Arrest of spermatogenesis in mice expressing an active heat shock transcription factor 1

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In mammals, testicular temperature is lower than core body temperature, and the vulnerable nature of spermatogenesis to thermal insult has been known for a century. However, the primary target affected by increases in temperature is not yet clear. We report here that male mice expressing an active form of heat shock transcription factor 1 (HSF1) in the testis are infertile due to a block in spermatogenesis. The germ cells entered meiotic prophase and were arrested at pachytene stage, and there was a significant increase in the number of apoptotic germ cells in these mice. In wild-type mice, a single heat exposure caused the activation of HSF1 and similar histological changes such as a stage-specific apoptosis of pachytene spermatocytes. These results suggest that male infertility caused by thermal insult is at least partly due to the activation of HSF1, which induces the primary spermatocytes to undergo apoptosis.

Keywords: apoptosis/heat shock/spermatogenesis/transcription/transgenic mouse

Introduction

Spermatogenesis is a complex process by which spermatogonial stem cells differentiate into mature sperm, and this process involves remarkable structural and biochemical changes. The vulnerable nature of this process to thermal insult has been known for a century (Crew, 1922; Moore, 1951). The testicular temperature is maintained constantly lower than the core body temperature, and many clinical observations have suggested a link between testicular hyperthermia and reduced spermatogenesis (Kandeel and Swerdloff, 1988; Mieusset and Bujan, 1995). These include the suppression of spermatogenesis in disorders such as acute febrile diseases, cryptorchidism and varicocele. Testicular heating caused by taking a sauna or hot bath or by wearing close-fitting underwear could be detrimental to spermatogenesis (Lynch *et al.*, 1986).

Many attempts were made to reveal the germ cells degenerated by hyperthermia, and the primary spermatocytes

were shown to be most sensitive to increases in temperature (Steinberger and Dixon, 1959; Chowdhury and Steinberger, 1970). More recently, examination of rodent testis exposed to a single heat stress or of experimental cryptorchidism showed that germ cell degeneration induced by high temperature was accompanied by apoptosis mainly of the pachytene spermatocytes in a stage-specific manner (Henriksen *et al.*, 1995; Yin *et al.*, 1997; Lue *et al.*, 1999). Several possible mechanisms of germ cell death by thermal injury have been suggested. These include decreases in the synthesis of DNA, RNA and proteins in germ cells, decreased capillary blood flow that supplies germ cells, and indirect effects of proteins synthesized by Sertoli and Leydig cells (see review by Mieusset and Bujan, 1995). However, the molecular targets affected by increased temperature and molecular events underlying the activation of germ cell death remain poorly understood.

Heat shock induces activation of heat shock transcription factor (HSF), which regulates expression of heat shock genes (Wu, 1995), the major products of which act as molecular chaperones by facilitating protein folding and assembly (Hartl, 1996). HSF is also essential for development in *Drosophila* without affecting *Hsp* gene expression, suggesting multiple functions of the HSF system (Westwood *et al.*, 1991; Jedlicka *et al.*, 1997). In mammals, the HSF gene family consists of three members, HSF-1, -2 and -4, DNA binding domains of which are highly conserved (Morimoto, 1998; Nakai, 1999). HSF1 is a major heat stress-responsive factor expressed ubiquitously (Baler *et al.*, 1993; Sarge *et al.*, 1993; Nakai *et al.*, 1997). HSF2 and HSF4 expression are regulated developmentally in a tissue-specific manner (Rallu *et al.*, 1997; Tanabe *et al.*, 1999), and expression of the former is upregulated during testis development (Sarge *et al.*, 1994; Alastalo *et al.*, 1998), suggesting the involvement of the HSF system in normal spermatogenesis.

In general, cells are protected from thermal insult by inducing a set of heat shock proteins (Hsps) through HSF activation. Cells pretreated with sublethal heat stress, in which Hsps are highly induced, are more resistant to lethal heat stress than untreated cells. Conversely, blockade of Hsp induction by disruption of the *HSF1* gene in mouse cells or the *HSF3* gene in chicken cells reduced the viable cell number after heat stress and induced apoptosis (McMillan *et al.*, 1988; Tanabe *et al.*, 1998). To examine the effects of activation of HSF1 after thermal insult on spermatogenesis, we generated mice expressing the active form of HSF1 in the testis. Unexpectedly, constitutive expression of an active form of HSF1 resulted in the blockade of spermatogenesis at pachytene stage and in increases in the number of apoptotic spermatocytes. This suggested that inhibition of spermatogenesis after thermal insult can be explained at least by the activation of HSF1.

