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Heat-Induced Apoptosis of Mouse Meiotic Cells Is Suppressed by Ectopic Expression of Testis-Specific Calpastatin

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ABSTRACT: Calpastatin is a naturally occurring inhibitor of calpain, a protease involved in apoptotic cell death. A testis-specific isoform of calpastatin (tCAST) has been identified that is transcribed in haploid germ cells but not in spermatocytes. To investigate the possible function(s) of tCAST, we tested the hypothesis that the ectopic expression of calpastatin in spermatocytes would suppress the death of these cells in response to an apoptosis-inducing stimulus in vivo. To this end, the 5′-flanking region of the mouse *ldhc* gene was linked to tCAST, and transgenic mice were generated. Immunohistochemical analysis revealed that, in contrast to control sections in which the signal for tCAST was seen in round spermatids, intense staining was visualized in pachytene spermatocytes in the transgenic animals, indicating that the strategy we used to generate the transgenic animals resulted in the ectopic expression of tCAST in spermatocytes. We then tested the effect of a short period of heating on germ cell apoptosis in the testes of wild-type and transgenic mice. Pachytene spermatocytes were the major germ cell type seen to undergo apoptosis after heat treatment. There were no differences in the number of apoptotic germ cells per seminiferous tubule between wild-type and tCAST transgenic control mice; thus, there was no apparent effect of the transgene on normal apoptosis. Heating resulted in increased numbers of TUNEL-positive germ cells in both wild-type and tCAST transgenic mice, as well as increased testicular DNA fragmentation. Heating the tCAST transgenic mouse testes resulted in significantly fewer apoptotic cells per seminiferous tubule than in wild-type mice at both 8 and 24 hours after treatment. Thus, as hypothesized, the ectopic expression of tCAST in pachytene spermatocytes suppressed germ cell apoptosis.

Key words: *ldhc* gene, tCAST, cell death, calpain, spermatocytes.
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Calpains, a family of Ca^{2+}-dependent cysteine proteases that are ubiquitously expressed in mammalian cells (Carafoli and Molinari, 1998), are involved in a number of physiologic processes, including membrane-associated events such as cytoskeletal reorganization, enzyme mobilization, and receptor activation (Saido et al, 1994; Sorimachi et al, 1997). Calpains also have been shown to play an important role in the apoptotic death of some cells (Carafoli and Molinari, 1998; Ray et al, 1999; Wood and Newcomb, 1999; Gao and Dou, 2000; Nakagawa and Yuan, 2000; Shiraishi et al, 2000; Blomgren et al, 2001; Choi et al, 2001). In such cells, the precursor form of calpain, procalpain, moves from the cytosol to intracellular membranes to become activated (Wood and Newcomb, 1999); the up-regulation of activated calpain then catalyzes substrates such as transcription factors, cytoskeletal proteins, membrane-associated proteins, signal transduction factors, and/or calmodulin-dependent proteins (Kato et al, 2000; Wang, 2000), leading to cell death.

Calpastatin is a naturally occurring inhibitor of calpain that regulates its proteolytic activity (Barnoy et al, 1998; Barnoy and Kosover, 2003). The interaction of calpastatin with calpain has been shown to prevent apoptosis in vitro (Chi et al, 1999; Lu et al, 2002) by preventing the activation of procalpain and the catalytic activity of calpain and thus the proteolysis of substrates by activated calpain (Carafoli and Molinari, 1998; Kato et al, 2000). A testis-specific isoform of calpastatin (tCAST) was identified by Li and Goldberg (2000) in the mouse. The gene was shown to be transcribed in haploid germ cells and not in spermatocytes, and the protein was shown to associate with intracellular membrane structures of the postmeiotic cells. Thus, whereas calpain is expressed in premeiotic cells (Umemoto et al, 2001), tCAST, under normal circumstances, is not (Li and Goldberg, 2000).

