues, such as liver, ovary, and lung (27). FasL expression, however, is generally more restricted to lymphoid organs (13, 27). Interestingly, FasL mRNA expression is abundant in rat and mouse testis, with a suggested localization to Sertoli cells (13, 27).

In testis, the Fas system has been implicated in maintaining immune privilege (24). By transplanting Sertoli cells lacking functional FasL across immunological barriers, Bellgrau *et al.* showed that FasL was critical to prevent immune rejection (24). This concept has been supported by experiments in which cotransplantation of myoblasts transfected with FasL prevented islet allograft rejection (28). According to this hypothesis, FasL-expressing Sertoli cells eliminate Fas-positive activated T cells, providing general protection against rejection in the testicular environment. However, Fas expression by germ cells suggests another role for the Fas system within the seminiferous epithelium. Here, we demonstrate that Fas signal transduction is responsible for germ cell apoptosis and that this pathway is a key regulatory system for spermatogenesis.

Materials and Methods

Animals

C57BL/6 (B6), B6.MRL-*Fas*$^{lpr/lpr}$ (B6-*lpr*/*lpr*) mice (Jackson Laboratory, Bar Harbor, ME) and male Fischer rats (Charles River Laboratories, Wilmington, MA) were used. All animals were given water and chow (Pro-Lab rat, mouse and hamster chow no. 3000, Farmer's Exchange, Framingham, MA) *ad libitum*. The animal room climate was kept at a constant temperature (68–70 F) at 35–70% humidity with a 12-h alternating light-dark cycle.

Experimental protocol

Three different treatments were performed to study germ cell apoptosis in testis. Mono-(2-ethylhexyl) phthalate (MEHP) treatment was performed as described by Richburg *et al.* (5). Fischer rats (28-day-old) received a single dose of MEHP (2 g/kg BW) in corn oil by gavage. After 12 h, testes were removed and processed for frozen sections and isolation of RNA. 2,5-Hexanedione (2,5-HD) treatment was performed as described by Blanchard *et al.* (4). Adult Fischer rats, weighing 150–175 g, were treated with 1% 2,5-HD (Aldrich Chemical Co., Milwaukee, WI) in the drinking water. At various times after initiating exposure (0, 2, 4, and 5 weeks), rats were killed to obtain testes for terminal deoxynucleotide transferase-mediated deoxy-UTP nick end labeling (TUNEL) staining (29) and isolation of RNA. For MEHP and 2,5-HD treatment, at least three animals per time point were used. For the heat exposure experiment (9), mice (30–38 days old) were anesthetized with sodium pentobarbital (40 mg/kg BW; Abbott Laboratories, North Chicago, IL), and their scrotal testes were immersed in a water bath (44 $\pm$ 0.5 C) for 15 min. At various times after immersion (0–12 h), mice were killed, and their testes were removed.

FasL/Fas immunostaining and TUNEL

For immunostaining of FasL and Fas, 8-μm frozen cross-sections from 28-day-old rat testis were prepared, fixed in acetone for 10 min at -20 C, rinsed in PBS, and then incubated in 1% H_2O_2 for 15 min to quench endogenous peroxidases. Sections were blocked for endogenous biotin and then blocked with 5% normal goat serum and 1% BSA in PBS (PBS$^+$). Polyclonal antibodies to FasL and Fas (sc-956 and sc-716, respectively; Santa Cruz Biotechnology, Santa Cruz, CA) were added to the sections at 0.2 μg/ml in PBS$^+$ for 1 h at room temperature. Primary antibody was detected using a biotin-conjugated goat antirabbit IgG secondary antibody (Calbiochem, San Diego, CA) and the ABC-Elite Kit (Vector Laboratories, Burlingame, CA) with 0.05% diaminobenzidine substrate. Inhibitor peptides (2 μg/ml; Santa Cruz Biotechnology) were used to verify the specific staining of FasL or Fas. For TUNEL staining, the standard protocol for frozen sections was followed (ApopTag, Oncor, Gaithersburg, MD). To quantitate the incidence of apoptosis at each time point, the number of TUNEL-positive cells within a seminiferous tubule cross-section was counted, and the data were represented as the percentage of seminiferous tubules containing more than three apoptotic cells of the total number of seminiferous tubules counted in a cross-section. Because, in control rat testis, the percentage of tubules with more than 3 TUNEL-positive cells is less than 5%, an increase in apoptosis is easily determined using this counting approach. In the heat treatment experiment, a different cut-off (>4 TUNEL-positive cells/seminiferous tubule cross-section) was applied due to the somewhat higher incidence of apoptosis in control mouse testis. For the MEHP and 2,5-HD experiments, about 300–400 essentially round tubules were counted/time point.