Fig. 1. Structure and expression of a truncated form of human HSF1 in cells. **(A)** Schematic representation of hHSF1ΔRD (Δ221–315) transgene. Expression of the transgene was driven by the human β-actin promoter region, 5' untranslated region (5'nt) and the first intron (int). SV40 late region polyadenylation signal (poly A) was located downstream of the hHSF1ΔRD cDNA insert. **(B)** Analysis of expression of the transgene by Western blotting using anti-HSF1β antibody. Expression vector of the hHSF1ΔRD was transfected into human erythroblast K562 cells, and cells stably expressing hHSF1ΔRD were isolated. The levels of endogenous hHSF1 (open arrow) and hHSF1ΔRD (closed arrow) in these cells (clones a–d) were compared with those of parental K562 cells (K562) and mock-transfected cells (clone x). **(C)** DNA binding activities in whole cell extracts of cells expressing various amounts of hHSF1ΔRD. Gel shift assay was performed using a ³²P-labeled HSE probe. The magnitude of DNA binding activity was dependent on the level of hHSF1ΔRD protein. Non-specific binding is indicated by ns. **(D)** Analysis of expression of major heat shock genes by Northern blotting. Levels of heat shock genes in normally growing cell lines were compared with those in parental K562 cells (cont). For comparison, K562 cells were heat shocked at 42°C for 1 h and mRNA levels of heat shock genes were analyzed (hs). Probes used were human cDNAs for Hsp90α, Hsp40 and Hsp27, human genomic DNAs for Hsp70 and 28S rRNA, and a mouse cDNA for Hsp110. **(E)** Growth curves of cells expressing hHSF1ΔRD. A total of 5 × 10⁴ cells were inoculated into 90 mm dishes and cell numbers were counted for up to 96 h. Each experiment was performed in triplicate.

Results

Characterization of cells expressing an active form of HSF1

We constructed an expression vector for an active form of human HSF1 (hHSF1ΔRD) driven by the human β-actin

Fig. 2. Generation of mice expressing a truncated form of human HSF1. Analysis of expression of the transgene in adult mice by Western blotting. Whole tissue extracts from the brain (lanes 1 and 8), heart (2 and 9), lung (3 and 10), liver (4 and 11), spleen (5 and 12), stomach (6 and 13) and kidney (7 and 14) in two strains of transgenic mice (B4-6 and B6-6) were prepared, and expression of hHSF1ΔRD (arrows) was examined using antiserum against HSF1. As a control, K562 cells expressing hHSF1ΔRD were analyzed (lanes 15 and 16). To compare the levels of hHSF1ΔRD in male reproductive organs including the testis (lanes 20–22) and epididymis (lanes 23–25) and in the heart (lanes 17–19) in wild-type (WT) and transgenic mice, the same amounts of protein were analyzed as described above. Asterisks indicate non-specific bands.

promoter (Gunning *et al.*, 1987) by deleting the regulatory domain (amino acids 221–315) that suppresses the C-terminal activation domain of human HSF1 (Figure 1A) (Green *et al.*, 1995). First, we established human erythro-leukemia K562 cell lines stably expressing different levels of hHSF1ΔRD (Figure 1B) to examine expression of a set of heat shock genes and the effects of hHSF1ΔRD on cell fate including differentiation and death. High levels of hHSF1ΔRD protein were associated with high DNA binding activities (Figure 1C). Increases in the expression of target heat shock genes were observed when the level of hHSF1ΔRD was as high as that of endogenous mHSF1 (Figure 1D, clone b), and marked elevation of heat shock genes was observed in cells expressing higher levels of hHSF1ΔRD (clones c and d). Growth rates (Figure 1E) and proportions of cells in each stage of the cell cycle (data not shown) in hHSF1ΔRD-expressing clones were similar to those in parental cells.

Generation of transgenic mice expressing high levels of an active form of HSF1 in the testis and heart

We then examined the effects of overexpression of the active HSF1 in the testis as well as other tissues in mice. Twenty transgenic F₀ male and female mice were mated with wild-type mice to generate F₁ progeny. Among them, two male F₀ mice did not sire any offspring when mated with wild-type females. In only 10 of 18 F₀ female mice, the transgene was transmitted to F₁ progeny and the lines were maintained by mating female mice heterozygous for the transgene with