To begin to elucidate the possible function(s) of tCAST in the testis, we wished to test the hypothesis that the

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ectopic expression of calpastatin in spermatocytes would suppress the death of these cells in response to an apoptosis-inducing stimulus in vivo. To this end, transgenic mice were generated in which tCAST was ectopically expressed in spermatocytes. The strategy used to produce these mice was based on a previous report by Goldberg and his colleagues (Li et al, 1998), who showed that by linking the 5′-flanking region of the mouse *ldhc* gene 100 bp upstream from the transcription start site to a reporter gene (*Escherichia coli lacZ*), the *ldhc* promoter was able to drive the expression of β-galactosidase in a testis-specific manner in transgenic mice, with the gene product expressed only in pachytene spermatocytes. By linking this 5′-flanking region to tCAST, transgenic mice were produced in which tCAST was misexpressed in pachytene spermatocytes. We hypothesized that the expression of the tCAST gene in these cells would inhibit the germ cell apoptosis induced by a short period of testicular heating, a procedure shown previously to result in significant increases in the apoptotic death predominantly of primary spermatocytes (Yin et al, 1998; Lue et al, 1999, 2000; Rockett et al, 2001; Bailey et al, 2002; Miura et al, 2002; Sinha Hikim et al, 2003).

The results that we have presented demonstrate a significant decrease in the number of apoptotic germ cells in the tCAST transgenic animals compared to controls in response to heat. Thus, as hypothesized, the ectopic expression of calpastatin was able to protect germ cells from heat-induced apoptotic death in vivo.

Materials and Methods

Animals

Adult male CD1 wild-type mice were obtained from Charles River Laboratories (Raleigh, NC). Age-matched male CD1 calpastatin transgenic mice were produced at Northwestern University (Evanston, Ill) by injection of the linearized vector pNAssβ mtCT into the pronuclei of single-cell stage embryos (Li and Goldberg, 2000). All procedures were performed in accordance with protocols approved by the Animal Care and Use Committees of Northwestern University and of Johns Hopkins University (Baltimore, Md).

Immunohistochemical Analysis

A glutathione *S*-transferase fusion protein containing the testis domain of calpastatin was expressed in bacteria and used to immunize rabbits. Antiserum specificity was confirmed by Western blotting and absorption with the fusion protein for control sections (data not shown). No cross-reactivity with somatic calpastatin was observed.

Mouse testes were fixed in Bouin solution and embedded in paraffin. Sections of 5 μm were rehydrated with phosphate-buffered saline and incubated with 3% H_2O_2 in methanol to quench endogenous peroxidase activity. Slides were then incubated with rabbit anti-tCAST antiserum (1:1000) using the Histostain-SP Kit according to the manufacturer's instructions (Zymed Laboratories Inc, South San Francisco, Calif).

Heat Treatment

A total of 20 adult tCAST transgenic mice and 20 wild-type mice were randomly assigned to 4 groups of 5 mice each. Mice were sacrificed at 8 or 24 hours after testicular heating, and the wild-type controls were sacrificed at the same times. Thus, for each of the tCAST and wild-type mice, the groups were 1) heat, 8 hours; 2) control, 8 hours; 3) heat, 24 hours; and 4) control, 24 hours. To heat the testes, the lower half of the torso of a mildly sedated mouse was submerged in a 43°C heated water bath for 15 minutes. Control animals were sedated but were not exposed to warm water. At 8 or 24 hours after the 15-minute exposure to heat, mice were euthanized, and both testes were removed. The right testis was immersion-fixed in 10% neutral-buffered formalin for 24 hours at 4°C and embedded in paraffin for TUNEL analysis (see below). The left testis was decapsulated, snap frozen in liquid N_2, and stored at −70°C for subsequent DNA laddering analysis (see below).

TUNEL Analysis

We first ascertained whether apoptotic cells were distributed evenly throughout the testes by exhaustive sectioning. To this end, approximately 60 sections of 6 μm were cut per testis, including proximal, medial, and distal regions, and cells were examined for in situ end labeling of DNA strand breaks (ApopTag Peroxidase TUNEL Kit; Intergen, Purchase, NY) according to the manufacturer's instructions. Briefly, slides from 3 animals per group were digested with proteinase-K (Roche Diagnostics, Indianapolis, Ind) at a concentration of 20 μg/mL for 10 minutes and quenched with 3% hydrogen peroxide in methanol. Slides were incubated for 5 minutes in equilibration buffer and then were placed in a humidified chamber (37°C) with terminal deoxynucleotidyl transferase (TdT) enzyme (50 μL, 1 hour, 37°C). The slides were then incubated (30 minutes) with antidioxigenin peroxidase enzyme conjugate in a humidified chamber and developed with 3,3′-diaminobenzidine peroxidase substrate. The sections were counterstained with either 0.5% methyl green (Vector Laboratories, Burlingame, Calif) or periodic acid-Schiff.

