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HSP70 Protects against TNF-Induced Lethal Inflammatory Shock

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Summary

The heat shock (HS) response is a universal response activated after exposure to various stimuli. The major HS protein (HSP) is the 72 kDa HSP70 with strong homology in different eukaryotic species. We demonstrate that HS treatment of mice leads to a strong induction of HSP70 in several organs and confers significant protection against lethality induced by tumor necrosis factor (TNF). HS prevents high production of interleukin-6 and nitric oxide and reduces severe damage and apoptosis of the enterocytes in the bowel. Mice deficient in the inducible *hsp70.1* gene were no longer protected by HS treatment. We show that HS can be applied successfully in an antitumor protocol based on TNF and interferon-γ, leading to a significant inhibition of lethality but not to a reduction of antitumoral capacity.

Introduction

Heat shock (HS) proteins (HSPs) are conserved molecules present in all prokaryotes and eukaryotes studied so far. Under normal physiological conditions, the expression of these proteins is very low (Craig and Gross, 1991). In stress situations, a very strong synthesis of these proteins has been observed (Lindquist and Craig, 1988). These stress stimuli can either be physiological (growth factors and hormonal stimulation), environmental (HS, heavy metals, and ultraviolet radiation), or pathological (inflammation, autoimmune reactions, and viral, bacteriological, or parasitic infections). The main function of HSPs is to operate as intracellular chaperones for aberrantly folded or mutated proteins and to provide cytoprotection against the stress conditions mentioned above. For this reason, the presence of a cellular stress response in cancer cells reduces their sensitivity to chemical stress caused by insufficient tumor perfusion or chemotherapy (Jolly and Morimoto, 2000).

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One of the major HSPs is HSP70, named after its molecular mass of approximately 70 kDa. The subfamily, consisting of at least seven members in the mouse, contains both constitutive and inducible forms (Lindquist and Craig, 1988). As constitutive member in the mouse, the 70 kDa HS cognate HSC70 (Giebel et al., 1988), as well as the 75 kDa and 78 kDa glucose-regulated proteins GRP75 (Domanico et al., 1993) and GRP78 (Kozutsumi et al., 1989), has been described. HSP70.1 and HSP70.3 are both inducible (Hunt et al., 1993), while the spermatocyte-specific HSP70.2 is expressed during the meiotic phase of spermatogenesis (Allen et al., 1988). The testis-specific HSC70 (HSC70t) is expressed in postmeiotic spermatids (Matsumoto and Fujimoto, 1990). Recently, several reports have dealt with the protecting capacities of HSP70 against various toxic stimuli. HS was demonstrated to prevent lethality induced by lipopolysaccharide (LPS) in rats and mice (Ryan et al., 1992; Hotchkiss et al., 1993). This protection also correlated with HSP70 upregulation in several organs (Hotchkiss et al., 1993). Ischemic preconditioning of the liver also leads to strong HSP70 induction, which results in resistance to subsequent ischemia-reperfusion injury of the liver in the rat (Kume et al., 1996).

HSP70-transgenic mice were generated a few years ago (Marber et al., 1995). In these mice, the rat *hsp70* gene was placed under control of an hCMV-IE enhancer and β-actin promoter, resulting in strong constitutive expression in cardiac muscle, skeletal muscle, and brain. These mice display resistance against several models of heart ischemic injury (Marber et al., 1995; Hutter et al., 1996; Trost et al., 1998). Also by means of adenoviruses, the protecting capacity of HSP70 against myocardial ischemia was demonstrated (Mestril et al., 1996). Another argument for a protective role of HSP70 against LPS toxicity was provided by the construction of HS transcription factor-1 (HSF-1) knockout mice (McMillan et al., 1998). HSFs regulate the stress-inducible synthesis of HSPs (Morimoto, 1998). HSF-1 knockout mice show an increased sensitivity to LPS-induced toxicity and lethality (Xiao et al., 1999). Finally, there are numerous reports clearly stating a protecting role of HSP70 in vitro by means of HS induction or HSP70 overexpression (Jäättelä et al., 1989; Larrick and Wright, 1990; Margulis et al., 1991) against toxicity induced by several cytokines.

In this study, we evaluated the role of HSP70 induction by means of whole-body HS in the toxicity and lethality induced by TNF in mice. TNF is a pleiotropic cytokine mainly produced by activated macrophages and has a strong antitumor activity both in vitro and in vivo (Aggarwal and Natarajan, 1996). Systemic administration of TNF at relatively high doses induces a systemic inflammatory response syndrome, characterized by bowel necrosis, liver damage, and severe hypotension, leading to death (Tracey and Cerami, 1993). We demonstrate that application of HS in mice induces HSP70 in several organs and significantly leads to attenuation of TNF-induced lethality. We further show that HSP70 induction prevents interleukin-6 (IL-6) and nitric oxide (NO) induc-

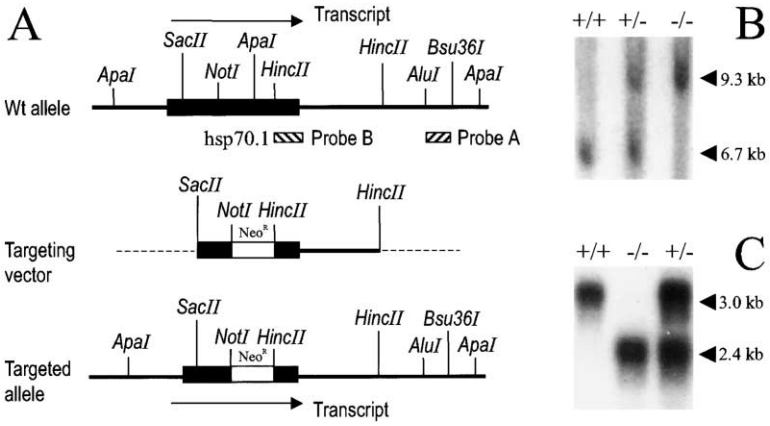

Figure 1. Generation of *hsp70.1*$^{-/-}$ Mice

(A) Targeting strategy.