Table 1. Fertility and testis weight of HSF1ΔRD transgenic male mice

Strain	Genotype	Fertility ^a (%)	Testis weight (mg) ^b				
			2 weeks	3 weeks	4 weeks	8 weeks	16 weeks
B4-6	+/+	100 (6/6)	9.9 ± 0.3	17.8 ± 0.7	23.1 ± 1.5	75.2 ± 3.8	82.2 ± 1.5
	Tg/+	0 (0/6)	8.8 ± 0.5	15.9 ± 0.7	16.0 ± 1.3	35.5 ± 1.0	36.1 ± 1.6
B6-6	+/+	100 (5/5)	8.8 ± 0.3	12.8 ± 0.5	16.0 ± 1.9	73.2 ± 0.5	82.6 ± 1.3
	Tg/+	0 (0/4)	8.7 ± 0.6	11.0 ± 0.3	11.8 ± 0.5	29.3 ± 0.4	33.1 ± 1.5

^aMale mice (8 weeks old) of each strain were mated with wild-type females for 8 weeks. Percentage of fertility and numbers of female mice mated are shown.
^bTransgenic female mice were mated with wild-type males and testis weights of offspring (2–6 offspring) were compared between littermates at the time points indicated in each strain. The average litter size was 4.8 pups per litter and the proportion of transgenic pups was 1:1. Values shown are mean ± SD.

Fig. 3. Gross anatomy of male reproductive organs (A and B) and the heart (C and D) of 4-month-old wild-type (+/+) (A and C) and transgenic B6-6 (Tg/+) (B and D) mice. Note the ~50% reduction in size of testis in transgenic mice. T, testis; Eh, head of epididymis; Et, tail of epididymis; Ad, adipose tissues.

wild-type male mice. Two lines (B4-6 and B6-6), in which positions of integration sites were different, expressed high levels of hHSF1ΔRD protein. Despite having a ubiquitous

promoter, the product of the transgene was expressed in a tissue-restricted fashion. The hHSF1ΔRD protein was detected by Western blotting in the testis, heart and stomach

Fig. 4. Transgenic mice showed defective spermatogenesis. Histological analysis of testis sections from 4-month-old wild-type (A) and transgenic mice (B–F). The diameter of seminiferous tubules in transgene-positive mice was reduced by 20–30%, and the number of interstitial Leydig cells was markedly increased (A and B). Many degenerating pachytene spermatocytes with condensed nuclei and eosinophilic cytoplasm (C and D) and giant cells (E) were seen. (G and H) Immunostaining of endogenous HSF1 and ectopically expressed hHSF1 Δ RD. Cryosections of testis from wild-type (G) and transgenic (H) adult mice were immunostained with antiserum against HSF1 as the first antibody and peroxidase-conjugated goat anti-rabbit IgG as the second antibody. Signals were detected using a DAB substrate kit. Magnification: A and B, $\times 200$; C–H, $\times 400$.

(Figure 2, lanes 2, 6 and 9), but not in other tissues including the ovary (data not shown). The levels of hHSF1 Δ RD proteins were similar in testes of the two lines, but the level in the heart of B6-6 mice was much higher than that of B4-6 mice. The body weights of these two lines were normal (data not shown).

Transgenic male mice were infertile

Surprisingly, transgenic male mice mated with wild-type females did not sire any offspring despite normal copulatory behavior as shown by the formation of vaginal plugs in the female mice (Table I). Their littermates that did not have the transgene sired offspring normally (Table I) and transgene-positive female mice were fertile (data not shown). At autopsy, the testes of transgene-positive male mice were grossly smaller than those of transgene-negative mice (Figure 3), whereas the size of the epididymis in these male mice, in which hHSF1 Δ RD

was not expressed (Figure 2), was normal. On average, the weight of the testes from transgene-positive adult (16-week-old) mice was $\sim 42\%$ of that of transgene-negative littermates (Table I). These results demonstrate the profound effects of expression of active HSF1 on testis development. In contrast to testis development, the heart of B6-6 mice appeared macroscopically normal (Figure 3) and no histological abnormality was observed (data not shown).

Arrest of spermatogenesis at pachytene stage in transgenic mice

Histopathological analysis of the testes of transgene-positive mice showed that the size of the seminiferous tubules was reduced (20–30% in diameter) although the number of tubules per testis appeared normal (Figure 4A and B). The interstitial Leydig cells surrounding the tubules appeared to be increased in number, probably because of the decrease in the number of germ cells. In the seminiferous tubules, consecutive spermatogenic cycles are classically depicted as waves of differentiating germ cells. In wild-type mice, tubules containing elongated spermatids in the inner layer were easily detected, whereas these tubules were hardly detectable in transgene-positive mice (Figure 4A and B). Even in the tubules containing round spermatids, they were few in number per section. Most seminiferous tubules in mice expressing the transgene were rich in primary spermatocytes in pachytene stage and only a small number of round spermatids was observed (Figure 4C–F). There were many degenerating pachytene spermatocytes in which the nucleus was condensed and the cytoplasm was stained strongly with eosin (Figure 4C and D) and some giant cells were also observed (Figure 4E). Analysis of apoptosis by TUNEL assay revealed that 20–30% of seminiferous tubules in cross-sections of testes from transgene-positive mice contained a cluster of apoptotic cells (Figure 5B, arrowheads and D), whereas few apoptotic cells were detected in wild-type mice (Figure 5A and C). This observation revealed that the induction of apoptosis in the presence of an active form of HSF1 is stage specific at pachytene.