After ascertaining that apoptotic cells were, indeed, evenly distributed throughout the testes, the apoptotic cells were quantified by counting TUNEL-stained sections from the center (medial) region of the right testis of wild-type and transgenic mice. For each animal, 100 seminiferous tubule cross sections were counted, with 3 animals per group. The results were expressed as the mean number of apoptotic germ cells per seminiferous tubule cross section per group for each of the 2 postheating time points, 8 and 24 hours. The apoptotic cell types were identified on the basis of the characteristics of their nuclei, surrounding cells, and the stage of the cycle of the seminiferous epithelium, according to previously described criteria (Russell et al, 1990).

High- and Low-Molecular-Weight DNA Isolation

DNA was isolated from frozen tissues (TACS Apoptotic DNA Laddering Kit; Trevigen; Gaithersburg, Md) according to the manufacturer's instructions. Briefly, 100 mg of frozen testis tis-

sue was homogenized in 100 μL of sample buffer and 20 μL of 10× tissue buffer. Tissue was incubated at 50°C for 2 hours, after which 100 μL of lysis buffer was added to the tissue. After 10 minutes at room temperature, 700 μL of extraction solution 2 was added, followed by 400 μL of extraction buffer 3. After vortexing for 10 seconds, the lysate was centrifuged at 12 000 × *g* for 5 minutes at 4°C. The upper aqueous layer was recovered while the organic phase was discarded. Sodium acetate (0.1 vol) was added to the aqueous phase, followed by an equal volume of 2-propanol, and the DNA was precipitated overnight at −20°C. The samples were centrifuged again at 12 000 × *g* for 10 minutes at 4°C, and the supernatant was removed without disturbing the pellet. The pellet was washed with 1 mL of 70% cold ethanol and centrifuged at 12 000 × *g* for 10 minutes. The pellet was resuspended in 100 μL of DNase-free water, and the DNA was quantified by measuring the optical density of each sample at 260 nm. Undiluted DNA was adjusted with DNase-free water to bring the final concentration of DNA to 1 μg/μL. DNA (0.4–2 μg) from each sample was 3′ end labeled with [α-32P]dideoxy-CTP (10 μCi/μL; Amersham Life Sciences, Arlington Heights, Ill) using DL Klenow enzyme (5 U/μL). The labeled samples were separated on a 1.5% Trevigel (Trevigen) agarose gel, dried without heat, and exposed to Hyperfilm-MP (Amersham) at −70°C for 2–12 hours.

Statistical Analysis

Data are expressed as mean ± SEM. The differences among groups were assessed by 1-way analysis of variance. In cases of $P < .05$, individual group differences were determined using the Scheffé *F* test. Means were considered statistically different at $P < .05$.

Results

No apparent testicular abnormalities were observed in the transgenic mice (data not shown). Figure 1 shows cross sections of seminiferous tubules of wild-type (A) and transgenic (B) littermates. The sections were incubated with an antiserum that specifically detected tCAST, followed by a horseradish peroxidase–conjugated secondary antibody. Relatively light staining for tCAST was seen in the haploid round spermatids of the testes from both wild-type (A) and transgenic (B) mice. In striking contrast to the wild-type mice, intense staining for tCAST was seen in pachytene spermatocytes in the testes of the transgenic animals (B).

Figure 2 shows TUNEL-stained heat-treated (Figure 1A through C) and control (Figure 1D through F) testes from wild-type mice, taken from the proximal (A, D), medial (B, E), and distal (C, F) regions of the testes. In comparison to the testes from the no-heat controls, in which little germ cell apoptosis was seen in any region of the testis (Figure 1D through F), substantial apoptosis was evident in all regions of the testes 24 hours after heat treatment, with apoptotic cells distributed throughout each