Antisense oligonucleotide treatment in vitro

Sertoli germ cell cocultures were prepared from 21-day-old rat testes, as previously described (30). Cells (1.5 $\times$ 10^6 cells/35-mm plate) were plated in DMEM mixed 1:1 with Ham's F-12 medium (Life Technologies, Gaithersburg, MD) plus 1 ng/ml epidermal growth factor, 10 μg/ml ITS$^+$ premix (containing insulin, transferrin, selenious acid, BSA, and linoleic acid; Collaborative Research, Bedford, MA), and 50 μg/ml gentamicin (DMEM/F12$^+$). Cocultures were incubated at 32 C on laminin (Life Technologies)-coated plates (3 μg/ml). This procedure yielded a monolayer of Sertoli cells attached to the plates and germ cells, most of which were located on top of Sertoli cells. Taking advantage of the distinctive appearance of germ cells by phase contrast microscopy, germ cells were easily counted. Phosphorothioate oligonucleotides (Eppendorf, Madison, WI) were added (10 μg/ml) 30 h after isolation. For quantitative reverse transcription-PCR (RT-PCR), total RNA was isolated from the cells 18 h after the addition of oligonucleotides. The FasL antisense oligonucleotide (5′-GGTAATTCACGGGCTGCTGCAT-3′) was complementary to the translation initiation site of the rat FasL gene. Controls included a FasL sense oligonucleotide (5′-ATGCAGCAGCCCGTGAATTACC-3′) complementary to the FasL antisense oligonucleotide and a scrambled oligonucleotide (5′-GCTGCATCGGGCTGGTAATTCA-3′), in which the antisense sequence was conserved but rearranged. To quantitate survival, about 2000 germ cells in 9 different areas/treatment group were counted by phase contrast microscopy before the addition of oligonucleotides. Two days later, germ cells in same areas were counted again, and the survival rate was calculated.

Jo-2 antibody treatment in vitro

Sertoli germ cell cocultures were prepared from testes of 21-day-old mice, as previously described with minor modifications (30). Cells (1.5 $\times$ 10^6 cells/35-mm plate) were initially plated in DMEM/F12$^+$. The cocultures were incubated at 32 C on laminin-coated plates (3 μg/ml). Two days after incubation, culture medium was collected from the plates, and the cells were washed with DMEM/F12$^+$. As the Sertoli cells remained attached to the plates after washing, germ cell-enriched cultures resulted from centrifugation and replating (1 $\times$ 10^5 cells/35-mm plate) of the cells in the culture media and the washing solutions. Jo-2 antibody (PharMingen, San Diego, CA) or hamster IgG antibody (PharMingen) as a control were added to the culture medium (5 μg/ml), and the viable cells were counted by trypan blue exclusion after 0, 3, 6, and 12 h For each time point, plates were prepared in triplicate.

Quantitative RT-PCR

Total RNA was isolated from tissues using TRIreagent (Molecular Research Center, Cincinnati, OH). First strand complementary DNA was

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made using 1 μg total RNA in the presence of Superscript II reverse transcriptase (Life Technologies) and random primer. One microliter of reverse transcription reaction buffer was used as a template for the subsequent PCR reaction. PCR products of rat FasL (238 bp), rat Fas (969 bp), mouse Fas (314 bp), and β-actin (389 bp) were amplified using the following primers: rat FasL, 5′-GGAATGGGAAGACACATATGG-AACTGC-3′ and 5′-CATATCTGGCCAGTAGTGCAGTAATTC-3′; rat Fas, 5′-CTGTGGATCATGGCTGTCCTGCCT-3′ and 5′-CTCCAGAC-TTTGTCCTTCATTTTC-3′; mouse Fas, 5′-GAGAATTGCTGAAGAC-ATGACAATCC-3′ and 5′-GTAGTTTTCACTCCAGACATTGTCC-3′; and β-actin, 5′-AGGCATCCTGACCCTGAAGTAC-3′ and 5′-TCT-TCATGAGGTAGTCTGTCAG-3′.

All PCR products were verified by restriction enzyme analysis. For quantitative analysis, β-actin mRNA, as an internal control, was coamplified with FasL or Fas mRNA by using β-actin primers (0.1–0.2 μM) and FasL/Fas primers (1 μM). PCR products were collected between 25–40 cycles, and the exponential increase in PCR products was confirmed. Conditions for coamplification of rat FasL and β-actin were 94 C for 1 min, 65 C for 1 min, and 72 C for 1 min for 35 cycles in 1.0 mM $MgCl_2$; conditions for coamplification of rat Fas and β-actin were 94 C for 1 min, 65 C for 1 min, and 72 C for 1 min for 35 cycles in 1.5 mM $MgCl_2$; conditions for coamplification of mouse Fas and β-actin were 94 C for 1 min, 53 C for 1 min, and 72 C for 1 min for 35 cycles in 1.5 mM $MgCl_2$.

Western blot analysis

Tissues were weighed and decapsulated into homogenation buffer (10 mM Tris-HCl, 1 mM EDTA, 2.5% SDS, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). After being homogenized and boiled at 95 C for 5 min, samples were centrifuged at 10,000 × *g* for 2 h. Supernatants were collected, and the protein concentration was determined by Bradford assay. Proteins (0.5–1 μg/μl) were loaded into SDS-PAGE gel (Phast Gel, Pharmacia, Uppsala, Sweden) and electrophoresed, then transferred to nitrocellulose. Two anti-Fas antibodies (sc-716 and sc-1024; Santa Cruz Biotechnology; 0.2 μg/ml) were used. For detection of primary antibody, an ECL detection Kit (Amersham, Aylesbury, UK) was used. Crude lysates from CTLL-2, a mouse cytotoxic T cell line, were used as a positive control.