(B) Southern blot analysis of representative mouse tail DNA digested with ApaI and hybridized with probe A.

(C) Northern blot analysis of representative HS-induced mouse embryonic fibroblast mRNA hybridized with probe B. The *hsp70.1-NeoR* fusion mRNA was calculated to be 2.4 kb based on the sequence of the targeting vector.

tion in the serum as well as apoptosis and tissue damage of the intestinal tract. We provide direct proof for the involvement of HSP70 using *hsp70.1*-deficient mice. Finally, we provide evidence for a potential application of HS in antitumor treatment based on TNF and interferon-γ (IFN-γ).

Results

Generation of *hsp70.1*$^{-/-}$ Mice

The *hsp70.1* genomic structure, the targeting vector, and the targeted allele are shown in Figure 1A. After restriction digest of genomic DNA with ApaI and hybridization with probe A, a 6.7 kb fragment was obtained for wild-type (wt) mice. The targeted allele provided a fragment of 9.3 kb due to loss of the unique ApaI site in the *hsp70.1* gene by homologous recombination with the targeting vector (Figure 1A). The latter was constructed with the neomycin-resistant (*NeoR*) gene without promoter as positive selection marker. Thus, the *NeoR* gene can be expressed from the *hsp70.1* gene promoter, provided accurate homologous recombination had occurred. The frequency of the homologous recombination was 2/78 of G418-resistant clones. Two cloned embryonic stem (ES) cells were injected into blastocysts. The resulting chimera mice bred so that *hsp70.1* heterozygous mice (−/−) were obtained. After eight generations of backcross with C57BL/6J mice, *hsp70.1* homozygous mice (+/−) were generated from crossbreeding between heterozygotes. *Hsp70.1* (+/+), (+/−), and (−/−) genotypes were born according to expected Mendelian ratios of 1:2:1. Screening of ES cells and mice was performed by Southern blot, yielding a single fragment of 6.7 kb for wt, a 9.3 kb fragment for knockout, and both fragments for heterozygote mice (Figure 1B). To further confirm the targeted disruption of the *hsp70.1* gene, we examined *hsp70.1* mRNA transcription by Northern blot analysis (Figure 1C). Cultures of wt, homozygous, and heterozygous embryonic fibroblasts were cultured at 42°C for 1 hr, followed by 12 hr incubation at 37°C. Probe B, specific for the *hsp70.1* gene, hybridized with the 3.0 kb mRNA for the wt allele and the 2.4 kb mRNA for the targeted allele. These results confirm that correct homologous recombination had occurred in ES cells. *Hsp70.1* gene knockout mice developed normally and were fertile. Gross and histopathological observations revealed no apparent differences between knockout and wt mice under specific pathogen-free conditions.

HS Treatment of Mice Induces HSP70 in Several Organs

A number of different methods to induce HS have been described previously. Some of these methods demonstrate HSP70 induction in several organs in rats and mice (Hotchkiss et al., 1993; Kume et al., 1996). First, we evaluated the increase in core body temperature during the HS induction method used. Mice (n = 12) were HS treated for 20 min. The rectal body temperature was monitored from HS start until 10 min after HS end and was recorded every 2 min. The body temperature increased in a linear way up to 10 min after HS start and then displayed a plateau level of about 41.5°C until HS end. .6 min after HS end, all mice had regained their normal body temperature of approximately 37°C (Figure 2A). To test whether the HS conditions used had led to a strong HSP70 induction, mice were HS treated for 20 min; 2, 6, 12, 24, 48, 72, or 96 hr thereafter, mice were killed. Seven organs (liver, lung, duodenum, jejunum, colon, heart, and kidney) were removed and homogenized in glycerol buffer. The homogenates were analyzed by Western blot for HSP70 presence (Figure 2B). HSP70 induction in the liver was clear at the earliest time point. The expression was maximal between 6 and 24 hr following HS and diminished after 48 hr. In control mice (not subjected to HS), no detectable HSP70 was present. A similar HSP70 induction profile was also observed in lung samples, high levels being reached from 6 to 24 hr after HS. In the duodenum, HSP70 was detected only between 6 and 12 hr after HS. A very high expression was also observed in the jejunum 6 and 12 hr after HS. In the colon, the expression was low at 2 hr but remained strong 6 to 48 hr after HS. In the heart, there was only induction 24 hr after HS, while no HSP70 could be detected in the kidney (results not shown). These data demonstrate that the method used for HS application results in a high increase in core body temperature and in a strong HSP70 induction in several organs.

HS Induction Prevents TNF-Induced Lethality

Based on our data on HSP70 induction, we evaluated the effect of HSP70 upregulation on TNF-induced lethality. In most of the organs tested, the induction is maximal