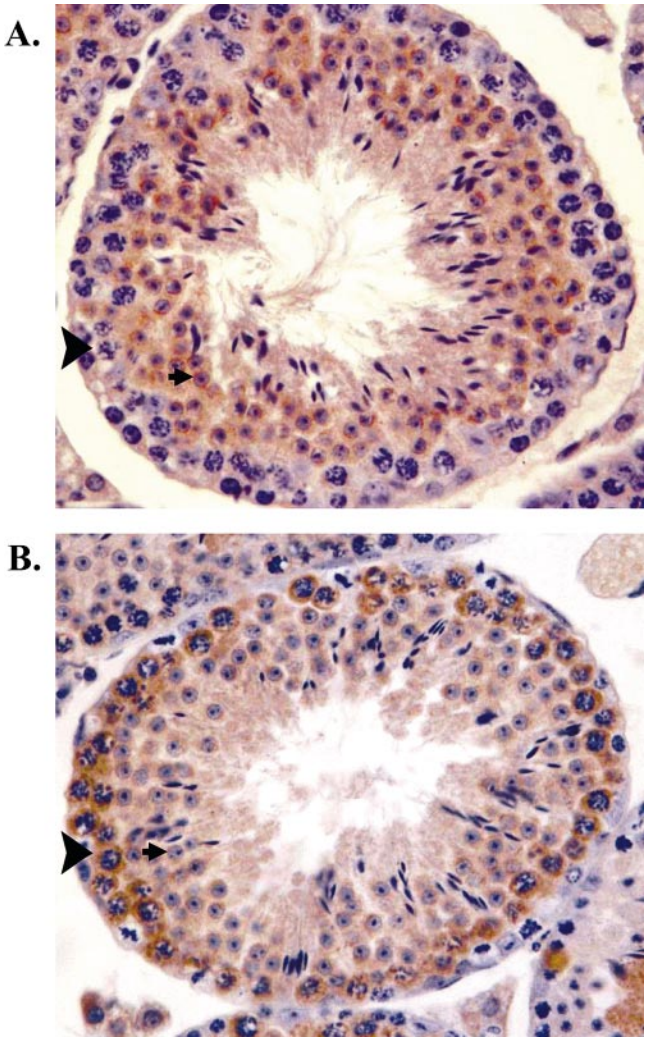

Figure 1. Ectopic expression of a testis-specific isoform of calpastatin (tCAST) in seminiferous tubule cross sections from the testes of wild-type **(A)** and tCAST transgenic **(B)** mice. Sections were incubated with tCAST primary antiserum and then horseradish peroxidase–conjugated secondary antibody. tCAST staining (brown) in spermatids was seen (small arrows) in the testes of both wild-type and transgenic mice **(A, B)**. The large arrowheads point to spermatocytes. tCAST staining of the spermatocytes was seen only in the testes of the transgenic mice **(B).** 400× magnification.

region (ie, not localized to a given part of any testis section). Most of the apoptotic cells were pachytene spermatocytes.

To determine whether tCAST expression had an effect on heat-induced germ cell apoptosis, the number of apoptotic cells in the testes of wild-type and transgenic animals, with and without heat treatment, was compared. Figure 3 quantifies TUNEL-positive cells in wild-type and transgenic control (room temperature) and heated testes at 8 hours (Figure 2A) after heat treatment. In the wild-type animals, heat treatment resulted in significantly increased numbers of TUNEL-positive cells per tubule compared to the room temperature controls. Increases in