Results

Expression of FasL and Fas in the testis

Immunohistochemistry of 28-day-old rat testicular cross-sections was performed to localize FasL and Fas protein. FasL staining (Fig. 1A) gave a basal and spoke-like pattern, characteristic of localization to Sertoli cells, whereas Fas staining (Fig. 1C) was limited to germ cells, mostly spermatocytes. Rat FasL and Fas proteins were also detected by Western blots using crude lysates of testis (data not shown). TUNEL analysis was performed to detect programmed cell death *in situ* (Fig. 1E). As with Fas immunohistochemistry, the TUNEL technique (Fig. 1, C and E) stained only spermatocytes with a similar incidence. To further explore this association, we modulated the frequency of apoptosis by toxicant treatment. Rats were treated with MEHP, a widely studied Sertoli cell toxicant known to increase germ cell apoptosis within 12 h of exposure (5, 31). After injury, FasL expression became more pronounced in areas of Sertoli cell cytoplasm adjacent to spermatocytes (Fig. 1B), and the number of spermatocytes expressing Fas markedly increased (Fig. 1D). This up-regulated expression of FasL and Fas correlated with increased apoptosis detected by TUNEL analysis (Fig. 1F) and nuclear DNA fragmentation (data not shown).

Up-regulation of FasL and Fas in injury-associated germ cell apoptosis

TUNEL-positive cells on cross-section from control and MEHP-treated testis were counted to quantify the incidence of apoptosis (Fig. 2A). After exposure, the number of TUNEL-positive cells was dramatically increased. Quantitative RT-PCR was performed on total RNA from control and MEHP-treated testes to evaluate the expression level of FasL and Fas mRNA. The induction of FasL and Fas in the MEHP-exposed testis was evident compared with that in controls (Fig. 2B). 2,5-HD was used as another model of toxicant-induced testicular injury. 2,5-HD exposure in the rat produces a well characterized sequence of Sertoli cell dysfunction leading to death of the dependent germ cell population by apoptosis (32–34). The incidence of germ cell apoptosis was quantitated by counting TUNEL-positive cells (Fig. 2C). By 2 weeks after the start of exposure, the incidence of germ cell apoptosis started to increase and peaked at 5 weeks. Using quantitative RT-PCR, FasL and Fas mRNA expression were shown to be dramatically induced by 2,5-HD exposure (Fig. 2D), and the levels of expression corresponded with the increased incidence of apoptosis.

Induction and inhibition of germ cell apoptosis in vitro

To test the function of the Fas-mediated pathway in testis, we transiently blocked FasL expression with an antisense oligonucleotide (35) specific to FasL mRNA in Sertoli-germ cell cocultures and assessed germ cell survival (Fig. 3). Cocultures of rat Sertoli cells and germ cells contain approximately 15% germ cells, most of which die within 1 week (36, 37). Many germ cells in cocultures were stained by the TUNEL technique (data not shown); therefore, this coculture system may be useful for the study of germ cell apoptosis. Oligonucleotides were added to Sertoli-germ cell cocultures, and the level of FasL mRNA was determined after 18 h. Only antisense oligonucleotide-treated cultures showed a marked decrease in FasL mRNA (Fig. 3B), as determined by quantitative RT-PCR. After 2 days of antisense oligonucleotide exposure, germ cell survival in cocultures was increased by about 40% compared with that in untreated controls or cocultures treated with scrambled or sense oligonucleotides (Fig. 3A).

Next, we induced apoptosis of germ cells by incubating with an anti-Fas antibody (Jo-2), an antibody that is known to initiate apoptosis in some Fas-expressing cell types (11, 22). Mouse germ cells were partially purified through differential plating after isolation of Sertoli-germ cell cocultures. The survival of germ cells was determined 3, 6, and 12 h after coincubation with Jo-2 antibody. Fewer germ cells (by ~20–30%) incubated with Jo-2 antibody (5 μg/ml) survived compared to nontreated cells or control IgG antibody-treated cells (Fig. 4).

