Key Apoptotic Pathways for Heat-Induced Programmed Germ Cell Death in the Testis

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Short-term exposure (43 C for 15 min) of the rat testis to mild heat results within 6 h in stage- and cell-specific activation of germ cell apoptosis. Initiation of apoptosis was preceded by a redistribution of Bax from a cytoplasmic to paranuclear localization in heat-susceptible germ cells. Here we show that the relocation of Bax is accompanied by cytosolic translocation of cytochrome *c* and is associated with activation of the initiator caspase 9 and the executioner caspases 3, 6, and 7 and cleavage of poly(ADP) ribose polymerase. Furthermore, early in apoptosis, a significant amount of Bax also accumulates in endoplasmic reticulum, as assessed by Western blot analyses of fractionated testicular lysates. In additional studies using the FasL-defective *gld* mice, we have shown that heat-induced germ cell apoptosis is not blocked, thus providing evidence that the Fas signaling system may be dispensable for heat-induced germ cell apoptosis in the testis. Taken together, these results demonstrate that the mitochondria- and possibly also endoplasmic reticulum-dependent pathways are the key apoptotic pathways for heat-induced germ cell death in the testis. (*Endocrinology* 144: 3167–3175, 2003)

GERM CELL DEATH has long been recognized as a significant feature of mammalian spermatogenesis (1). In adult rats this loss is incurred mostly during spermatogonial development (up to 75%) and to a lesser extent during maturation divisions of spermatocytes and spermatid development (2). A growing body of evidence demonstrates that both spontaneous (during normal spermatogenesis) and increased germ cell death triggered by various regulatory stimuli, including deprivation of gonadotropins and intratesticular testosterone by GnRH antagonist (3, 4) or by estradiol treatment (5), exposure to local testicular heating (6–8), Sertoli cell toxicant (9), and chemotherapeutic agents (6, 10) in rats occur via apoptosis. Recent studies in humans have demonstrated that both spontaneous (11) and increased germ cell death in conditions of abnormal spermatogenesis involve apoptosis and implicate a prominent role of programmed germ cell death in male fertility (12–15). However, the mechanisms by which these proapoptotic stimuli activate germ cell apoptosis are not well understood. Understanding the molecular components of the apoptotic program in cells is an essential step toward the development of novel therapeutic regimens to control accelerated apoptosis during abnormal spermatogenesis as well as more targeted approaches to male contraception.

Two major pathways, intrinsic and extrinsic, are involved in the process of caspase activation and apoptosis in mammalian cells (16–20). The intrinsic pathway for apoptosis involves the release cytochrome *c* into the cytosol where it binds to apoptotic protease-activating factor-1 (Apaf-1), a mammalian homolog of the *Caenorhabditis elegans* cell death protein CED 4. Once activated (possibly through oligomerization) by cytochrome *c*, Apaf-1 binds to procaspase 9 via the caspase recruitment domain at the amino terminus in the presence of deoxy-ATP, resulting in activation of the initiator caspase 9 and subsequent proteolytic activation of executioner caspases 3, 6, and 7. The active executioners are then involved in the cleavage of a set of proteins, including poly-(ADP) ribose polymerase (PARP), lamin, actin, and gelsolin, and causes morphological changes to the cell and nucleus typical of apoptosis. Members of the Bcl-2 family of proteins play a major role in governing this mitochondria-dependent apoptotic pathway, with proteins such as Bax functioning as an inducer and proteins such as Bcl-2 as suppressors of cell death (16–20). The extrinsic pathway for apoptosis involves ligation of the death receptor (such as Fas) to its ligand (FasL). Binding of FasL to Fas induces trimerization of Fas receptors, which recruit Fas-associated death domain (FADD) through shared death domains. FADD also contains a death effector domain in its N-terminal region. The Fas/FADD complex then binds to the initiator caspase 8 or 10 through interactions between the death effector domain of the FADD and these caspase molecules. Caspase 8 or 10 then activates the effector or executioner caspases 3 and 7, resulting in cellular disassembly. Cross-talk between these pathways occurs at multiple levels. A third subcellular compartment, the endoplasmic reticulum (ER), has also shown to be involved in apoptotic execution of renal tubular epithelial cells and cortical neurons (21). Both pathways converge on caspase 3 and other executioner caspases and nucleases that drive the terminal events of programmed cell death.

Recently, we analyzed the temporal and stage- and cell-specific changes in the activation of germ cell apoptosis in the

rat after mild testicular hyperthermia (22). Germ cell apoptosis, involving exclusively early (stages I–IV) and late stages, was activated within 6 h of heating. Cell types most susceptible to heat included pachytene spermatocytes and early spermatids at stages I–IV and pachytene, diplotene, and dividing spermatocytes at stages XII–XIV. We also noted in that study that the initiation of apoptosis was preceded by a redistribution of Bax from a cytoplasmic to paranuclear localization within 0.5 h after heating, as assessed by immunocytochemistry, and hypothesized that such relocation of Bax might represent an essential step in an apoptotic pathway leading to germ cell death triggered by mild testicular hyperthermia. The rapid induction of selective germ cell apoptosis after a single heat exposure thus provides an excellent *in vivo* model system for studying underlying mechanisms of programmed germ cell death in the testis. In this study, using the rat model of testicular hyperthermia, we characterize the key molecular components of the effector pathway(s) for germ cell death in the testis. Our results demonstrate that the mitochondria- and possibly also ER-dependent pathways play a major role in heat-induced germ cell apoptosis.