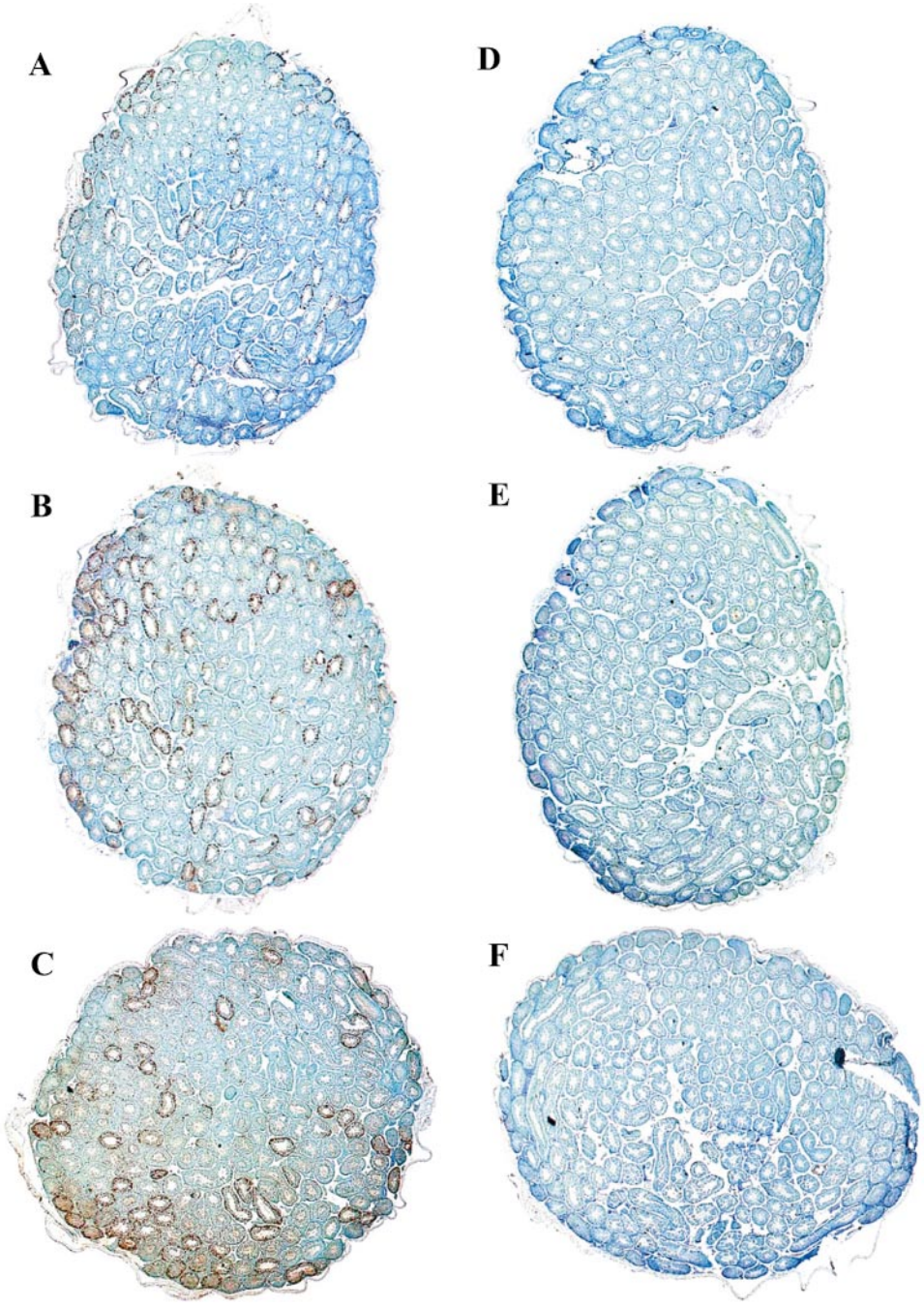

Figure 2. TUNEL staining of representative testis cross sections from heat-treated **(A–C)** and control (nonheated) **(D–F)** testes from wild-type mice. Sections were taken 24 hours after a single heat treatment at 43°C for 15 minutes. TUNEL-positive cells are stained brown.

TUNEL-positive cells also were observed 8 hours after heating the testes of tCAST transgenic mice in comparison to its controls, though the increases did not reach significance. Thus, though the numbers of apoptotic cells per tubule were increased by heat in both wild-type and transgenic mice, the number of apoptotic cells per tubule in the tCAST testes (about 5) was only half that in the heated wild-type testes. Given the location of the TUNEL-stained cells and the morphology of the intact surrounding cells, it was apparent that most of the dying cells were pachytene spermatocytes. The reduced number of apoptotic primary spermatocytes in the testes of the transgenic mice was consistent with the expression of the transgene in these cells, as shown in Figure 1.

Figure 4 quantifies TUNEL-positive cells in wild-type and transgenic control (room temperature) and heated tes-