Up-regulation of Fas in lpr testis after heat exposure

lpr mice show apparently normal spermatogenesis and are fertile. To examine germ cell apoptosis in *lpr* mice, TUNEL analysis was performed on testicular cross-sections from control and *lpr* mice. The incidence of apoptosis in control

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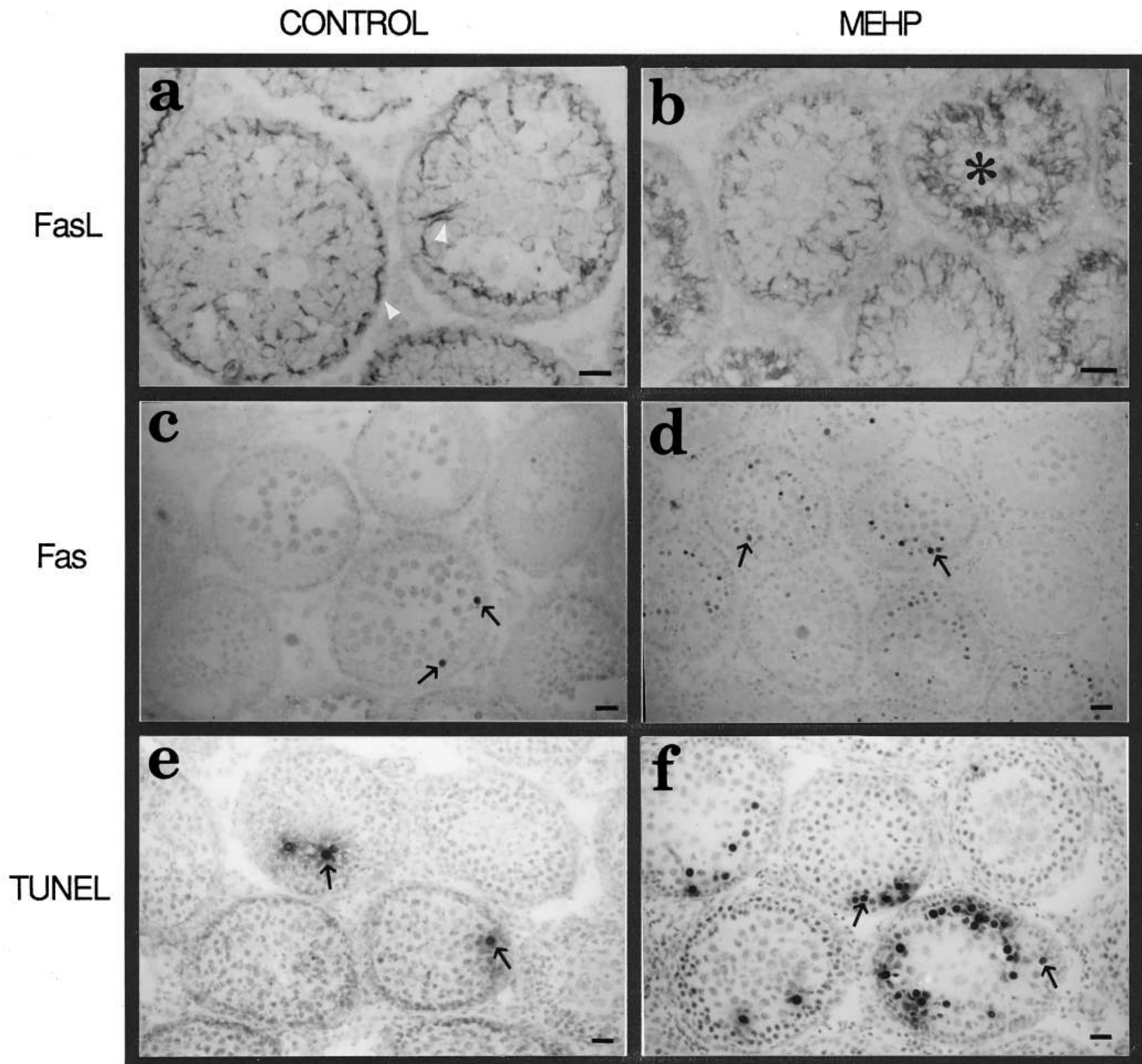

FIG. 1. Localization of FasL and Fas protein in rat testis. A and B, FasL staining (*arrowheads* in A, *asterisk* in B). C and D, Fas staining (*arrows* in C and D). E and F, TUNEL staining (*arrows* in E and F). Compared with sections from untreated rats (A and C), FasL staining was more intense and diffuse, and more spermatocytes were Fas positive in MEHP-treated testes (B and D). An increased number of TUNEL-positive cells, spermatocytes, was seen after MEHP exposure (compare E and F). The staining patterns for Fas and TUNEL are similar. *Scale bar* = 25 μm.

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and *lpr* mice was similar (data not shown). Testes from control and *lpr* mice were exposed to heat to modulate the rate of apoptosis. Animals were killed at different times after heat treatment, and germ cell apoptosis was evaluated by TUNEL staining. Massive germ cell apoptosis occurred in both control and *lpr* mice after heat exposure, and the temporal onset and incidence of apoptosis were the same (Fig. 5A), suggesting that germ cells from control and *lpr* mice behave similarly after heat exposure. Then, the expression of Fas after heat exposure was determined by examining both mRNA levels (Fig. 5B) and protein levels (Fig. 5C). Surprisingly, similar expression of Fas was detected in testes from control and *lpr* mice. Furthermore, Fas expression was dramatically up-regulated in both after heat exposure.