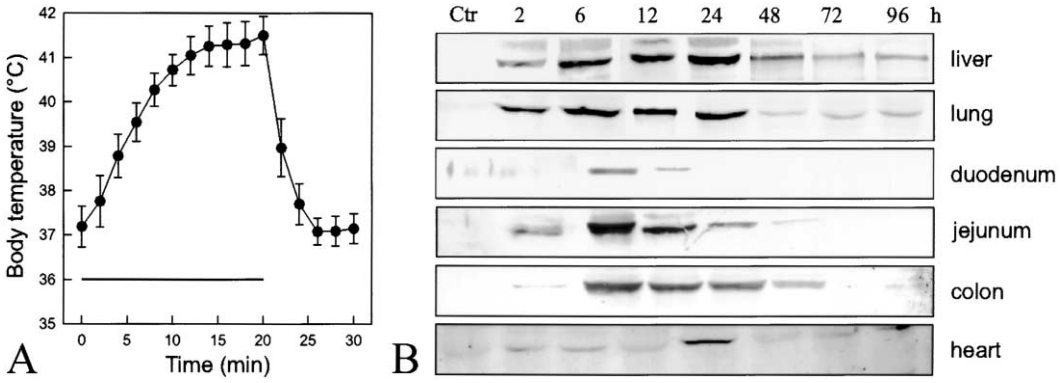

Figure 2. Increase in Body Temperature and HSP70 Induction in Tissue Homogenates of Mice after Whole-Body HS

(A) Rectal body temperature was monitored and recorded (●, n = 12) during HS until 10 min after HS (black line, HS induction period).
(B) Mice were subjected to HS and killed at different time points. Expression of HSP70 was analyzed by Western blotting.

from 6 to 24 hr after HS. Therefore, we subjected mice to HS and challenged them i.v. with 15 μg TNF, i.e., a 100% lethal dose, 6 hr (n = 8), 12 hr (n = 45), or 24 hr (n = 6) later. Mice were also challenged with TNF 2 hr (n = 6) or 48 hr (n = 6) after HS (low or no expression of HSP70). As controls, mice (n = 44) were challenged with TNF without prior HS. We show that mice are significantly protected from TNF-induced lethality when treated with TNF 12 hr after HS (Figure 3): a statistically significant difference was observed, both in survival time ($p < 0.0001$) and in final lethality (12/45 dead HS-pretreated mice versus 44/44 dead control mice; $p < 0.0001$). When mice were challenged with TNF 6 hr after HS, a highly significant delay in lethality ($p = 0.0066$) was found. However, eventually all mice died as a result of TNF toxicity. No significant protection was observed when mice were challenged 2, 24, or 48 hr after HS. From these data we conclude that mice are maximally protected 12 hr after HS treatment.

Effect of HS on TNF-Induced Hypothermia, NO, and IL-6

Injecting mice with a high (lethal) dose of TNF results in massive production of NO (leading to hypotension) and IL-6. High levels of nitrate and nitrite (stable end products of NO) and IL-6 were observed in the serum after TNF challenge (Van Molle et al., 1997). It is believed that sustained levels of IL-6 (Libert et al., 1990) and NO are representative of a lethal outcome. First, we studied the effect of HS treatment on the drop in temperature induced by TNF injection. Mice were HS pretreated (n = 12) and challenged i.v. 12 hr later with 15 μg TNF. Control mice (n = 12) received TNF without prior HS. HS-pretreated mice have significantly less TNF-induced hypothermia compared to mice challenged with TNF without prior HS treatment ($p < 0.001$ from 6 to 28 hr after the challenge) (Figure 4A). In both groups the body temperature dropped, but HS-pretreated mice recovered between 12 and 24 hr after the challenge. By 48 hr after the TNF challenge, all HS-pretreated surviving mice had regained normal body temperatures. In order to study the effect of HS on TNF-induced NO and IL-6, mice were pretreated with HS (n = 30) and challenged 12 hr later i.v. with 15 μg TNF. Control mice (n = 30) were challenged i.v. with TNF without HS pretreatment. One, three, six, nine, twelve, or twenty-four hours after the challenge, blood was taken by cardial puncture (six mice per time point) and serum was prepared. HS pretreatment significantly reduced the NO production (Figure 4B). A highly significant difference was found 9 hr ($p = 0.0019$), 12 hr ($p < 0.0001$), and 24 hr ($p < 0.0001$) after the challenge for HS-pretreated versus control mice. We also observed a strong inhibiting effect of HS on TNF-induced IL-6 production. IL-6 induction (Figure 4C) was significantly lower in HS-pretreated mice than in control mice at 3 hr ($p = 0.0233$), 6 hr ($p = 0.0025$), 9 hr ($p = 0.0042$), 12 hr ($p = 0.0002$), and 24 hr ($p = 0.0001$) after TNF challenge. These data demonstrate that HS pretreatment significantly prevents TNF-induced NO and IL-6 production.

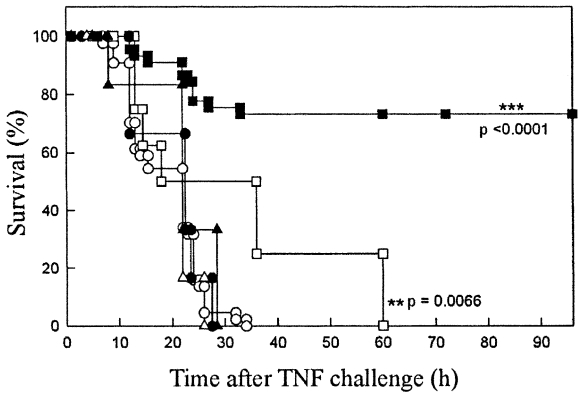

Figure 3. HS Treatment 12 Hr before TNF Challenge Prevents Lethality

Mice were subjected to HS and challenged i.v. with 15 μg TNF 2 hr (●, n = 6), 6 hr (□, n = 8), 12 hr (■, n = 45), 24 hr (△, n = 6), or 48 hr (▲, n = 6) after HS. Controls were challenged with TNF without prior HS (○, n = 44).