Materials and Methods

Animals and experimental protocol

Adult (90-d-old) male Sprague Dawley rats (350–375 g) purchased from Charles River Laboratories, Inc. (Wilmington, MA), were housed in a standard animal facility under controlled temperature (22 C) and photoperiod (12 h of light, 12 h of darkness) with food and water *ad libitum*. Heating of the scrota of the adult rats was performed as described previously (7). Briefly, after rats were anesthetized with an ip injection of sodium pentobarbital (40 mg/kg body weight), their scrota were immersed in a thermostatically controlled water bath at 43 C (treated) for 15 min, and animals were killed 0.5, 2, and 6 h after heat exposure. Control rats were immersed in a water bath maintained at 22 C and killed 6 h after exposure. To further characterize the involvement of the Fas system, we examined the incidence of heat-induced germ cell apoptosis in FasL-defective generalized lymphoproliferative disease (*gld*) mice (23). In these mice there is a point mutation near the COOH terminus of the coding region. This mutation results in the replacement of phenylalanine with leucine and abolishes the ability of FasL to bind to Fas. Seven- to 8-wk-old male wild-type (C57BL6J) and *gld* (B6Smn.C3H-*FasL^gld*) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Similar to our rat model, scrota from group 6 wild-type and *gld* mice were exposed to 22 C (control) or 43 C (treated) for 15 min, and the mice were killed 2 and 6 h after heat exposure. Animal handling and experimentation were performed in accordance with the recommendation of the American Veterinary Medical Association and were approved by the Harbor-University of California-Los Angeles Research and Education Institute animal care and use review committee.

Blood collection and tissue preparation

Both control and experimental animals were injected with heparin (130 IU/100 g body weight, ip) 15 min before a lethal injection of sodium pentobarbital (100 mg/kg body weight, ip) to facilitate testicular perfusion using a whole body perfusion technique (24). Blood samples were collected from each animal by cardiac puncture immediately after death, and plasma was separated and stored at −20 C for subsequent hormone assays. After perfusion with saline, one testis was removed, decapsulated, and weighed. Portions of testicular parenchyma were snap-frozen in liquid N_2 and stored at −70 C for subsequent analysis. The remaining portions of testicular parenchyma were used for subcellular fractionation and Western blotting. The contralateral testes were then fixed by vascular perfusion with either 5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) or Bouin's solution (Sigma-Aldrich Corp., St. Louis, MO).

The testes were removed and processed for routine paraffin embedding for either *in situ* detection of apoptosis or immunohistochemistry. Portions of glutaraldehyde-fixed testes were further diced into small pieces, postfixed into 1% osmium tetroxide, and embedded in Epon 812. Thin sections from selected tissue blocks were cut with an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Hitachi 600 electron microscope (Hialeah, FL) (24).

Assessment of apoptosis

In situ detection of cells with DNA strand breaks was performed in glutaraldehyde-fixed, paraffin-embedded testicular sections by the terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling (TUNEL) technique (4, 7, 8, 11) using an ApopTag-peroxidase kit (Intergen, Purchase, NY). Enumeration of the nonapoptotic Sertoli nuclei with distinct nucleoli and an apoptotic germ cell population was carried out in both wild-type and *gld* mice at stages I–IV, V–VI, VII–VIII, IX–X, and XI–XII using a BH-2 microscope (Olympus Corp., New Hyde Park, NY) with a ×100 oil immersion objective. For each mouse, at least 10 tubules/stage group were used. These stages were identified according to the criteria proposed by Russell *et al.* (1) for paraffin sections. The rate of germ cell apoptosis (apoptotic index) was expressed as the number of apoptotic germ cells per Sertoli cell (4, 7, 8, 10–12, 22).

Immunohistochemical analyses

Bouin's fixed, paraffin-embedded testicular sections were immunostained as described previously (22, 25). Primary antibodies included rabbit polyclonal cleaved caspase 9 (1:50; this antibody detects only the cleaved product p38 and p17 of active caspase 9), caspase 6 (1:50; this antibody recognizes only the p18 subunit of active caspase 6), and caspase 7 (1:50; this antibody recognizes only the p20 subunit of active caspases 7) antibodies (Cell Signaling Technology, Beverly, MA), and a rabbit polyclonal caspase 3 antibody (1:1000; CM1) that recognizes only the cleaved product of p18 and p12 subunits of active caspase 3, but not the inactive zymogen (26). Immunoreactivity was detected using biotinylated goat antirabbit immunoglobulin G secondary, antibody followed by avidin-biotinylated horseradish peroxidase complex visualized with diaminobenzidine tetrahydrochloride according to the manufacturer's instructions (Rabbit Unitect Immunohistochemistry Detection System, Oncogene Science, Inc., San Diego, CA). Slides were counterstained with hematoxylin.

Immunofluorescence and confocal studies

Activation of the initiator caspase 9 and the executioner caspase 3 in germ cells undergoing apoptosis was also detected by confocal microscopy using double immunostaining for the active (red) caspase 9 (1:50) or 3 (1:1000) and DNA fragmentation (green). *In situ* detection of cells with DNA strand breaks was performed in Bouin's fixed, paraffin-embedded testicular sections using an ApopTag-fluorescein kit (Intergen). In brief, after deparaffinization and rehydration, tissue sections were incubated with proteinase K for 15 min at room temperature, washed in distilled water, and then treated with 2% hydrogen peroxide in PBS for 5 min at room temperature to quench endogenous peroxidase activity. Sections were incubated with a mixture containing digoxigenin-conjugated nucleotide and terminal deoxynucleotidyl transferase in a humidified chamber at 37 C for 1 h and subsequently treated with antidigoxigenin-fluorescein for 30 min in the dark. After fluorescein staining, slides were washed in PBS and incubated with blocking serum for 20 min to reduce nonspecific antibody binding. For staining of caspases, slides were then incubated in a humidified chamber for 1 h with rabbit polyclonal active caspase 3 or 9 antibody, followed by goat antirabbit Texas Red-labeled secondary antibody for 45 min at room temperature. Slides were washed and then mounted in ProLong Antifade (Molecular Probes, Inc., Eugene, OR). For controls, sections were treated only with secondary antibody, and no signals were detected. Confocal imaging was performed using a TCS-SP-MP confocal microscope (Leica Corp., Deerfield, IL).