To examine cells expressing hHSF1 Δ RD protein, we performed immunostaining analysis using specific antiserum for HSF1. In the testis of wild-type mice, immunostaining for HSF1 was similar in most testicular cells (Figure 4G). In contrast, in the testis of transgenic mice, staining was stronger in the cells in the inner layer (Figure 4H). This result suggested that hHSF1 Δ RD protein was highly expressed in spermatogenic cells, but not in Sertoli cells or interstitial Leydig cells.

We next analyzed the expression of the marker genes for testis development. Histone H1t, a variant of histone H1, and proacrosin normally begin to be expressed at middle pachytene stage (Kashiwabara *et al.*, 1990; Bartell *et al.*, 1996). These transcripts were strongly expressed in the testes of transgene-positive adult mice (Figure 6). The transcript of Sperm-1, a member of the POU-domain gene family, normally begins to be expressed at 36–48 h before meiosis I (Andersen *et al.*, 1993) and was expressed in the testes of transgene-positive adult mice (Figure 6). In contrast, message of cyclin A1 normally increases at late pachytene stage and reaches a peak at diplotene stage (Sweeney *et al.*, 1996). The level of this transcript in the

Fig. 5. *In situ* detection of apoptosis by TUNEL staining. Apoptotic cells were stained brown. Sections were counterstained with methyl green. Apoptotic cells were rare in the testis of wild-type mice (A and C). In contrast, apoptotic cells were abundant in 20–30% of seminiferous tubules (B and D), which are indicated by arrowheads. Magnification: A and B, $\times 100$; C and D, $\times 400$.

testes of transgene-positive mice was much lower than that in wild-type mice (Figure 6). Transcripts of *Gapd-s* and *Hsc70t* are normally expressed in post-meiotic germ cells (Matsumoto and Fujimoto, 1990; Welch *et al.*, 1992) and their levels were much lower in the testes of transgene-positive mice than in wild-type littermates (Figure 6). Although absence of *Hsp70-2* was reported to cause developmental arrest at pachytene stage (Dix *et al.*, 1996), this transcript was expressed in the testes of transgene-positive mice. As *Sprml* is not expressed in the testis of the *Hsp70-2*-null mice (Dix *et al.*, 1997), the developmental stage of testis in hHSF1 Δ RD transgenic mice may proceed further. These results confirmed that spermatogenesis is mainly blocked at late pachytene stage.

Spermatogenesis in juvenile transgenic mice

The level of HSF1 was constant during normal testis development (Figure 7A, lanes 1–3 and 7C). In contrast, the HSF2 level was low before puberty and became high in adult mice. This increase was due to the high level of expression of HSF2 mRNA in late pachytene spermatocytes and spermatids (Sarge *et al.*, 1994). HSF4 expression was not detected in the testes (data not shown). Expression of the transgene was increased during testis development similarly to the expression of HSF2 (Figure 7A, lanes 4–6 and 7C), indicating that hHSF1 Δ RD was expressed in spermatogenic cells. Constitutive HSE binding activity was clearly observed in the testis of 4-week-old mice (Figure 7B). Expression of Hsps in wild-type adult (16-week-old) mice was lower than that in juvenile (4-week-old) mice (Figure 7C), whereas in transgenic adult mice the levels of expression of Hsps remained high

(Figure 7C). This high level of expression was due to enrichment of premature spermatocytes and activation of heat shock genes in the presence of an active form of HSF1.

The progression of the first wave of spermatogenesis was compared between transgene-expressing and wild-type littermates. At 2 weeks postnatally, pachytene spermatocytes were abundant in most seminiferous tubules in d-type and transgene-positive mice. Apoptotic cells were hardly detectable in tubules at this developmental stage (Figure 7D, a and b). After the premeiotic stage, spermatocytes undergo first and second meiotic divisions and become haploid spermatids. Subsequently, round spermatids were observed in seminiferous tubules of wild-type mice at 3 weeks, whereas the haploid cells were rare in transgene-positive mice (Figure 7D, c and d). Apoptotic pachytene spermatocytes appeared by 3 weeks in transgene-positive mice. A cluster of apoptotic cells (more than three apoptotic cells) was observed in 12% of seminiferous tubules (data not shown). By 4 weeks, 34% of tubules contained a cluster of apoptotic pachytene cells in the inner cell layer (Figure 7D, f). These results further confirmed that active HSF1 inhibited the progression of spermatogenesis at pachytene stage and led apoptosis of pachytene spermatocytes.