Testicular expression of Fas in lpr mice

To confirm normal expression of Fas in *lpr* testis, different organs from control and *lpr* mice were collected, and the expression level of Fas mRNA was determined by quantitative RT-PCR (Fig. 6). Primers were designed to amplify the 3′-region to the transposon insertion locus in the Fas gene (22). Consistent with previous data (21), high expression of Fas mRNA in liver and thymus from control mice was ob-

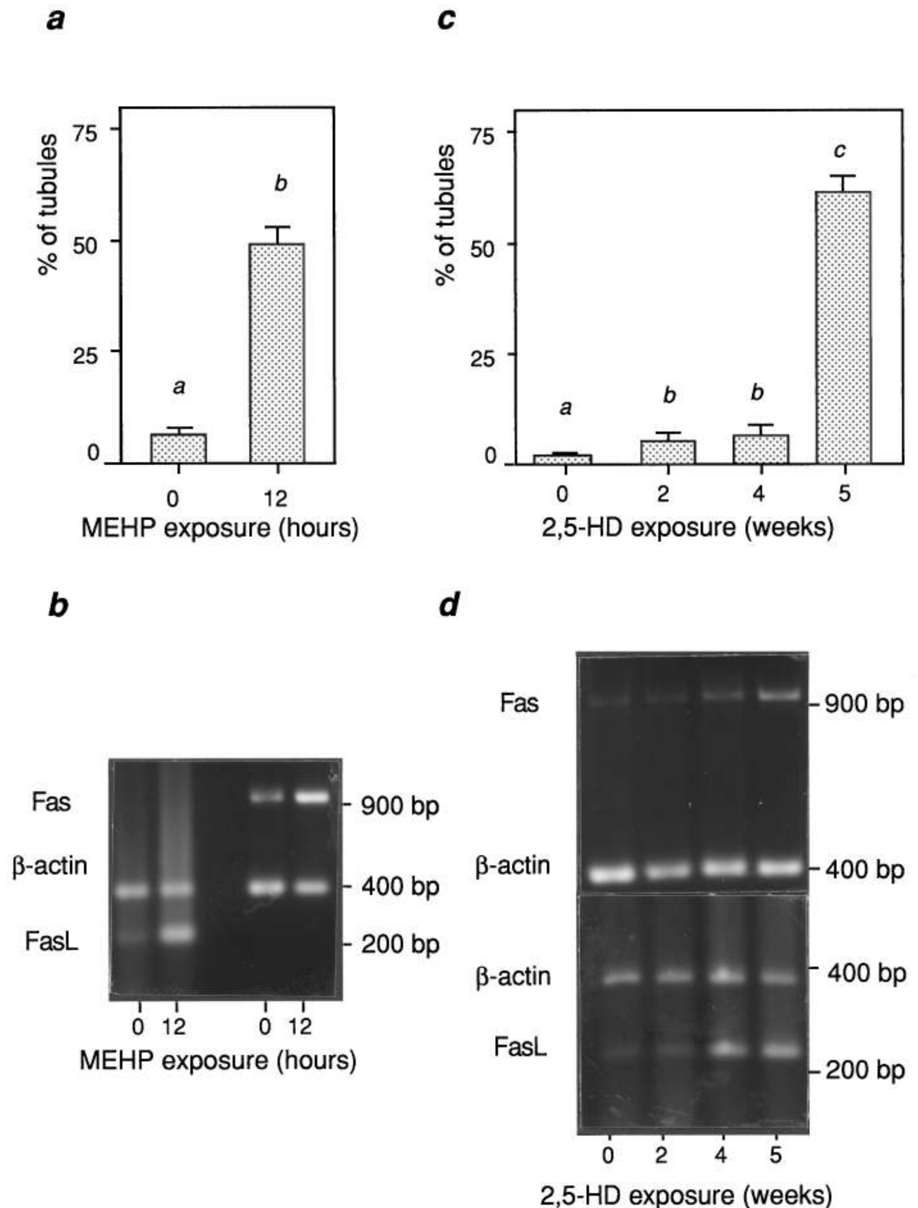

FIG. 2. Quantitation of TUNEL-positive cells and mRNA expression of FasL/Fas after toxicant exposure. A and C, The incidence of apoptosis at each time point was determined by counting the number of TUNEL-positive cells within a seminiferous tubule cross-section. The *abscissa* represents the percentage of seminiferous tubules that contained more than three apoptotic cells of the total seminiferous tubules counted in a cross-section, and the *ordinate* represents toxicant exposure time. *Bars* represent the mean ± SEM. Significant differences ($P < 0.05$, by ANOVA and Fisher's protected least significant differences test) are indicated by different letters. B and D, Quantitative RT-PCR of FasL and Fas mRNA after toxicant exposure. Increased expression of both genes is seen after MEHP or 2,5-HD exposure. β-Actin was used as an internal control for quantitative RT-PCR.

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served, with little or no expression in their *lpr* counterparts. Fas mRNA was also detected in brain from control, but not from *lpr*, mice. Again, testes showed similar expression of Fas mRNA in control and *lpr* mice.