HS Prevents TNF-Induced Bowel Damage

Challenging mice with TNF results in severe bowel swelling and damage (Tracey et al., 1986; Piguet et al., 1998). To evaluate the HS effect on TNF-induced bowel damage, mice were subjected to HS (n = 12), followed after 12 hr by an i.v. challenge with 15 μg TNF. Control mice (n = 12) were challenged i.v. with 15 μg TNF without prior HS and killed after 1 hr. The small intestine (duodenum,

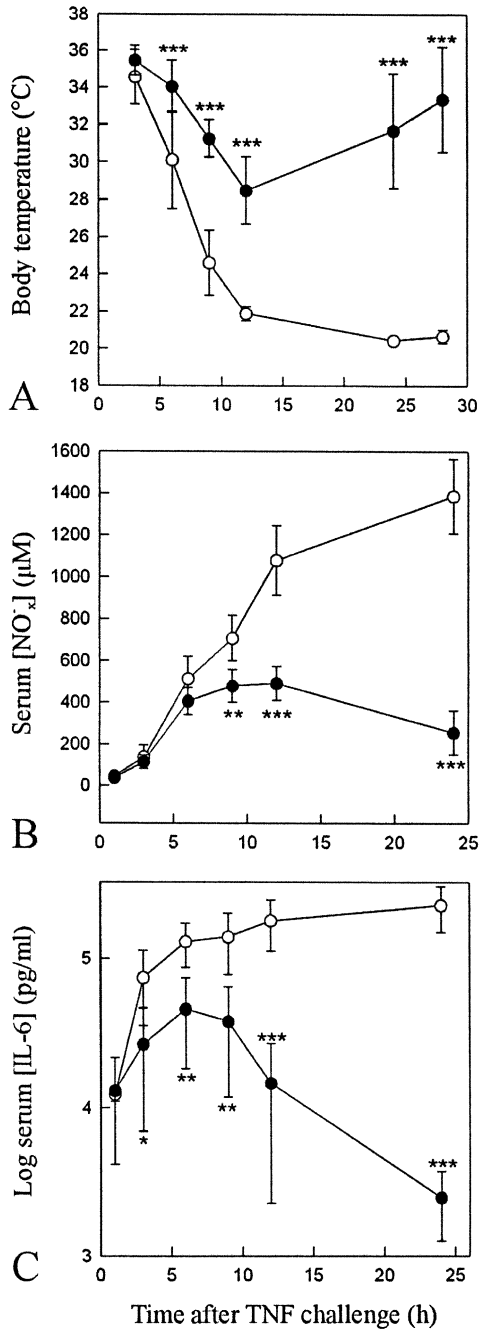

Figure 4. Effect of HS on TNF-Induced Hypothermia as well as TNF-Induced NO and IL-6 Production

(A) Mice were subjected to HS and challenged i.v. after 12 hr with 15 μg TNF (●, n = 12). Control mice were challenged i.v. with 15 μg TNF without prior HS (○, n = 12). Rectal body temperature was measured at the indicated time points after TNF treatment.

(B–C) Mice were subjected to HS and challenged i.v. after 12 hr with 15 μg TNF (●, n = 30). Control mice were challenged i.v. with 15 μg TNF without prior HS (○, n = 30). Blood was withdrawn and analyzed for NO_x^- and IL-6 concentrations 1, 3, 6, 9, 12, or 24 hr after TNF administration (n = 6 per time point).

jejunum, and ileum) was removed and weighed with its content (n = 12), which was significantly high ($p < 0.0001$ compared to mice treated with PBS). HS pretreatment greatly prevented exudation ($p < 0.0001$ compared to mice treated with TNF without prior HS) (Figure 5A). The small intestine is known to increase dramatically in weight at early time points (0.5–1.5 hr) after TNF challenge due to excessive fluid exudation (Piguet et al., 1998).

In order to investigate in more detail the TNF-induced tissue damage and HS-induced protection, parts of the jejunum were removed and 20% homogenates (n = 6) were made. The extent of apoptosis was determined using a DNA fragmentation ELISA. The degree of background apoptosis in PBS-treated mice was regarded as 100%, which is a measure for normal physiologically occurring apoptosis. TNF significantly increased the extent of apoptosis in the jejunum ($p = 0.0029$ as compared to PBS treatment), while HS pretreatment significantly inhibited this tissue damage ($p = 0.0232$ as compared to TNF treatment without prior HS) (Figure 5A). Since activation of caspases is a typical hallmark for apoptosis, we also determined the caspase activity in the homogenates. Cleavage of the chromogenic substrate acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin (ac-DEVD-amc) is a measure of caspase-3 and caspase-7 activity, both known as executioner caspases. In homogenates of mice treated with PBS (n = 6), there was some background caspase-like activity, but that activity was significantly increased after TNF challenge ($p = 0.0053$ as compared to PBS treatment) (Figure 5A). HS pretreatment prevents apoptosis, since DEVDase activity was significantly lower as compared to a TNF challenge without prior HS ($p = 0.0030$).

To investigate the TNF-induced tissue damage at the cellular level, parts of the jejunum were removed and stained with hematoxylin/eosin, and analyzed by immunohistochemistry for HSP70 induction or analyzed for apoptosis by TUNEL assay. Treatment with TNF (Figure 5C) resulted in severe damage of the jejunum compared to untreated animals (Figure 5B). The villi are flattened due to loss of their upper part with denudation at the top. The crypts are distended and contain mucus and debris. We also observed loss of goblet cells. When mice were HS-pretreated 12 hr before the TNF challenge, the damage was markedly reduced (Figure 5D). There is less shortening of the villi, and the crypts retain their normal architecture. Tissue sections were also analyzed by TUNEL assay to stain apoptotic cells. In control mice, no TUNEL staining was found (Figure 5E); in contrast, the top of the villi of TNF-treated mice showed positive TUNEL staining, as visualized by brown nucleus staining (Figure 5F, arrows). In HS-pretreated mice, TUNEL staining was completely absent (Figure 5G). With immunohistochemistry we only observed detectable HSP70 in the jejunum from HS-treated mice (Figure 5J), while only background staining was detected in PBS-injected mice (Figure 5H) and TNF-injected mice (Figure 5I) without prior HS. The expression was maximal at the top of the villi, demonstrating that HSP70-expressing cells were protected against TNF-induced apoptosis. These data demonstrate that HS pretreatment completely prevents the TNF-induced tissue damage (apoptosis) at the site of the jejunum.