Subcellular fractionation and Western blotting

Cytosolic, mitochondrial, and ER fractions were prepared as a modification of the procedure previously described (27). Briefly, saline-perfused testes were homogenized using a Dounce homogenizer in 3 ml buffer A (0.25 M sucrose, 50 mM HEPES, 10 mM NaCl, 10 mM EDTA, and 2 mM dithiothreitol) supplemented with protease inhibitors (Complete Protease Inhibitors, Roche, Indianapolis, IN). The crude homogenates were centrifuged at $1,000 \times g$ for 10 min at 4 C, and the resultant supernatant was centrifuged at $10,000 \times g$ for 15 min at 4 C to sediment the low speed fraction containing mainly mitochondria. The mitochondria were washed twice in buffer A and pelleted. The cytosolic and high speed fractions were isolated after centrifugation of the $10,000 \times g$ supernatant fraction at $100,000 \times g$ for 60 min at 4 C. The resulting supernatant was the cytosolic fraction, and the pellet was resuspended in buffer A to yield the high speed or ER fraction. The purity of the cytosolic, mitochondrial, and ER fractions was assessed by Western blotting using antibodies to cytochrome c oxidase subunit IV (0.2 μg/ml; Molecular Probes, Inc.), porin 31HL, Grp78 (1:500), and Erp72 (Calbiochem-Novabiochem Corp., San Diego, CA). Western blotting was performed using rat testicular lysates and subcellular fractions as described previously (22). SDS-PAGE gels were loaded with 50 μg protein/lane and run at 160 V. Membranes were probed using a rabbit polyclonal antibody to cytochrome c (1:200), Bax (1:200), Bcl-2 (1:200), PARP (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Grp78 (1:500; Calbiochem), or a mouse monoclonal antibody to VDAC1 or porin 1 (1:500; 31 HL, Calbiochem), Erp72 (1 μg/ml; Calbiochem), or β-actin (1:2000; Sigma-Aldrich Corp.). For immunodetection, membranes were incubated with SuperSignal Western blotting detecting reagents (Pierce Chemical Co., Rockford, IL) and exposed to x-ray film (Fuji Photo Film Co., Ltd., Stamford, CT). Band intensities were determined using Quantity One software from Bio-Rad Laboratories, Inc. (Hercules, CA).

Statistical analysis

Statistical analyses were performed using the SigmaStat 2.0 program (Jandel Corp., San Rafael, CA). Results were tested for statistical significance using the Student-Newman-Keuls test after one-way repeated measures ANOVA. Differences were considered significant at $P < 0.05$.

Results

Heat-induced stage- and cell-specific activation of germ cell apoptosis in rat testis

To delineate the apoptotic pathways involved in heat-induced germ cell death, we first examined the initiation of germ cell apoptosis after transient exposure to heat. Spontaneous apoptosis of germ cells in control rats was seen primarily in type A spermatogonia and a few spermatocytes late in meiosis. In contrast, in conjunction with our previous observations (22), extensive numbers of TUNEL-positive cells were observed exclusively in the early and late stages 6 h after mild testicular hyperthermia. The effect of heat on spermatogenesis was not only stage specific, but also cell type specific. Cell types most susceptible to heat included pachytene spermatocytes and early spermatids at stages I–IV and pachytene, diplotene, and dividing spermatocytes at stages XII–XIV. Electron microscopic observations of testicular materials further confirmed the identity of various apoptotic germ cells characterized by light microscopy (Fig. 1). As expected from previous studies (22), we also found that the initiation of germ cell apoptosis was preceded by a redistribution of Bax from a cytoplasmic to paranuclear (into a crescent-shaped area close to the nuclear periphery) localization in the heat-susceptible germ cells.

Redistribution of Bax is accompanied by cytosolic translocation of cytochrome c

Cytosolic translocation of cytochrome c has been proposed to be an essential component in the mitochondria-dependent pathway for apoptosis in various extragonadal cell systems
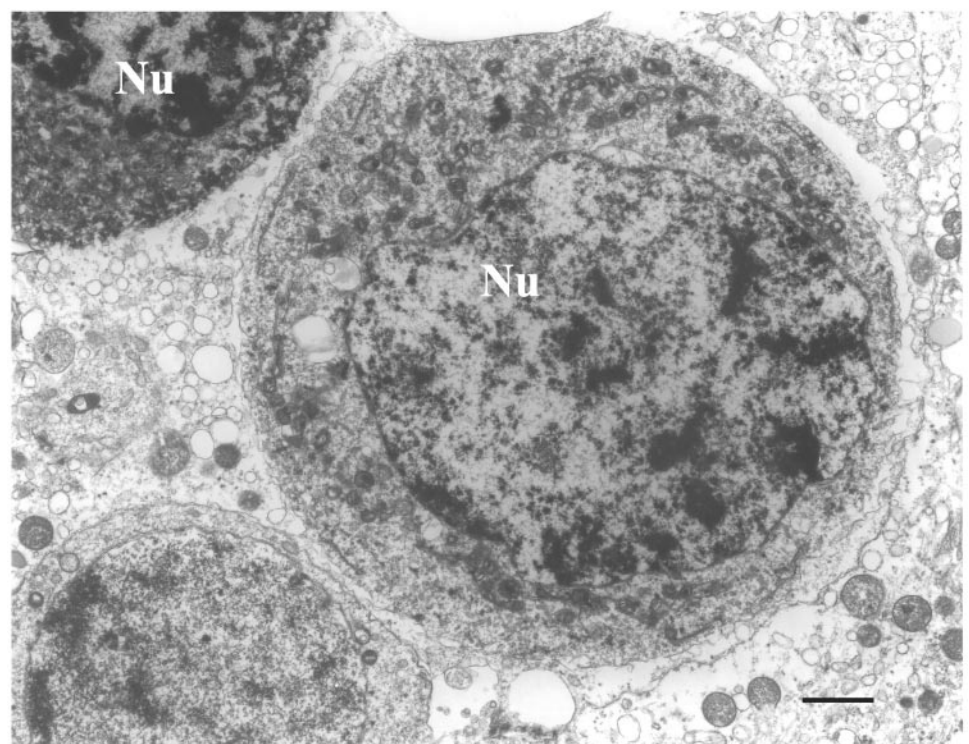

FIG. 1. Electron micrograph showing a late pachytene spermatocyte at early phase of apoptosis from a heat-treated rat 2 h after heating. Also shown is a portion of an advanced late pachytene spermatocyte in the *upper left corner*. Note the varying degrees of chromatin condensation and fragmentation typical of apoptosis in these cells. ν, Nucleus. *Scale bar*, 1 μm.