Discussion

In this study, we assessed the role of the Fas-mediated cell death pathway in rodent testis. First, we localized FasL to Sertoli cells and Fas to germ cells in rat testis. The similar staining patterns of Fas-positive cells and TUNEL-positive cells suggested that germ cell apoptosis was mediated by Fas expression. We demonstrated the up-regulation of FasL and Fas after exposure to two Sertoli cell toxicants, MEHP and 2,5-HD. Next, the functional importance of the Fas system in germ cell apoptosis was evaluated. Germ cell apoptosis *in vitro* could be modulated by blocking FasL expression or cross-linking Fas, suggesting that the Fas-mediated pathway is actively involved in signaling germ cell apoptosis. Lastly, we showed that Fas is normally expressed in testes from *lpr* mice and up-regulated after heat injury. The normal expression of Fas is unique to the *lpr* testis, as no or a much reduced amount of Fas was detected in liver, thymus, and brain from *lpr* mice.

The cell type-specific expression of FasL in Sertoli cells and of Fas in germ cells implies the presence of a paracrine mechanism similar to that of Fas-dependent cytotoxicity, in which cytotoxic T cell lymphocytes expressing FasL kill Fas-positive infected cells (23). As FasL staining in MEHP-exposed testis became intense and diffuse in areas of Sertoli cell cytoplasm surrounding spermatocytes, and the number of Fas-positive spermatocytes markedly increased after MEHP exposure, FasL may directly interact with Fas and induce apoptosis. The function of FasL as an initiator of germ cell death was evaluated in two ways: 1) transient blocking of

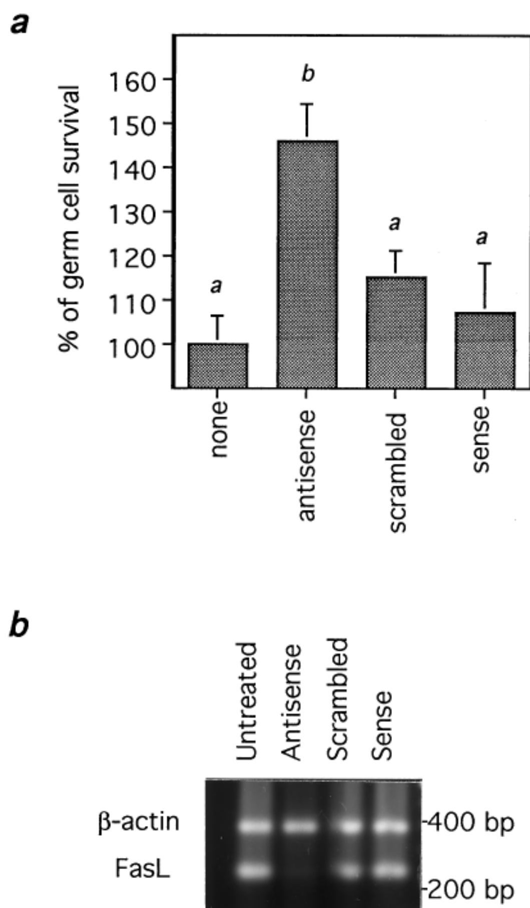

FIG. 3. Germ cell survival in Sertoli-germ cell cocultures after the addition of FasL antisense oligonucleotides. A, Germ cell survival with antisense oligonucleotide treatment. The survival rate after 2 days for the various oligonucleotide-treated groups is presented as a percentage of that of the untreated group. In the untreated group, about 35% of the germ cells survived after 2 days in culture. Only antisense oligonucleotide treatment conferred a significant survival advantage (46% greater than the untreated group). *Bars* represent the mean ± SEM. Significant differences ($P < 0.05$, by ANOVA and Fisher's protected least significant differences test) are indicated by different letters. The data shown are from a representative experiment of three independent experiments performed. B, FasL expression in cocultures treated with antisense, scrambled, or sense oligonucleotides. FasL mRNA expression was decreased in antisense oligonucleotide-treated cells, as determined by quantitative RT-PCR using β-actin as an internal control.

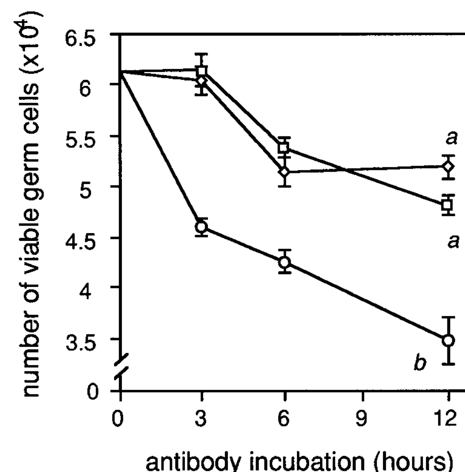

FIG. 4. Mouse germ cell survival after coincubation with anti-Fas antibody. Zero to 12 h after antibody treatment, the numbers of viable cells in the untreated group (*square*), the hamster IgG antibody-treated group (*diamond*), and the Jo-2 antibody-treated group (*circle*) were determined by trypan blue exclusion. *Bars* represent the mean ± SEM from triplicate samples. Significant differences ($P < 0.05$, by ANOVA and Fisher's protected least significant differences test) are indicated by different letters. The data shown are from a representative experiment of three independent experiments performed.