HS-Induced Protection against TNF Toxicity Is Absent in *hsp70.1*$^{-/-}$ Mice

To investigate whether induction of HSP70 is responsible for HS-conferred protection, we used *hsp70.1*$^{-/-}$

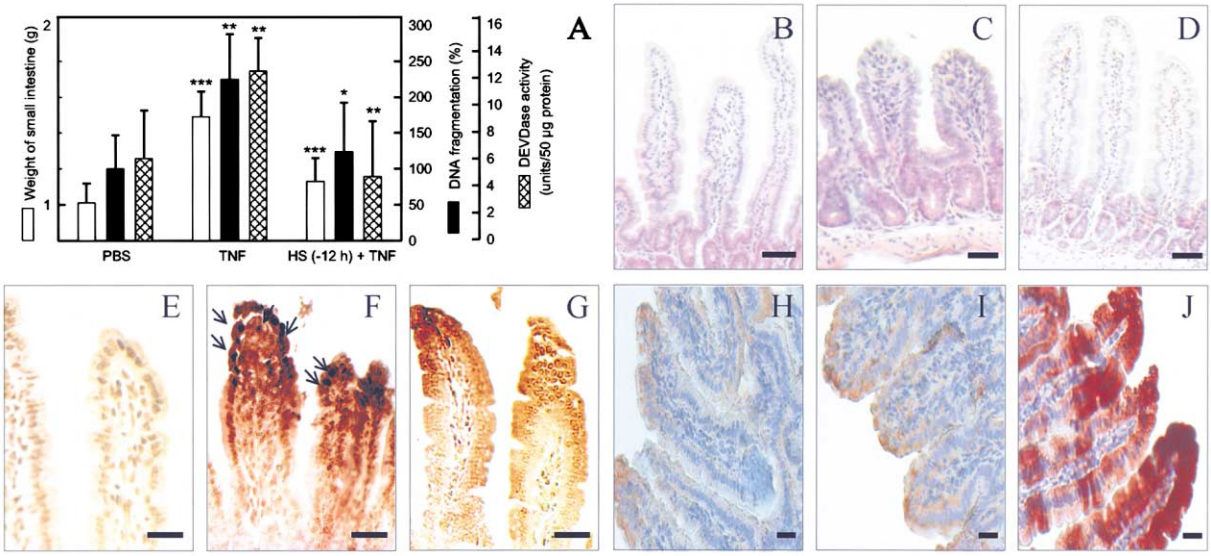

Figure 5. HS Pretreatment Prevents TNF-Induced Bowel Damage

Mice were kept at room temperature or subjected to HS and challenged i.v. after 12 hr with 15 μg TNF. Parts of the jejunum were homogenized for assessment of DNA fragmentation and DEVDase activity (A). Other parts of the jejunum were fixed and embedded in paraffin, followed by hematoxylin/eosin staining (B–D), TUNEL assay (E–G), or immunohistochemistry (H–J). PBS-treated mice (B, E, and H), TNF-treated mice (C, F, and I), and TNF-treated mice 12 hr after HS (D, G, and J) (scale bars, 10 μm).

mice. First, we analyzed the induction of HSP70 in these mice after HS treatment. Wt and *hsp70.1*$^{-/-}$ mice were HS treated and 12 hr later were sacrificed to remove liver, jejunum, and colon. While induction of HSP70 is very obvious in liver, jejunum, and colon of wt mice, no induction whatsoever was found 12 hr after HS (Figure 6A, inset).

Wt ($n = 13$) and *hsp70.1*$^{-/-}$ mice ($n = 12$) were subjected to HS and 12 hr later were challenged with 15 μg TNF. As control groups, we also treated wt ($n = 13$) and *hsp70.1*$^{-/-}$ ($n = 12$) mice without prior HS with 15 μg TNF (we observed no difference in sensitivity to TNF between wt and *hsp70.1*$^{-/-}$ mice; data not shown). HS treatment significantly prevented TNF-induced lethality in wt mice ($p < 0.0001$) (Figure 6A) but not in *hsp70.1*$^{-/-}$ mice ($p > 0.05$) (Figure 6B).

To study the role of HSP70 in the inhibition of TNF-induced hypothermia as well as in NO and IL-6 production, wt ($n = 6$) and *hsp70.1*$^{-/-}$ mice ($n = 6$) were HS subjected and challenged 12 hr later with 15 μg TNF. Wt ($n = 6$) and *hsp70.1*$^{-/-}$ mice ($n = 6$) without prior HS were challenged with TNF as control groups. As observed before, hypothermia, NO, and IL-6 levels were significantly inhibited by HS in wt mice (Figure 6C; $p < 0.004$) but not at all in *hsp70.1*$^{-/-}$ mice (Figure 6D, $p > 0.05$).

These data clearly demonstrate that induction of HSP70 by HS is responsible for inhibition of hypothermia as well as for NO and IL-6 induction.