Heat shock induces stage-specific germ cell apoptosis

A previous study showed that late pachytene spermatocytes were the first germ cells to degenerate (Chowdhury and Steinberger, 1970), and mild hyperthermia within 1 day resulted in marked activation of germ cell apoptosis predominantly at early and late stages (Lue *et al.*, 1999).

Fig. 6. Analysis of expression of genes specific to spermatogenic cells. Total RNAs were isolated from the testes of 16-week-old wild-type mice and two transgenic lines. Northern blotting was performed using specific probes as described (Dix *et al.*, 1997). The arrow indicates a band of testis-specific isoform of β -actin.

If activation of endogenous HSF1 is a major trigger for the induction of germ cell apoptosis, the number of apoptotic germ cells in the testis of transgenic mice would not be increased by heat shock. To test this possibility, scrotal regions of wild-type and transgenic mice were submerged in a water bath maintained at 43°C for 15 min and allowed to recover at room temperature for 24 h. This treatment activated the endogenous HSF1 in the testis of wild-type mice (Figure 8A). As reported previously (Chowdhury and Steinberger, 1970; Lue *et al.*, 1999), histological examination and TUNEL staining of the testis of wild-type mice showed stage-specific activation of germ cell apoptosis (Figure 8B, a and c). Pachytene spermatocytes and round spermatids at early stages and pachytene spermatocytes at late stages were degenerated at 24 h after hyperthermia. Clusters of apoptotic cells were detected in 25–30% of seminiferous tubules (Figure 8B, c). In the testis of transgenic mice exposed to hyperthermia, seminiferous tubules with vacuoles were observed (Figure 8B, b, an arrowhead). These vacuoles may have

been created by elimination of the degenerated germ cells (see Figure 4C and D), which could be accelerated by heat stress. There were no other marked histological alterations in the testis of transgenic mice exposed to hyperthermia compared with those without hyperthermia. Clusters of apoptotic cells were observed in 30–35% of seminiferous tubules (Figure 8B, d, arrows). The increase in the proportion of tubules containing clusters of apoptotic cells may be due to activation of apoptosis of pachytene spermatocytes at an early stage (Figure 8B, d). These observations are in good agreement with the hypothesis that activation of HSF1 would be a major trigger for the induction of apoptosis of germ cells, especially of late pachytene spermatocytes.

Discussion

Extensive studies have revealed that a major role of HSF1 is to protect cells from thermal stress by inducing a set of Hsps. Cells preconditioned with sublethal heat shock can survive otherwise lethal heat stress, a phenomenon known as thermotolerance. *HSF1*-null mouse embryo fibroblasts did not show induction of Hsps in response to heat shock. Consequently, thermotolerance was not acquired and heat shock-induced apoptosis was accelerated (McMillan *et al.*, 1998). *HSF3*-null chicken B lymphocyte DT40 cells show hardly any induction of Hsps after heat shock, and also fail to acquire thermotolerance (Tanabe *et al.*, 1998). Furthermore, *Drosophila* in which *HSF* was mutated did not acquire thermotolerance (Jedlicka *et al.*, 1997). All of these observations conclusively indicated that HSF is essential for acquisition of thermotolerance by inducing Hsps and that HSF supports cell survival against thermal stress. In this study, we unexpectedly found an opposite role of HSF1 through the analysis of transgenic mice expressing an active form of human HSF1. In germ cells at a specific stage, an active form of HSF1 induced cell death by an apoptotic mechanism and inhibited spermatogenesis. Late pachytene spermatocytes were revealed to be target cells for HSF1-induced cell death. These cells have been shown to be highly susceptible to thermal stress (Chowdhury and Steinberger, 1970). This heat susceptibility may be due to the acceleration of apoptosis by activated HSF1 by thermal stress.