FasL by antisense oligonucleotides, and 2) mimicking FasL using an agonist of Fas, Jo-2 antibody. The fact that germ cell apoptosis *in vitro* could be modulated by interrupting or potentiating the Fas-mediated pathway argues that the Fas system is a key regulator of germ cell apoptosis, at least *in vitro*.

lpr and *gld* mice show apparently normal spermatogenesis and are fertile (38), although spermatogenesis in these mice has not been thoroughly studied. However, our *in vitro* results suggest that disruption of FasL or Fas *in vivo* should result in hyperplasia of early germ cells or abnormal spermatogenesis due to inadequate germ cell apoptosis. There are three possible explanations for this discrepancy: 1) the Fas system is not necessary for initiating apoptosis during spermatogenesis; 2) an alternative pathway is activated in the mutant mice, which assumes the Fas function of apoptosis initiation; or 3) a salvage pathway is activated in the mutant mice that restores the Fas system. There are many examples of knock-out mice in which an essential gene is knocked out, but apparently normal phenotypes are seen (39, 40). It is generally assumed that an alternative pathway(s) takes over the function of the knocked out gene in these circumstances. The apparently normal spermatogenesis in *gld* mice and Fas knock-out mice (41) may be explained in this context. Although the third possibility initially seemed unlikely, our data support activation of a salvage pathway that "rescues" Fas expression in the *lpr* testis. The incidence of testicular germ cell apoptosis in control and *lpr* mice was similar, and after heat exposure, up-regulation of Fas, corresponding to the increased incidence of apoptosis, was similar in both control and *lpr* mice. These data indicate that the *lpr* testis is not Fas deficient, suggesting that a salvage mechanism(s) restores Fas expression.

The transposon element inserted into intron 2 of the Fas gene in the *lpr* mouse results in premature transcript termination or a larger chimeric transcript that incorporates the transposon (19, 20), both of which are nonfunctional. In thymus and liver from *lpr* mice, functional Fas mRNA and protein were expressed at less than 10% of the normal level (21). However, our data show normal expression of testicular Fas in *lpr* mice. Two anti-Fas antibodies used in our experiments were raised against peptides corresponding to amino acids 295–314 (for sc-1024) or 308–327 (for sc-716) mapping at the carboxyl-terminus of mouse Fas. These antibodies detected the same size of proteins in both control and *lpr* testes, excluding the possibility that the Fas protein seen in *lpr* testes is the product of an abnormal transcript. Among several organs tested, including liver, brain, and thymus,

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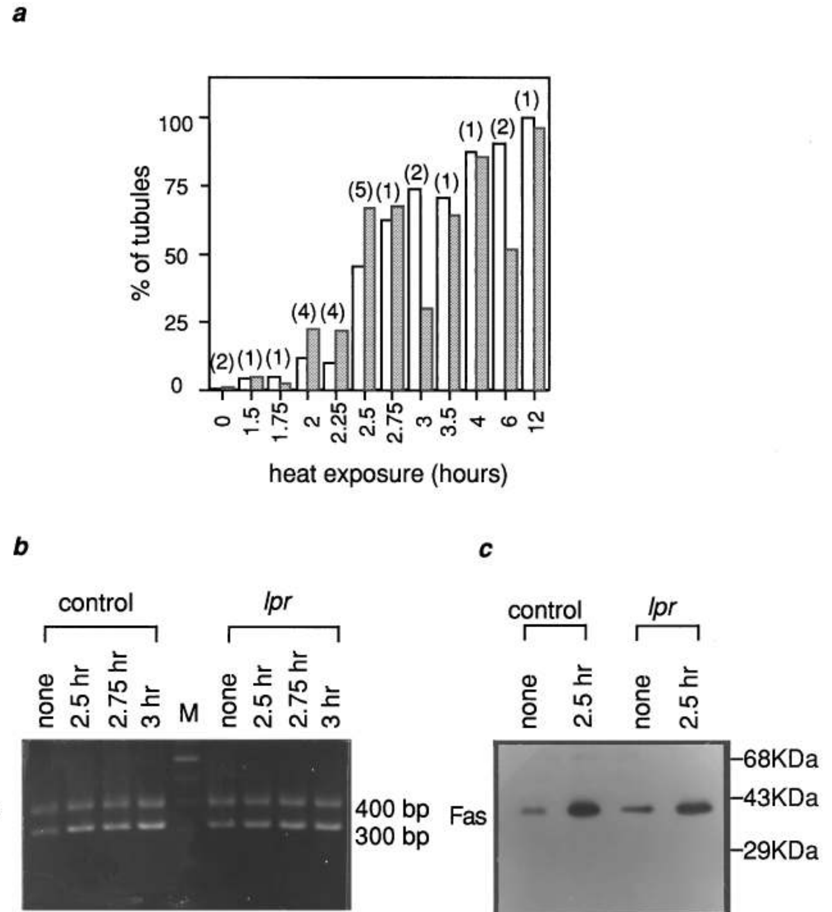