HS Application in an Antitumor Protocol

Inoculation of C57BL/6 mice with B16Bl6 melanoma cells is a syngeneic tumor model. Application of TNF in combination with IFN-γ induces regression of these tumors but is accompanied with high mortality (Brouckaert et al., 1986). In order to reduce or inhibit this toxicity, we evaluated the application of HS induction in an antitumor protocol. Since this experiment involves a 10 day HS treatment, we investigated whether ten consecutive HS treatments led to a feedback on HSP70 induction, resulting in lack of protection. To that end, mice ($n = 11$) were HS treated for 10 consecutive days and were injected i.v. with 15 μg TNF 12 hr after the tenth HS treatment. As controls, mice were treated with 15 μg TNF 12 hr after a single HS treatment ($n = 12$) or without HS treatment ($n = 12$). To analyze HSP70 induction, liver, jejunum, and colon were removed from parallelly HS-treated mice at the time of TNF injection (12 hr after HS). A single HS treatment induced high HSP70 levels; after ten consecutive HS treatments, the expression of HSP70 was still high, even higher as compared to a single HS treatment (Figure 7A, inset). Furthermore, the increased number of HS treatments led to a significant protection against TNF lethality, although statistically not better than a single HS treatment (Figure 7A). We also observed a better protection against TNF-induced hypothermia. Nine and twelve hours after TNF challenge, a significant difference in body temperature was found between mice treated ten times or only once with HS ($p = 0.0041$ and $p < 0.0001$ for 9 and 12 hr, respectively) (Figure 7B). These data indicate that repeated HS treatments do not lead to downmodulation of *hsp70* expression or tolerization against HSP70 induction and that increased HSP70 levels provide a better protection (dose response between HSP70 levels and degree of protection).

In antitumor experiments, mice were inoculated s.c. with B16Bl6 cells on day 0 and divided randomly into three groups on day 9. One group ($n = 10$) received daily s.c. injections of PBS for 10 days. The HS group ($n = 11$) was treated s.c. with 10 μg TNF in combination with 5000 IU IFN-γ 12 hr after HS for 10 consecutive days. Control mice ($n = 10$) received ten daily injections

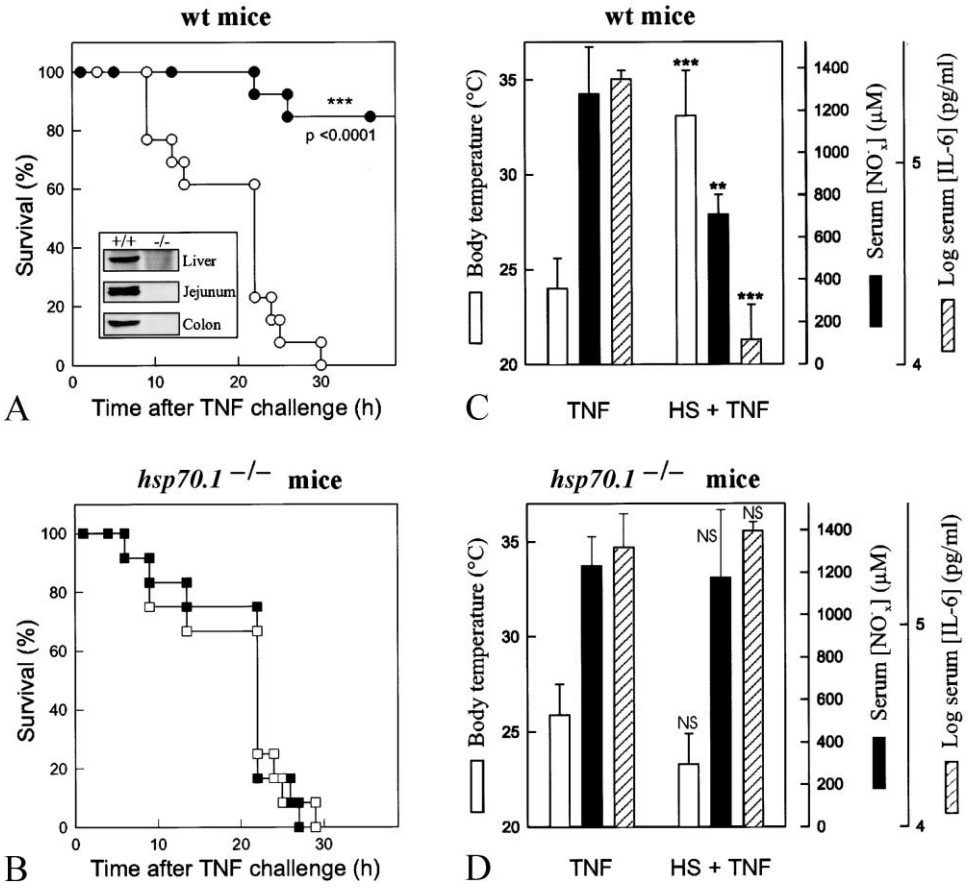

Figure 6. HS-Induced Protection Is Absent in *hsp70.1*$^{-/-}$ Mice

(A–B) Wt mice (●, n = 13) and *hsp70.1*$^{-/-}$ mice (■, n = 12) were HS treated and challenged i.v. after 12 hr with 15 μg TNF. Control wt mice (○, n = 13) and *hsp70.1*$^{-/-}$ mice (□, n = 12) were injected with 15 μg TNF without prior HS. Liver, jejunum, and colon were removed from parallelly HS-treated animals 12 hr after HS. (inset) Western blot for HSP70 12 hr after HS.