Spermatogenesis involves marked structural and biochemical changes, such as synaptonemal complex formation and condensation of chromatin at a temperature lower than core body temperature. Hsps, which act as molecular chaperones, are highly expressed and there are many testis-specific isoforms such as Hsp70-2 (Dix *et al.*, 1996), Hsc70t (Matsumoto *et al.*, 1990) and Apg-1 (Kaneko *et al.*, 1997). These lines of evidence suggested that the process of spermatogenesis is supported by many molecular chaperones. This process should be susceptible to thermal stress that sequesters molecular chaperones. Embryonic weight was reduced when normal female mice were mated with males exposed to thermal stress (Jannes *et al.*, 1998), indicating that embryo quality is linked to sperm quality, and injury caused by thermal stress is passed on to the next generation. Therefore, it is reasonable that germ cells exposed to thermal stress are actively eliminated to protect these cells from descending to the next generation. This phenomenon caused by the activation of HSF1 may be

Fig. 7. Spermatogenesis in juvenile mice. (A) Transgene expression in testis of mice at 2 (lanes 1 and 4), 4 (lanes 2 and 5) and 8 (lanes 3 and 6) weeks after birth. Whole testis extracts were prepared and aliquots of proteins were subjected to Western blot analysis using antiserum against HSF1 (upper panel) or HSF2 (lower panel). Asterisks indicate non-specific bands. (B) Acquisition of DNA binding activities in juvenile mice. Tissue extracts prepared as described above were subjected to gel shift assay using a ^{32}P -labeled HSE oligonucleotide as a probe. Free probe (Free) is indicated at the bottom. (C) Analysis of expression of heat shock genes as well as *HSF* genes during testis development. Total RNAs were isolated from the testes of 2-, 4- and 16-week-old wild-type (+/+) and transgenic (Tg/+) mice. Northern blot analysis was performed using mouse cDNA probes for Hsp110, Hsp70.1 or β -actin, and human cDNA probes for HSF1, HSF2, Hsp90 α , Hsp40 or Hsp27. The transcript of the hHSF1 Δ RD transgene was expressed at a low level at 2 weeks and showed marked increases during testis development. Testis-specific isoforms of Hsp40 (arrow) and actin (star) are indicated. (D) Histological analysis of testis sections from mice at 2 (a and b), 3 (c and d) and 4 weeks (e and f). At 2 weeks, spermatocytes at pachytene stage were abundant in seminiferous tubules in testes of both wild-type and transgenic mice (a and b). Round spermatids that accomplished meiotic cell division appeared in tubules of 3-week-old wild-type mice (c). Apoptotic pachytene spermatocytes with dense nuclei (arrowheads) were seen in the testis of transgenic mice (12% of tubules) (d). At 4 weeks in transgenic mice, clusters of apoptotic pachytene spermatocytes were observed in 34% of tubules (f). Magnification: $\times 400$.

analogous to the elimination of cells with DNA damage from various stresses such as UV irradiation, by an apoptotic mechanism induced by p53 (Polyak *et al.*, 1997).

HSF1 is a good candidate for acceleration of apoptosis of germ cells because it can sense thermal stress directly as well as indirectly. Gel-purified or bacterially expressed mammalian HSF1 was activated by heat shock *in vitro* (Goodson and Sarge, 1995; Larson *et al.*, 1995) and purified *Drosophila* HSF was reversibly activated by heat shock *in vitro* (Zhong *et al.*, 1998). However, HSF1 is not activated by sensing the absolute environmental temperature. Threshold temperature for the activation of human HSF1 was reprogrammed to a decreased temperature of $\sim 10^\circ\text{C}$ when expressed in *Drosophila* (Clos *et al.*, 1993). Interestingly, HSF1 in germ cells is activated by a lower temperature threshold than somatic cells in the testis (Sarge, 1995). In the isolated pachytene spermatocyte HSF1 is markedly activated at temperatures such as 35°C , which are lower than the core body temperature (Sarge, 1995). The germ cell-specific environment may be responsible for lowering the temperature set point for HSF1 activation. The unique feature of HSF1 regulation in germ cells may facilitate monitoring of changes of temperature to decide whether germ cells should be killed.

Forced expression of Hsps in cells has been reported to affect many cell fates. Overexpression of Hsp70 suppressed growth of *Drosophila* cells at normal temperature (Feder *et al.*, 1992). In yeast cells carrying deletions in both *SSA1* and *SSA2* Hsp70 genes, increased expression of other Hsps by HSF was suggested to inhibit cell growth (Halladay and Craig, 1995). These observations suggested that high concentrations of Hsps are detrimental to growth at normal temperature. Furthermore, many studies have demonstrated that overexpression of Hsps affected processes of cell death (Galea-Lauri *et al.*, 1996; Mosser *et al.*, 1997). In all of these cases, however, the proportions of each Hsp were not physiological. As major Hsps act co-ordinately as molecular chaperones, forced overexpression of one Hsp would cause an imbalance of the molar ratios of Hsps and subsequently many signaling cascades may be affected. We observed no detrimental effect on growth and hemin-induced erythroid differentiation of human K562 cells (Figure 1E, data not shown) or cell death of human thymic HPB-ALL cells induced by anti-Fas antibody (data not shown) (Lee *et al.*, 1998), as well as cells in the mouse heart, when Hsps were highly expressed by expressing an active form of HSF1. Our observations revealed that at least in some cells and