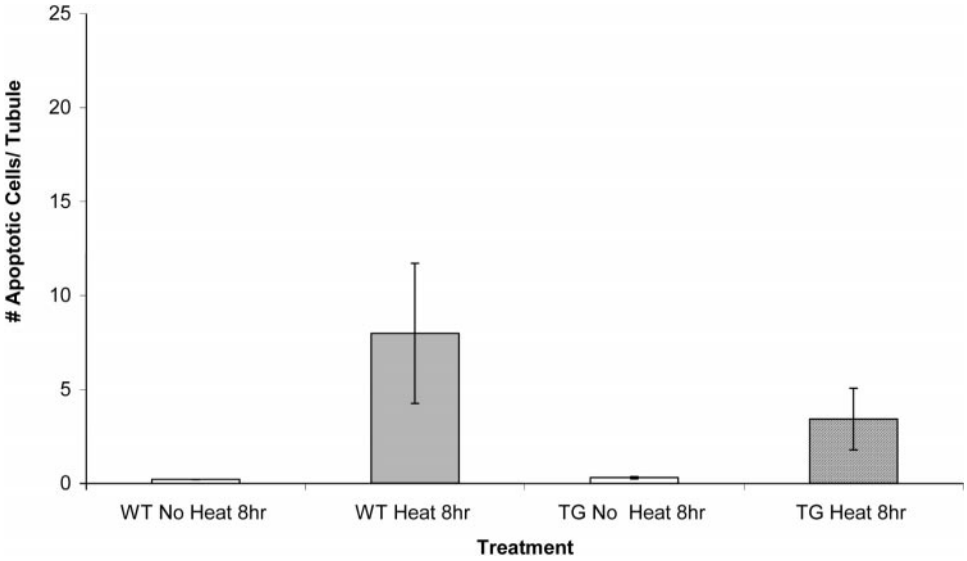

Figure 3. Mean number of TUNEL-positive apoptotic germ cells per seminiferous tubule 8 hours after 43°C, 15-minute heat treatment of wild-type (WT) and transgenic (TG) mice and their respective controls (no heat). Values are mean ± SEM.

tes at 24 hours after heat treatment. As expected from the results at 8 hours, the numbers of TUNEL-positive cells in the heated wild-type and transgenic testes were greater than in their respective controls. The number of apoptotic cells per tubule in the wild-type heated testes, 20, was about twice the number found at 8 hours. In contrast, the number in the heat-treated transgenic testes, about 5, was unchanged from 8 hours.

To gain further insight into the magnitude of the differences in the response of germ cells to heat treatment in the wild-type vs tCAST transgenic mice, we examined the percentages of seminiferous tubules containing 0, 1–10, or more than 10 apoptotic cells in the control and heat-treated testes. The results of this analysis are shown in the Table.

Controls (no heat)—In the testes of wild-type and transgenic mice not exposed to heat, 100% of the tubules contained 0–10 apoptotic cells per tubule, among which most tubules (80%–90%) had no apoptotic cells, and the remainder had 1–5.

Heat, 8 hours—In the wild-type mice that received an 8-hour heat treatment, the percentage of tubules without apoptotic cells dropped to half that in the controls, about 40%, and the percentage of tubules with 1–10 and greater than 10 apoptotic cells rose to 32% and 26%, respectively. The results with the transgenic mice that received 8-

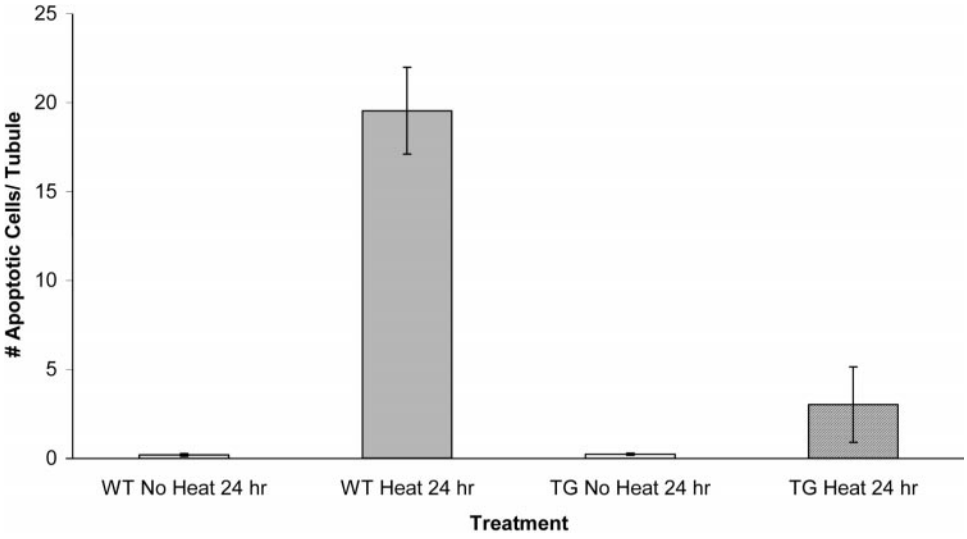

Figure 4. Mean number of TUNEL-positive apoptotic germ cells per seminiferous tubule 24 hours after 43°C, 15-minute heat treatment of wild-type (WT) and transgenic (TG) mice and their respective controls (no heat). Values are mean ± SEM.