(C–D). Wt and *hsp70.1*$^{-/-}$ mice (both n = 6) were HS treated and challenged i.v. after 12 hr with 15 μg TNF. Wt and *hsp70.1*$^{-/-}$ mice (both n = 6) were injected with 15 μg TNF without prior HS. Twenty-four hours after challenge, body temperature was recorded, and blood was withdrawn to determine NO_x^- and IL-6 levels. NS, nonsignificant.

with TNF and IFN-γ without prior HS. The combination of TNF and IFN-γ was administered s.c. in the vicinity of the tumor and not i.v., since the former application allows the best tumor regression. The tumor size index (TSI) was recorded from day 10 (start treatment) until day 19 (stop treatment). Lethality was followed up to 1 week after the last day of treatment (no further deaths occurred). Mice treated with PBS displayed drastic tumor growth, while TNF/IFN-γ treatment led to complete regression of the tumor ($p < 0.001$ from day 14 as compared to PBS-treated mice) (Figure 7C). HS pretreatment did not inhibit this antitumor activity. From day 14, the TSI was significantly different from the HS and PBS groups ($p < 0.001$), while no statistical differences were observed between the two TNF/IFN-γ-treated groups. HS induction had a significantly protective effect against lethality induced by a TNF/IFN-γ treatment (Figure 7D). A significant difference, both in survival time ($p = 0.0002$) and final lethality ($p = 0.0037$), was found compared to TNF/IFN-γ-treated mice without prior HS. These data demonstrate that HS induction not only prevents TNF-induced lethality but also allows application of TNF in combination with IFN-γ as a safe antitumor strategy.

Discussion

TNF is successfully used in the clinic in combination with IFN-γ and melphalan for isolated limb perfusion (Eggermont, 1998). Different pathologies have also been associated with TNF. A role for TNF has been demonstrated in inflammatory bowel disease, since application of a monoclonal antibody directed against TNF led to improvement of the disease, both in an animal model (Watkins et al., 1997) and in patients with Crohn's disease (D'Haens et al., 1999). TNF inhibitors and anti-TNF antibodies have also proven their efficacy in treating arthritis (Lorenz, 2000; Emery, 2001). In diabetes it is still doubtful whether TNF plays a deleterious role (Hunger et al., 1997) or a protective role (Cope et al., 1997).

We are studying several ways to inhibit the toxicity induced by TNF. In the past, we have demonstrated that acute phase proteins confer protection against TNF-induced toxicity and lethality (Libert et al., 1994, 1996; Van Molle et al., 1997, 1999). In this report, we evaluated the protective capacities of HS induction against TNF. Subjecting mice to a HS treatment for 20 min at 42°C resulted in a remarkable increase in body temperature

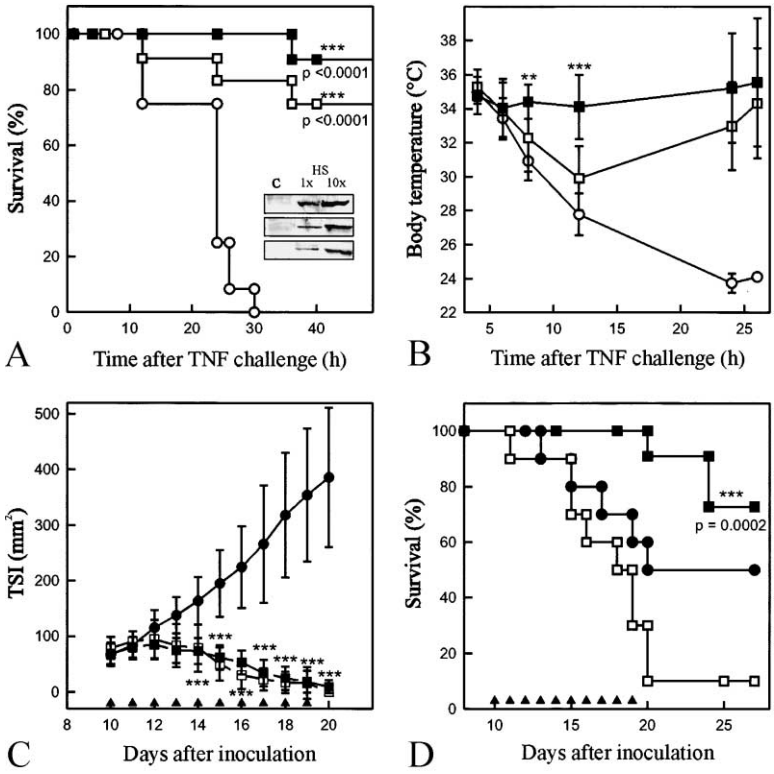

Figure 7. Repeated HS Treatments Do Not Lead to Downregulation of HSP70 Expression, Still Confer Protection against TNF Lethality, and Allow Application in an Antitumor Protocol

(A–B) Mice were subjected once (□, n = 12) or ten consecutive times (■, n = 11) to HS and challenged i.v. 12 hr later with 15 μg TNF. Controls were challenged with 15 μg TNF without prior HS (○, n = 12). Induction of HSP70 12 hr after (last) HS was analyzed by Western blot (inset: liver, jejunum, colon).

(C–D) Mice were inoculated s.c. with 6×10^5 B16Bl6 cells on day 0. From day 10 until day 19, mice were injected s.c. with PBS (●, n = 10), 10 μg TNF + 5000 IU IFN-γ without HS (□, n = 10) or 10 μg TNF + 5000 IU IFN-γ 12 hr after HS (■, n = 11).

and a strong induction of HSP70 in lung, liver, colon, and jejunum. This was not shown before for jejunum and colon. Induction was high 6 hr after HS treatment and remained high until 24–48 hr. We also demonstrate that HS treatment confers significant protection against TNF-induced lethality when applied 12 hr but not 2, 24, or 48 hr before TNF administration. When mice were HS subjected 6 hr before the TNF challenge, we only observed a marginal protection.