(16–20). Accordingly, as a first step we examined the cytochrome *c* release during heat-induced programmed germ cell death. Testicular lysates were fractionated into cytosolic and mitochondrial fractions and analyzed by Western blotting. As shown in Fig. 2A, no cytochrome *c* was detected in cytosol from control testis. In contrast, cytosolic accumulation of cytochrome *c* was clearly evident at 0.5, 2, and 6 h after heat treatment. Mild testicular hyperthermia, on the contrary, had no effect on the total levels of cytochrome *c* in whole testicular lysates (Fig. 2B).

Translocation of Bax to ER during heat-induced germ cell apoptosis

We further examined the relocation of Bax into the ER. Testicular lysates from control and heat-treated rats were fractionated into cytosolic, mitochondrial, and ER fractions and analyzed by Western blotting (Fig. 3). The quality of fractionation was controlled by the presence of known component-specific proteins. In untreated rats, the majority of the testicular Bax was present in both cytosolic and mitochondrial fractions, with minimal amounts in the ER fraction. Upon exposure of the testis to mild heat, the amount of Bax increased significantly ($P < 0.05$) by 1.9-fold (over the values measured in controls) in the ER fraction within 0.5 h of heating, but was not different from the control values thereafter. This suggests a possible translocation of Bax to ER early during initiation of apoptosis. Bcl-2 was only detected in the mitochondrial fraction, and as expected from our previous
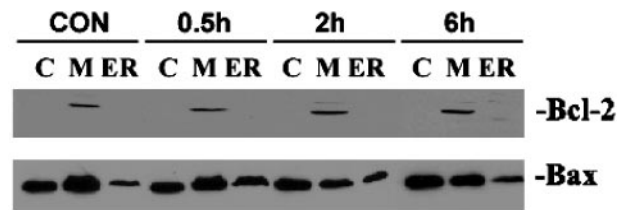

FIG. 3. Western blot analysis of Bax and Bcl-2 in cytosolic (C), mitochondrial (M), and ER fractions of testicular lysates from control and heat-treated rats at 0, 0.5, 2, and 6 h after heat treatment. Data are representative of four animals at each time point from one of three separate experiments.

studies, its levels were increased significantly ($P < 0.05$) by 2.0-fold (over the values measured in controls) at 2 and 6 h after local testicular heating.

Cytosolic translocation of cytochrome c is associated with activation of the initiator caspase 9 and the executioner caspases 3, 6, and 7 and cleavage of PARP during heat-induced programmed germ cell death

Because the release of cytochrome *c* from mitochondria into cytosol triggers caspase activation (reviewed in Refs. 16–20), we then examined the activation of the initiator caspase 9 and the executioner caspases 3, 6, and 7. Within 2 h of heat treatment, the initiator caspase 9 and the effector caspase 3 were activated in heat-susceptible late pachytenes, as evidenced by immunofluorescence staining of active caspases 9 and 3 (Figs. 4 and 5). We also found activation of caspases 6 and 7 during heat-induced programmed germ cell death (data not shown).

To further explore the potential involvement of these effector caspases in heat-induced germ cell apoptosis, we then examined the cleavage of their downstream substrate PARP. As shown in Fig. 6, no PARP cleavage product was detected in cytosol from untreated testis. In contrast, PARP cleavage product was clearly detected at 0.5, 2, and 6 h after heat treatment.

Testes of gld mice that express a nonfunctional form of Fas ligand are equally sensitive to heat-induced germ cell apoptosis

To evaluate the involvement of the Fas signaling system in heat-induced testicular germ cell apoptosis, scrota from wild-type and *gld* mice that harbor a loss of function mutation of FasL were exposed once to 22 or 43 C for 15 min, and the animals were killed 2 or 6 h after heat treatment. Testes from *gld* mice expressed both Fas and FasL as abundantly as those from wild-type mice (Fig. 7). Western blot analysis further showed no obvious alterations of FasL levels, whereas Fas levels appeared to be slightly lower in testes of *gld* mice 6 h after heat treatment (Fig. 7). Both groups of untreated mice exhibited a few TUNEL-positive cells involving primarily differentiating spermatogonia and a few spermatocytes late in meiosis (Fig. 8A). In contrast, a massive increase in the number of apoptotic germ cells predominantly at stages I–IV and XI–XII was readily observed in both wild-type and *gld* mice (Fig. 8B) as early as 2 h after local testicular heating. As shown in Fig. 9, the incidence of germ
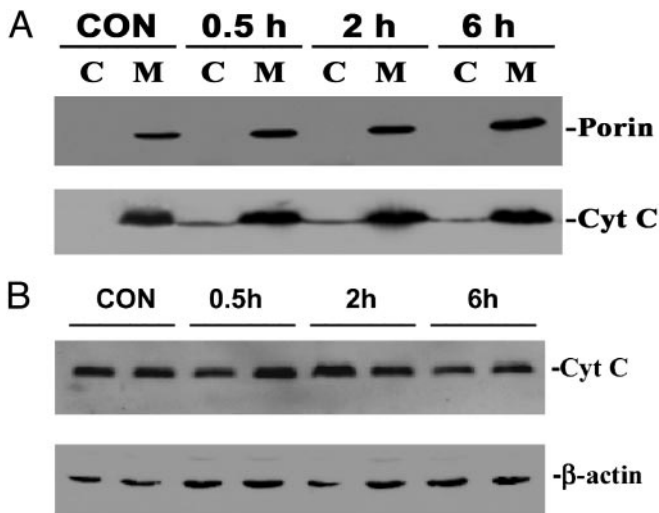