FIG. 5. Consequences of exposure of control and *lpr* mice to testicular immersion at 44 C for 15 min. A, Quantitation of TUNEL-positive cells on testicular cross-sections from control (*open bar*) and *lpr* mice (*solid bar*) after heat exposure. The incidence of apoptosis at each time point was determined by counting the number of TUNEL-positive cells within a seminiferous tubule cross-section. The *abscissa* represents the percentage of seminiferous tubules that contained more than four apoptotic cells of the total seminiferous tubules counted in a cross-section, and the *ordinate* represents hours after heat exposure. The *numbers in parentheses* represent the number of animals used at each time point. No significant differences ($P < 0.05$, by ANOVA and Fisher's protected least significant differences test) were observed between control and *lpr* groups at any time point. B, Quantitative RT-PCR of Fas mRNA after heat exposure. A 100-bp DNA marker (M) is shown. C, Western blot analysis of Fas protein (~43 kDa) after heat exposure.

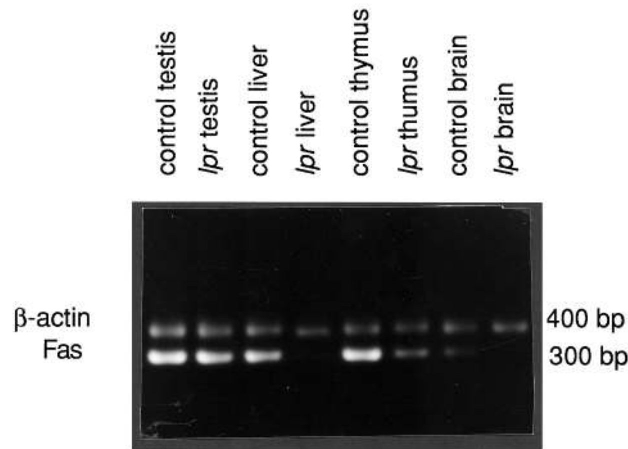

FIG. 6. Expression of Fas mRNA in different tissues from control and *lpr* mice, determined by quantitative RT-PCR using β-actin as an internal standard.

testis was the only organ that expressed Fas at the same level in control and *lpr* mice.

The unique testicular expression of Fas may be explained by the fact that the *lpr* mouse is a spontaneous mutant with a leaky mutation. As such, compensatory mechanisms unique to the testis may result in normal Fas expression. For example, overcrowding of germ cells in the seminiferous epithelium could result in insufficient Sertoli cell support. Germ cells may sense this inadequate environment and activate a self-elimination pathway, in which Fas is involved. In this situation, the transcriptional inefficiency of the Fas gene in *lpr* mice might be overcome, at least in testis, by repeated transcription of the Fas gene. If so, our results strongly argue for the importance of the Fas system in testicular homeostasis. Alternatively, the *lpr* mutation may have little or no influence on the expression of the Fas gene in *lpr* testis because of testis-specific mechanisms of transcriptional regulation. Many genes are known to be regulated in a germ cell-specific way (42). In this context, an unknown mechanism(s), developed by natural selection of a fertile *lpr* mutant, could result in normal expression of Fas in *lpr* testis. To fully assess these alternative explanations of normal Fas expression in the *lpr* testis will require an extensive series of experiments, including a determination of the relative ratios of different Fas transcripts, including premature transcript, chimeric transcript, and normal transcript seen in *lpr* mice. The ratio of premature transcript, chimeric transcript, and normal transcript seen in *lpr* mice may be tissue dependent, which may explain the unique Fas expression in *lpr* testis.

Cross-talk between Sertoli and germ cells via cell to cell contact or secretion of paracrine factors was supported by several observations (43, 44). Sertoli cells, the only supportive cells in the seminiferous epithelium, respond to and provide the hormonal and nutritional needs of germ cells. Therefore, alterations in Sertoli cell function may result in germ cell loss.

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The modes of action of MEHP and 2,5-HD are known to be different, but both selectively target the Sertoli cell (31). Thus, up-regulation of FasL in both models of toxicant-induced testicular injury suggests an endogenous activation of the Fas-mediated pathway, resulting from injured Sertoli cells within the seminiferous tubule. We hypothesize that in the normal state Sertoli cells express FasL and signal the killing of Fas-positive germ cells, limiting the size of the germ cell population to numbers they can support. After injury, Sertoli cells increase FasL expression to reach a new equilibrium state, which matches the reduced supportive capacity of the dysfunctional Sertoli cells with fewer germ cells. Up-regulation of Fas in germ cells is thus seen as a self-elimination process for cells that are destined to die because of inadequate support. The Fas-mediated pathway in the testis, in which active culling is initiated from Sertoli cells, may show a different aspect of paracrine control between Sertoli and germ cells, that is a negative selection process of germ cells as opposed to positive support.

Acknowledgments

MEHP was a gift from the National Institute of Environmental Health Sciences. We thank L. Braun and K. Miller for their helpful comments.

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