Fig. 8. Germ cell death induced by single exposure to heat. (A) Acquisition of HSF1 DNA binding activity. Testicles of anesthetized wild-type mice were submerged in a water bath at 22°C (lanes 1, 2, 5, 6, 9 and 10) or 43°C (lanes 3, 4, 7, 8, 11 and 12) for 15 min and whole tissue extracts were prepared immediately after heat shock. Gel shift assay was performed using a ^{32}P -labeled HSE oligonucleotide as a probe in the presence of preimmune serum (lanes 1–4), anti-HSF1 serum (lanes 5–8) or anti-HSF2 serum (lanes 9–12). (B) Histological analysis of germ cell death in wild-type (a and c) and transgenic (b and d) mice induced by heat shock. At 24 h after heat treatment at 43°C for 15 min, testes were dissected, fixed and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin and eosin (a and b) or stained for apoptotic cells by the TUNEL method (c and d). Non-specific TUNEL staining was observed in the interstitial Leydig cells. An arrowhead in b indicates a tubulus having many vacuoles. Seminiferous tubules containing a cluster of apoptotic cells are indicated by arrows in c and d. Magnification: a, b and d, $\times 200$; c, $\times 100$.

tissues, forced expression of a set of Hsps by an active form of HSF1 does not affect cell growth, differentiation or death. This is quite important for the therapeutic use of the heat shock response. Some compounds that induce heat shock response without serious side effects have been used clinically in wound healing and treatment of ischemia (Vigh *et al.*, 1997). Our results support the use of compounds that activate HSF1 and lead to increases in the production of a set of Hsps, although these compounds may lead to male infertility.

Our observations raise the question of how HSF1 induces apoptosis of germ cells. Germ cell apoptosis is regulated by regulators of cell death such as the Fas system, bcl-2 and bax (Knudson *et al.*, 1995; Furuchi *et al.*, 1996; Lee *et al.*, 1999). HSF1 may induce or repress unknown target genes involved in germ cell apoptosis. In *Drosophila*, activated HSF binds to the loci on polytene chromosomes involved in development (Westwood *et al.*, 1991), and HSF is required under normal growth conditions for oogenesis and early larval development (Jedlicka *et al.*, 1997). These observations suggest that HSF may regulate genes involved in cell growth, differentiation and death. Alternatively, HSF1 may modulate functions of other transcription factors that may regulate cell growth and death such as *c-myc* and tumor suppressor p53, which are known to be involved in germ cell apoptosis (Suzuki *et al.*, 1996; Yin *et al.*, 1998). Complex formation of HSF3 with c-Myb (Kanei-Ishii *et al.*, 1997) and of HSF1 with signal transducer and activation of transcription 1 (STAT1) (Stephanou *et al.*, 1999) have been reported previously.

A single exposure to mild heat causes apoptosis of early

and late pachytene spermatocytes and round spermatids (Chowdhury and Steinberger, 1970; Lue *et al.*, 1999), and experimental cryptorchidism induces apoptosis of almost the same subset of cells (Yin *et al.*, 1997). Our study confirmed that the same cell types were affected by a single heat and showed that expression of the active form of HSF1 induced mostly late pachytene spermatocytes. Mild heat exposure (43°C for 15 min) of the testis of the transgenic mice showed a small increase in the number of seminiferous tubules with a cluster of apoptotic cells (30–35%) compared with the untreated testis (25–30%). This increase may have been due to the apoptosis of early pachytene spermatocytes and of the small number of round spermatids induced by heat stress. Although the cell type of germ cells affected by heat is altered by the timing and temperature of heat exposure, we demonstrated that the activation of HSF1 is sufficient to induce apoptosis of at least late pachytene spermatocytes. In these cells, the death cascade may be more easily turned on by active HSF1 than in other germ cells or somatic cells. The balance of pro-apoptotic factors and anti-apoptotic factors would differ between cell types.

Materials and methods

Cell culture

Human erythroblastic K562 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. To induce erythroid differentiation, K562 cells were treated with 30 μM hemin for 3 days. Erythroid differentiation was estimated by benzidine staining. Briefly, 10 ml of saturated *o*-dianisidine (Nakalai Tesque Co., Kyoto, Japan) in 3% acetic acid was mixed with 1 ml of 30% hydrogen peroxide just before use.

Cell suspensions in phosphate-buffered saline (PBS) (100 µl) were mixed with the dianisidine solution (10 µl) and incubated for 30 min. Benzidine-positive cells were counted under a light microscope.