FIG. 2. Accumulation of mitochondrial cytochrome *c* in the cytosol. A, Representative Western blots of cytosolic (C) and mitochondrial (M) fractions of testicular lysates from control and heat-treated rats at 0, 0.5, 2, and 6 h after heat treatment show accumulation of cytochrome *c* only after heat treatment. No cytochrome *c* is detected in the cytosol from control testis. Porin 31 HL was used to determine the purity of the mitochondrial fraction. The absence of porin, a specific marker for mitochondrial outer membrane, in cytosolic extracts confirmed that the cytosolic preparations were free of mitochondrial contamination. Data are representative of four animals at each time point from one of three separate experiments. Mild testicular hyperthermia, on the contrary, had no effect on the total levels of cytochrome *c* in whole testicular lysates (B). The gels are representative of two animals at each time point from one of three separate experiments. β-Actin in the immunoblot is shown as a loading control.
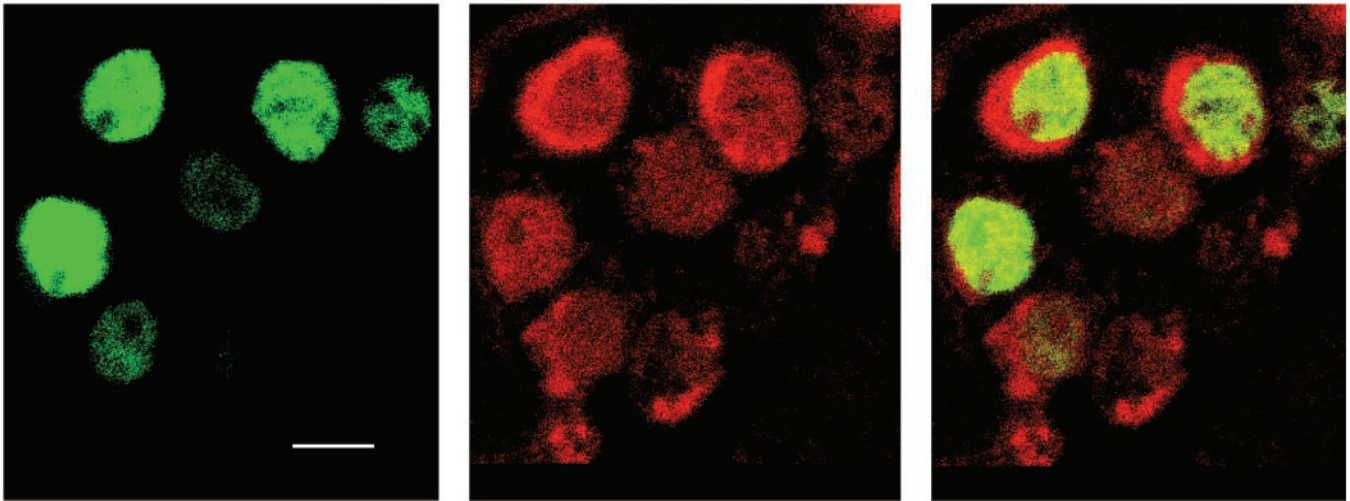
Fig. 4. Activation of caspase 9 in rat testicular germ cells undergoing apoptosis. Confocal images of late pachytene spermatocytes from a rat that had been exposed to short-term local testicular heating show TUNEL (*green*) and active caspase 9 (*red*) within 2 h of heating. In the merged panels, *green* corresponds to TUNEL, and *red* to active caspase 9; colocalization between TUNEL and active caspase 9 in the nucleus is detectable as *yellow*. *Scale bar*, 10 μm.
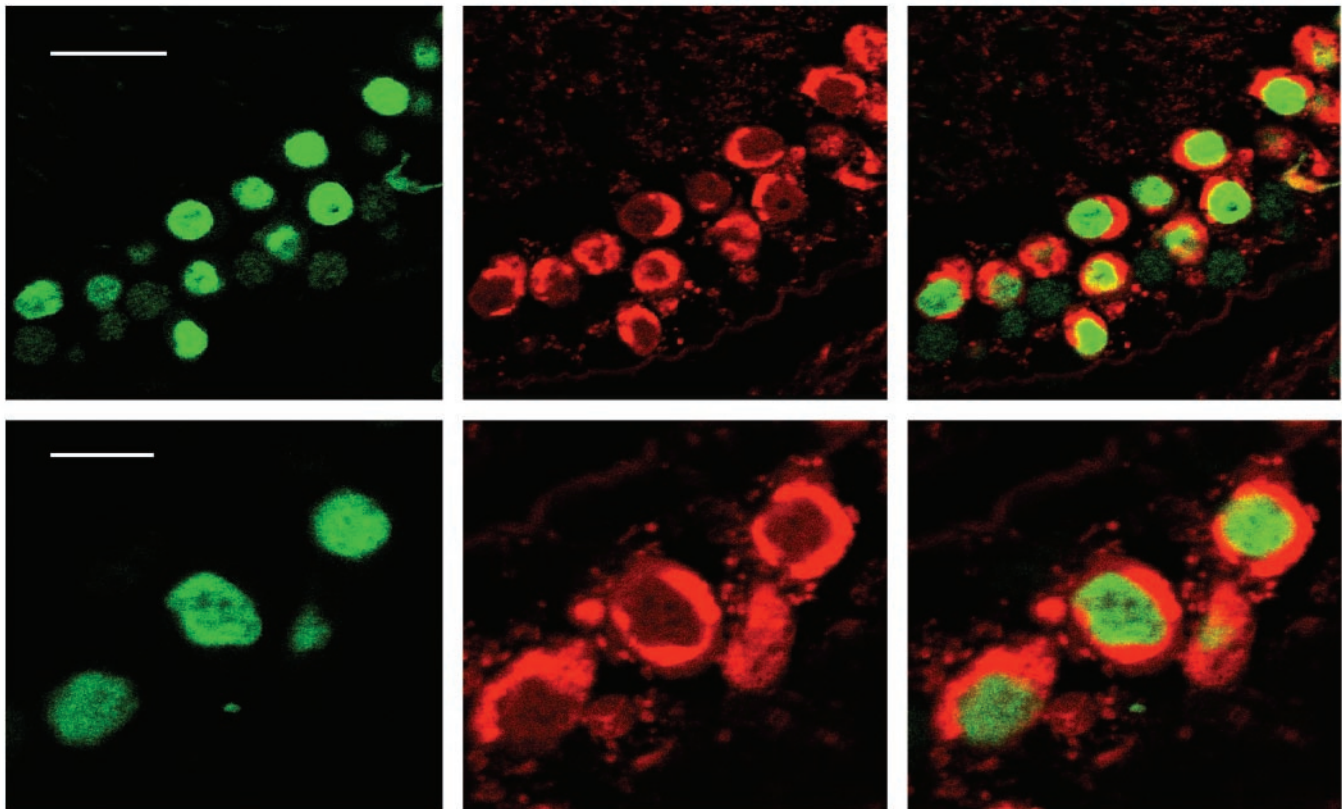