*Numbers of apoptotic cells per seminiferous tubule in control and heat-treated wild-type and tCAST transgenic mice**

Treatment Group	% Apoptotic Cells per Tubule		
	0	1–10	>10
Wild-type, control 8 h	88	12	0
Wild-type, heat-treated 8 h	42	32	26
Transgenic, control 8 h	83	17	0
Transgenic, heat-treated 8 h	75.3	14.7	10
Wild-type, control 24 h	86.7	13.3	0
Wild-type, heat-treated 24 h	16	28.7	53.3
Transgenic, control 24 h	88.3	11.7	0
Transgenic, heat-treated 24 h	62	32	6

* tCAST indicates testis-specific isoform of calpastatin.

hour heat treatments were far less dramatic; there was a small decrease in the percentage of tubules without apoptotic cells, about 75%, and the percentage of tubules with greater than 10 apoptotic cells per tubule rose, but only to 10%.

Heat (24 hours)—After heating the testes of wild-type mice for 24 hours, the percentage of tubules with greater than 10 apoptotic cells per tubule rose to more than 50%, while only 16% had 0 apoptotic cells. For the tCAST transgenic mice that received heat for 24 hours, 62% of the tubules had no apoptotic cells, and only 6% had greater than 10.

Figure 5 shows genomic DNA from the heat-treated (H) and control (C) testes of wild-type (WT) and transgenic (TG) mice. Twenty-four hours after heat treatment, extensive DNA laddering was seen in the wild-type testes in comparison to the control (unheated) testes. This was also true of the heat-treated transgenic testes compared to their controls. The extent of DNA fragmentation was clearly greater in the heat-treated wild-type mice than in the tCAST transgenic mice. Indeed, little DNA fragmentation was seen without heat treatment in either wild-type or tCAST transgenic animals. Fragmentation was also observed in the positive control, which was genomic DNA isolated from rat testicular germ cells after treatment of the animals with luteinizing hormone–suppressive testosterone and estrogen implants for 8 weeks (Kim et al, 2001).

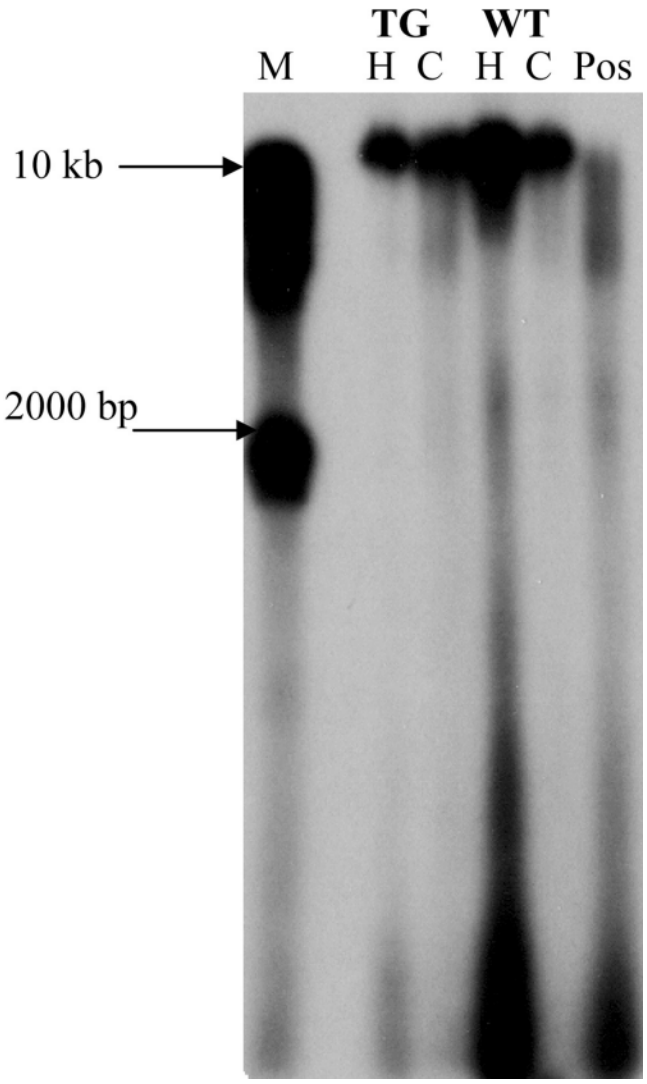

Figure 5. DNA fragmentation observed in the testes of transgenic (TG) and wild-type (WT) mice 24 hours after heat treatment (H) and non-heated controls (C). M indicates DNA laddering 1-kilobase (kb) marker; Pos, positive control. DNA isolated from rat testicular germ cells after treatment of the rats with testosterone- and estradiol-containing Silastic implants for 8 weeks.