Construction of plasmid and transgenic mice

A plasmid containing mutant hHSF1ΔRD was constructed by PCR-mediated site-directed mutagenesis consisting of two-step PCR, using two overlapping internal primers at the mutagenic site and two outer general primers each flanked by a *Hind*III site. The internal primers used were: hHSF1-9 (5'-GACAGTGGCTCAGCACATCGCCCTCTTCCGTGGAC-3') and hHSF1-10 (5'-GTCCACGGAAGATGGGCG-ATGTGCTGAGCCACTGTC-3'). The resulting mutant hHSF1ΔRD cDNA fragment was inserted downstream of the human β-actin promoter into the pHβAPr-1-neo expression vector. The construct was linearized by *Eco*RI. Transgenic mice were generated by microinjecting the DNA into fertilized eggs obtained by BDF1 (C57BL/6 × DBA/2) mating. The tail genomic DNA was prepared and examined to detect integration of the transgene by Southern blotting (founder mice) or PCR analysis (subsequent generations) using primers: hHSF1-11 (5'-TAGTCCACGGAAGATGGCG-3') and hHSF1-12 (5'-ATGCAGCACCCATGCTTC-CTG-3'). Breeding lines of mice were maintained by backcrosses to C57BL/6 mice.

Exposure of mouse testis to hyperthermia

Mice were anesthetized by intraperitoneal injection of 25 µg/kg sodium pentobarbital (Dainippon Pharmaceutical Co., Osaka, Japan) and the scrotal regions were submerged in a water bath at 43°C for 15 min. To prepare whole testis extracts, testes were immediately dissected, frozen in liquid nitrogen and stored at -80°C. For histological analysis, mice were recovered to room temperature and testes were dissected 24 h after hyperthermia and immediately fixed in Bouin's fixative (Muto Pure Chemical Co., Tokyo, Japan).

Western blot analysis and gel shift assay

Tissues and cultured cells were frozen in liquid nitrogen and stored at -80°C until further processing. Whole cell extracts were prepared as described (Nakai *et al.*, 1995) and tissue extracts were prepared by homogenization in 5 vol. of buffer C (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin, 1 µg/ml leupeptin and 0.5 mM dithiothreitol) using a Dounce homogenizer. Gel shift assay was performed using a self-complementary consensus HSE oligonucleotide (5'-CTAGAAGCTTCTAGAAGCTTCTAG-3') as a probe. Antiserum (2 µl of 1:10 dilution in PBS) was mixed with extracts (10 µg protein) in a volume of 10 µl and incubated for 10 min at room temperature. Binding reactions containing a ³²P-labeled HSE probe were applied to 4% native polyacrylamide gels and autoradiography was performed.

Histopathology and detection of apoptotic germ cells

The testes were dissected immediately after sacrifice, fixed in Bouin's fixative at 4°C for 16–24 h, embedded in paraffin and cut into sections 5 µm thick. Sections were stained with hematoxylin and eosin or stained for apoptotic cells by the TUNEL method using an apoptosis *in situ* detection kit (Wako Pure Chemical Co., Osaka, Japan) and examined microscopically.

Immunostaining

Immunostaining of cryostat sections was performed as described previously (Kawazoe *et al.*, 1999). Embryos were fixed in 4% paraformaldehyde and sequentially equilibrated with 12, 15 and then 18% sucrose in PBS. Samples were frozen with Tissue-Tek OCT compound (Sakura Finetechnical Co., Tokyo, Japan), and serial sections (12 µm) were cut. After drying, sections were treated with 4% paraformaldehyde at room temperature for 15 min, 100% methanol at -20°C for 30 min and 4% H₂O₂ at room temperature for 1 h. Then, sections were blocked with 5% dried milk, treated with preimmune serum or serum specific to HSF1 (anti-HSF1γ, 1:100 dilution in 5% dried milk) as the first antibody at room temperature for 1 h, and then treated with peroxidase-conjugated goat anti-rabbit IgG (1:100 dilution) (Cappel) at room temperature for 1 h. After washing with PBS, signals were detected using a DAB substrate kit (Vector Laboratories, Inc., CA).

RNA analysis

Total RNA was extracted from cells and testes using TRIZOL reagent (Gibco-BRL) according to the manufacturer's instructions. GeneScreen Plus membranes (NEN) containing 10 µg of total RNA were prepared and hybridized in hybridization solution (50% formamide, 6× SSC, 1%

SDS, 3× Denhardt's reagent, 400 µg/ml salmon sperm DNA) containing ³²P-labeled cDNA probes at 42°C overnight. Membranes were washed in 0.1× SSC and 0.1% SDS at 65°C. When membranes containing mouse RNA were hybridized using human cDNA probes, they were washed in 2× SSC and 0.1% SDS at 60°C.

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