Fig. 5. Activation of caspase 3 in rat testicular germ cells undergoing apoptosis. Low (in the *upper panels*) and higher magnified (in the *lower panels*) confocal images of late pachytene spermatocytes from a rat that had been exposed to short-term local testicular heating show TUNEL (*green*) and active caspase 3 (*red*) within 2 h of heating. *Scale bar*: *upper panels*, 25 μm; *lower panels*, 10 μm.

cell apoptosis (expressed as the number of apoptotic germ cell per Sertoli cell) at various stages of the seminiferous epithelial cycle was very low in both untreated wild-type (0.06–0.29) and *gld* (0.04–0.23) mice. Massive germ cell apoptosis occurred between 2 and 6 h after heat exposure,

predominantly at stages I–IV (1.14 $\pm$ 0.48 and 1.66 $\pm$ 0.55, respectively; $P < 0.05$) and XI–XII (2.03 $\pm$ 0.28 and 2.67 $\pm$ 0.47, respectively; $P < 0.05$) in wild-type males. Surprisingly, the temporal onset and incidence of germ cell apoptosis after heat treatment were similar in wild-type and *gld* mice, sug-
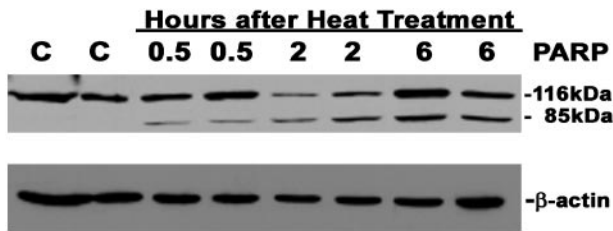
FIG. 6. PARP cleavage after mild testicular hyperthermia. Western blot analysis of rat testicular lysates at 0, 0.5, 2, and 6 h after heat treatment indicates PARP cleavage as early as 0.5 h of heating. The gels are representative of two animals at each time point from one of three separate experiments. β-Actin in the immunoblot is shown as a loading control.
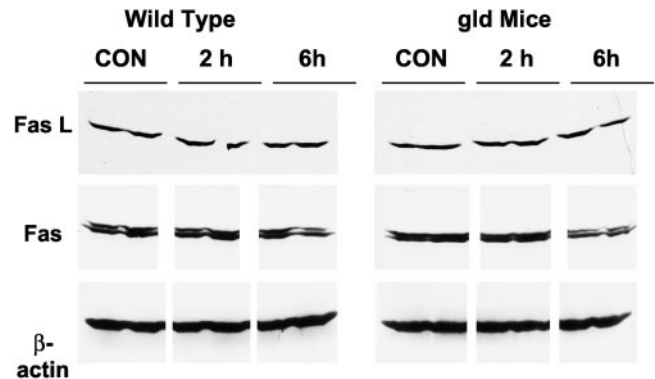

FIG. 7. Western blot analysis of Fas and FasL in testicular lysates from wild-type and FasL-defective *gld* mice at 0 (control), 2, and 6 h after heat treatment. Testicular lysates were analyzed by SDS-PAGE and immunoblotted with a rabbit polyclonal Fas (1:250; StressGene) or a mouse monoclonal FasL (1:500; BD Transduction Laboratories, Inc., Lexington, KY). There were no obvious alterations in FasL levels, whereas Fas levels appeared to be slightly lower in the testes of *gld* mice 6 h after heat treatment. The gels are representative of two animals at each time point from one of three separate experiments. β-Actin in the immunoblot is shown as a loading control.

gesting that germ cells of wild-type and *gld* mice are equally sensitive to heat-induced germ cell death.

Discussion

Programed germ cell death occurs spontaneously during spermatogenesis and can be induced in a stage- and cell-specific manner by a variety of proapoptotic stimuli, including mild testicular hyperthermia (7, 28). In a recent study we demonstrated that a single transient testicular hyperthermia (43 C for 15 min) induces, as early as 6 h, stage- and cell-specific activation of germ cell apoptosis in rats (22). In the present study using this model system, we characterize the signaling events in heat-induced germ cell apoptosis in the testis. The results of the present study show that the initiation of germ cell apoptosis (detected by TUNEL assay) after heat stress is preceded by cytosolic translocation of cytochrome *c*. The release of cytochrome *c* from mitochondria into the cytosol is further associated with activation of the initiator caspase 9 and the executioner caspases 3, 6, and 7, and cleavage of PARP.

The Bcl-2 family of proteins governs the mitochondria-dependent pathway for apoptosis (16–20). One of the intriguing aspects of apoptosis regulation by members of this family is their subcellular localization and translocation. Some Bcl-2 family members, such as Bcl-2 and Bak, constitutively localize to the mitochondrial membrane, whereas others, such as Bax and Bid, translocate from cytosol to mitochondria early during apoptosis (29–31). Furthermore, insertion of Bax into mitochondrial membranes has been shown to play an essential role in releasing cytochrome *c* from the mitochondrial membrane space to the cytosol in various cell systems (32–34). Thus, it is conceivable that the signal for cytochrome *c* release from mitochondria in heat-induced testicular germ cell apoptosis emanates from relocation of Bax to mitochondria.