Discussion

In the tCAST transgenic mice, the germ cell–specific *ldhc* promoter drives the ectopic expression of the tCAST transgene (Li et al, 1998; Miura et al, 2002). Immunohistochemical analysis of Bouin-fixed sections of the testes of these mice revealed that, in contrast to controls in which a signal was seen in round spermatids, staining for tCAST also was visualized in pachytene spermatocytes, as has also been reported for the testis-specific expression of β-galactosidase (Li et al, 1998; Miura et al, 2002). The transgenic mice had normal testicular morphology and testis weights, suggesting that the overexpression of tCAST did not interfere with normal spermatogenesis and testicular development. Consistent with this, there were no differences in the number of apoptotic germ cells per seminiferous tubule between unheated wild-type and tCAST transgenic control mice and thus no apparent effect of the transgene on normal apoptosis.

Based on the observation that calpastatin overexpression can prevent apoptosis in vitro (Chi et al, 1999; Lu et al, 2002), we tested the possible in vivo inhibitory effect of the ectopic expression of tCAST on germ cell apoptosis, with apoptosis induced by a 15-minute expo-

sure to heat and the appropriate test samples being examined 8 and 24 hours thereafter. Heating resulted in increased numbers of TUNEL-positive germ cells in both wild-type and tCAST transgenic mice, as well as increased DNA fragmentation, results that are consistent with previous studies reporting that a single 43°C, 15-minute heat treatment in mice resulted in increased germ cell apoptosis (Yin et al, 1997; Rockett et al, 2001; Miura et al, 2002). Our results also are consistent with previous studies showing that pachytene spermatocytes are the major germ cell type observed to undergo apoptosis after heat treatment (Yin et al, 1998; Lue et al, 1999, 2000; Rockett et al, 2001; Bailey et al, 2002; Miura et al, 2002; Sinha Hikim et al, 2003).

Heating the tCAST transgenic mouse testes resulted in significantly fewer apoptotic cells per seminiferous tubule than in wild-type mice at both 8 and 24 hours after heat treatment. These results indicate clearly that the ectopic expression of tCAST in pachytene spermatocytes prevented germ cell apoptosis during the time periods of maximal germ cell apoptosis. That the calpastatin gene is not normally expressed in pachytene spermatocytes may reflect a biologically useful adaptation to enhance the clearance of pachytene spermatocytes damaged by stressors.

The mechanism by which the misexpression of tCAST in pachytene spermatocytes functions to suppress heat-induced germ cell apoptosis is uncertain. Calpastatin is an endogenous inhibitor of calpain, and calpain has been shown to be involved in apoptosis. Given the observation that calpain is expressed in the mouse testis and that its staining localizes to rat spermatocyte nuclei after experimental testicular torsion (Umemoto et al, 2001), it seems likely that calpain is normally expressed in mouse spermatocytes or that it is induced to detectable levels in these cells after a stressor, such as heat. It is possible that the suppression of heat-induced spermatocyte apoptosis by tCAST overexpression is via the inhibition of calpain. It also is possible that the inhibition of pachytene spermatocyte apoptosis in tCAST transgenic mice resulted from an effect of calpastatin, a protease inhibitor, on other proapoptotic molecules in the mouse testis or on downstream proteolytic events in the apoptotic pathway. For example, p53, which induces apoptosis and is expressed in high levels in pachytene spermatocytes (Yan et al, 2000), has been suggested to be responsible for the initial phase of germ cell apoptosis in response to cryptorchidism (Yin et al, 1998), with Fas responsible for apoptosis during the later phases of heat-induced germ cell death (Yin et al, 2002). It is possible, therefore, that tCAST interacts with p53 or Fas to delay the onset of apoptosis after heat treatment and/or with other pro- or antiapoptotic proteins such as Bcl-xL, Bcl-2, or Bax, all of which have been shown to respond to local testicular heating (Rodriguez et al, 1997; Miura et al, 2002). Determining the exact role of tCAST in preventing heat stress–induced apoptosis is complicated by the fact that different intracellular pathways leading to apoptosis may be induced, depending on the method by which the testes are heated (eg, experimental cryptorchidism vs local testicular heating, as in our study).

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