The release of cytochrome *c* from mitochondria initiates caspase activation by binding to the caspase-activating protein Apaf-1 (reviewed in Ref. 35). Indeed, in the present study we found activation of the initiator caspase 9 and the executioner caspases 3, 6, and 7. Activation of the executioner caspases was also accompanied by cleavage of their downstream substrate PARP. A wide variety of experimental evidence, including gene ablation experiments in mice, has demonstrated that caspase 9 serves as the initiator caspase in the mitochondria-dependent apoptotic pathway (reviewed

in Refs. 20 and 35). Results from these gene ablation studies further underscore the importance and linearity of each component of the mitochondria-dependent apoptotic pathway. For example, compared with cell lines established from wild-type embryos, the Apaf-1 protein in cytochrome *c* knockout cells remained in the monomeric state in the presence of apoptotic stimuli (36). Also, in Apaf-1- or caspase 9-deficient cells no caspase 3 activation was detected in response to apoptotic stimuli even though cytochrome *c* was released into the cytosol (37–39). Taken together, these findings suggest the involvement of the mitochondria-dependent pathway for heat-induced germ cell apoptosis.

The involvement of other caspases, such as caspase 12, has also been documented (21). Caspase 12 is localized to the ER and activated by ER stress. A study of caspase 12 knockout mice revealed that caspase 12 is not essential for thymocyte apoptosis mediated by either mitochondria or Fas-mediated death pathway (21). These findings apparently point to the existence of an apoptotic pathway independent of mitochondria and death receptors. The question remains: if this is true, then where do the Bcl-2 family members fit in? We have demonstrated that early in apoptosis, a significant amount of Bax accumulates in the ER. At present we do not know the possible significance of our findings. In this context, it is important to note that cells lacking both Bax and Bak, but not cells lacking one of these components, are resistant to multiple stimuli, including those that act through stress signaling from the ER induced by thapsigargin, tunicamycin, or brefeldin (40). Thus, even ER stress-induced apoptosis requires Bax or Bak, which might reflect undefined roles of Bax or Bak at ER sites. These results, together with the present finding of translocation of Bax to ER early in apoptosis, suggest a possible involvement of ER in heat-induced testicular germ cell apoptosis.

Mechanisms regulating cell death are perhaps best exemplified in Fas- and FasL-induced apoptosis. The Fas/FasL signaling system has also been implicated in inducing germ
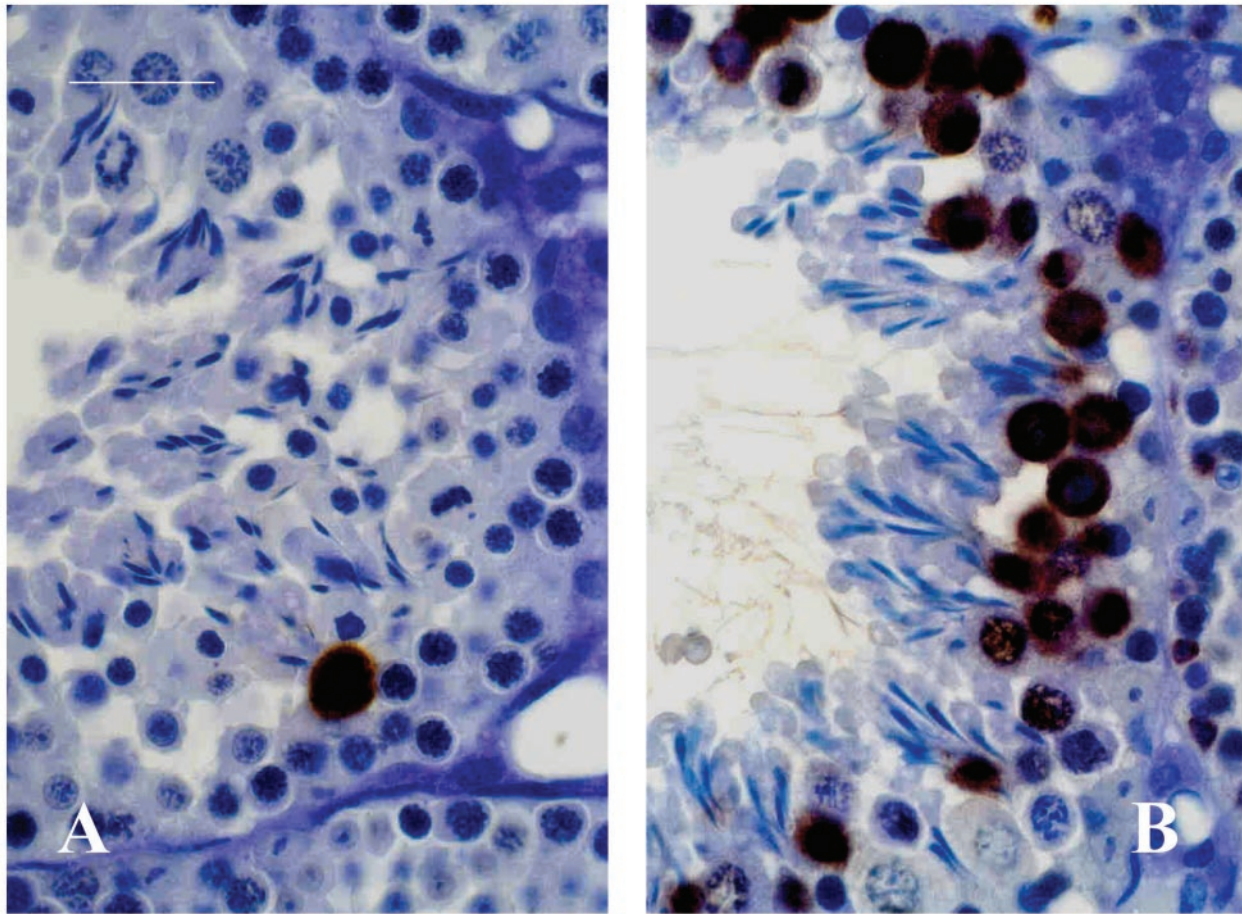
FIG. 8. *In situ* detection of germ cell apoptosis in *gld* mice after short-term local testicular heating. A, Portion of a stage XII tubule from an untreated *gld* mouse shows only an occasional apoptotic germ cell. B, Portion of a stage XII tubule from a heat-treated mouse shows a marked increase in the number of TUNEL-positive germ cells 6 h after treatment. *Scale bar*, 20 μm.

cell apoptosis in rats after testosterone withdrawal (41, 42) or exposure to the Sertoli cell toxicant mono-2-(ethylhexyl)phthalate (43). To evaluate the requirement for the Fas-mediated extrinsic pathway, we examined whether *gld* mice that express a nonfunctional form of FasL (23) would confer resistance to heat-induced germ cell apoptosis. We found that germ cells from wild-type and FasL-defective *gld* mice are equally sensitive to heat-induced germ cell death. It may be relevant in this connection to note that the *gld* mice are fertile and display apparently normal spermatogenesis (44). In fact, these mice showed a higher incidence of spontaneous germ cell apoptosis than that in wild-type males. These results together with earlier findings that FasL transcription occurs exclusively in murine meiotic and postmeiotic germ cells, whereas the protein is displayed on the surface of mature spermatozoa (45), suggest that heat-induced germ cell apoptosis in the *gld* testis is triggered by an alternate mechanism. Indeed, in a preliminary study we have provided evidence for the involvement of the mitochondria-dependent apoptotic pathway in heat-induced germ cell apoptosis in *gld* mice (46). Further support for this suggestion stems from the findings in our recent study involving *lpr*^{*cg*} mice that express a nonfunctional from of Fas (23) after heat stress (47). In that study to examine whether the *lpr*^{*cg*} mice

would confer resistance to heat-induced germ cell apoptosis, 7- to 8-wk-old *lpr*^{*cg*} and wild-type (MRL/MpJ) mice were exposed once to 22 C (control) or 43 C (heat-treated) for 15 min, and the animals were killed 2 and 6 h after heat exposure. Germ cell apoptosis was detected by the TUNEL assay and was quantitated as the number of apoptotic germ cells/ Sertoli cells (apoptotic index) at stages I–IV and XI–XII. Unlike untreated C57BL/6, a higher incidence of spontaneous germ cell apoptosis (expressed as numbers per Sertoli cell) was detected at stages I–IV and XI–XII in both MRL/MpJ wild-type (1.58 $\pm$ 0.22 and 1.84 $\pm$ 0.30, respectively) and *lpr*^{*cg*} (1.31 $\pm$ 0.26 and 2.01 $\pm$ 0.35) mice. No increase in apoptosis was detected in either wild-type or *lpr*^{*cg*} mice 2 h after heat treatment. By contrast, a significant ($P < 0.05$) increase in the incidence of germ cell apoptosis was seen 6 h after heat treatment at stages I–IV and XI–XII in both wild-type (4.17 $\pm$ 0.29 and 5.52 $\pm$ 0.79, respectively) and *lpr*^{*cg*} (3.61 $\pm$ 0.57 and 4.90 $\pm$ 0.06) mice. Most importantly, the incidence of germ cell apoptosis before and after heat treatment was similar in wild-type and mutant mice, suggesting that germ cells from wild-type and Fas receptor-mutant mice are also equally sensitive to heat-induced apoptosis. Of note, in a recent study using *lpr* and *gld* mice, Putcha *et al.* (48) provided evidence indicating that Fas/FasL signaling does not contribute to
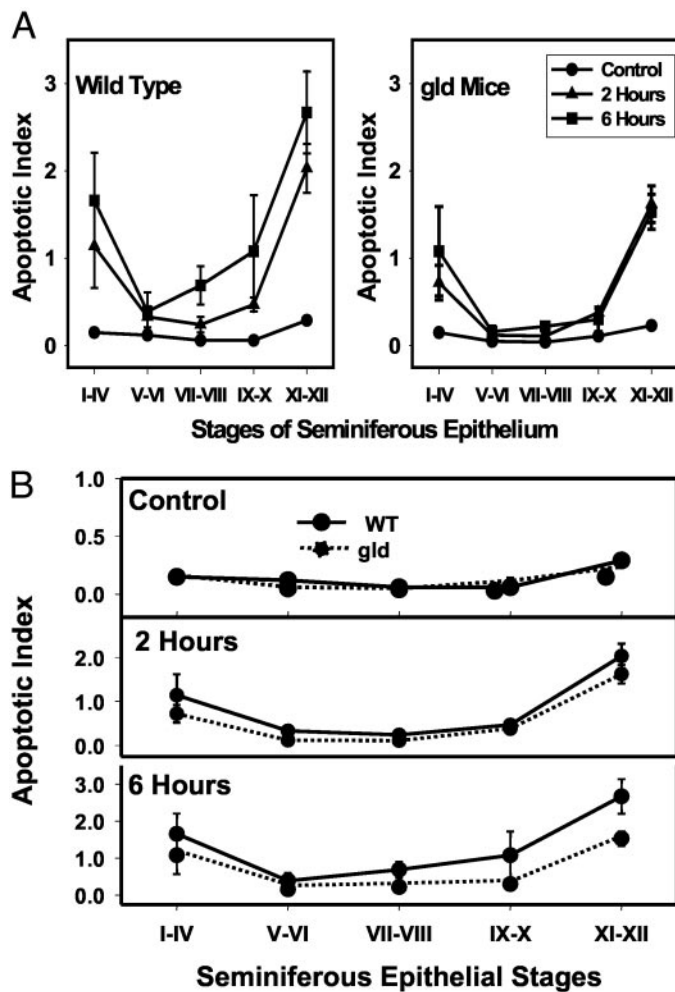
FIG. 9. A, Quantitative estimation of temporal and stage-specific changes in the incidence of germ cell apoptosis (apoptotic index, expressed as number per Sertoli cell) at designated stages of the seminiferous epithelium after short-term local testicular heating in wild-type and *gld* mice. Values are given as the mean ± SEM. The mean incidence of germ cell apoptosis was significantly increased ($P < 0.05$) at early (I–IV) and late (XI–XII) stages in both wild-type and *gld* mice at 2 and 6 h after heat treatment. B, A comparison of the mean apoptotic index of germ cells between wild-type and *gld* mice. Note a similar increase in the incidence of germ cell apoptosis at 2 and 6 h after heat treatment in both wild-type and *gld* mice.

trophic factor deprivation-induced neuronal apoptosis. Collectively, these results suggest that the Fas signaling system may be dispensable for heat-induced germ cell apoptosis in the testis.

Acknowledgments

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