When *hsp70.1*$^{-/-}$ mice were treated with a whole-body HS, we detected no HSP70 12 hr after HS. These data indicate that inactivation of one of the two inducible *hsp70* genes (in our case, *hsp70.1*) negatively influences *hsp70* induction. Traces of HSP70 were found 6 hr after HS in *hsp70.1*$^{-/-}$ mice, probably due to expression of the remaining *hsp70.3* gene. Reduced levels of HSP70 6 hr after HS in *hsp70.1*$^{-/-}$ mice and *hsp70.3*$^{-/-}$ mice have recently been shown, but without investigation of *hsp70* induction at later time points after HS (Huang et al., 2001). Since we show that *hsp70.1*$^{-/-}$ mice are no longer protected against TNF lethality after HS, we unambiguously prove that HSP70 induction is perhaps sufficient but certainly required for HS-mediated protection.

Administration of TNF to mice results in hypothermia and a strong induction of IL-6 and NO, which leads to hypotension and which is reflected in high levels of nitrite and nitrate and IL-6 in the serum (Van Molle et al., 1997). We here show that HS induction significantly inhibits hypothermia and NO induction. There is also a strong effect on IL-6 induction. While control mice display high levels of IL-6, HS-pretreated animals show significantly lower levels of IL-6, 3 hr after TNF challenge. It has been speculated that a strong IL-6 induction is representative of a lethal outcome (Libert et al., 1990). The reduced production of IL-6 observed in this study could be mediated by glucocorticoids, since corticosterone levels are increased 5-fold when rats are exposed to 41°C for 20 min (Alexandrova and Farkas, 1992) and since we previously demonstrated that treatment of mice with the corticosteroid dexamethasone reduced IL-6 levels induced by TNF (Libert et al., 1991) and prevented TNF-induced lethality (Bertini et al., 1988; Libert et al., 1991). However, in *hsp70.1*$^{-/-}$ mice we observed no inhibition by HS of TNF-induced hypothermia as well as of NO and IL-6, indicating that HSP70 most probably affects IL-6 and NO induction independently of glucocorticoids. It has recently been shown that HSP70 interacts with Bag-1 (Song et al., 2001). Under physiological conditions, Bag-1 interacts with Raf-1 (Wang et al., 1996). The latter interacts with mitogen-activated protein kinase, which leads to activation of transcription factors involved in cell growth and differentiation (Morrison and Cutler, 1997). Under HS conditions, HSP70 and Raf-1 have been proposed to compete for Bag-1 binding, resulting in displacement of Bag-1/Raf-1 by Bag-1/HSP70 and arrest of DNA synthesis (Song et al., 2001). Hence, HSP70 may also affect the activation of transcription factors involved in the induction of IL-6 and inducible NO synthase.

I.v. administration of relatively high doses of TNF in rats and mice results in tissue damage in the lungs, kidney, and intestine (Tracey et al., 1986). More specifically, the duodenum (Piguet et al., 1998) and the jejunum (our unpublished data) are important targets for TNF-induced toxicity, as observed by swelling, shortening of the villi, crypt loss, and epithelial cell apoptosis. When we subjected mice to HS 12 hr before a lethal TNF

challenge, we observed a clear reduction of the TNF-induced damage at the jejunum, accompanied with fairly reduced apoptosis of epithelial cells. We showed that HS significantly prevents exudation and inhibits apoptosis and caspase activation in the jejunum. We also demonstrated that TUNEL-positive cells are not present after HS preconditioning and that those cells express HSP70 as visualized by immunohistochemistry. It has recently been reported that HSP70 in vitro inhibits the recruitment of procaspase-9 to the Apaf-1/apoptosome complex, thereby preventing cytochrome c and ATP-induced apoptosis on cytosolic extracts of Jurkat T cells (Beere et al., 2000; Saleh et al., 2000). Hence, we may postulate that the protection conferred by HS at the jejunum is based on the inhibition of TNF-induced apoptosis of epithelial cells by HSP70 induction. In TNF-treated animals, apoptosis of epithelial cells finally leads to loss in architecture of the intestine. This might result in an increased uptake in the circulation of gut-derived endotoxins and in a drastic sensitization to TNF (Neilson et al., 1989; Fox et al., 1996). Therefore, mice may die from endotoxins released by bacteria. When mice are pretreated with HS, the architecture of the small intestine is completely preserved, thereby preventing such an increased uptake. A possible argument for this hypothesis is that pretreatment of mice with tetracycline added to the drinking water for 3 days (sufficient to sterilize the gut) significantly prevents TNF-induced lethality (2/9 for tetracycline-pretreated mice versus 8/10 for control mice after TNF challenge, $p = 0.023$; our unpublished data).

Considering that HS induction appears to have broad-range protection, we evaluated the application of HS in an antitumor protocol. It has been recognized for years that fever has beneficial effects on survival during infection (Covert and Reynolds, 1977; Kluger, 1991). Furthermore, systemic application of hyperthermia has been used to treat patients with specific types of malignancies (Robins et al., 1989). In this report, we show that a daily HS treatment does not inhibit the antitumor activity of TNF and IFN-γ. We further also show that repeated HS treatments lead not only to higher expression levels of HSP70 and no tolerance to HSP70 but also to a better protection against TNF toxicity. Mice that are HS-treated prior to TNF/IFN-γ treatment are less susceptible to loss in body weight and, more importantly, are significantly protected against the induced lethality. Daily treatment of mice with TNF and IFN-γ results in apoptotic and necrotic damage in several organs, which is prevented by HS-induced HSP70 (our unpublished data). It might seem contradictory that HS treatment and induction of HSP70 inhibit the lethality and not the antitumoral capacity of TNF and IFN-γ. Overexpression of HSP70 in tumor cell lines has been demonstrated to protect these cells against TNF toxicity (Jäättelä et al., 1989), though such studies only involved the direct effect of TNF. We have good reasons to believe that in vivo tumor killing by the combination of TNF and IFN-γ is, at least partially, mediated by the host and is not solely a direct cytotoxic effect on tumor cells (Brouckaert et al., 1986). Thus, it is possible that host-mediated effects are not inhibited by HSP70, in a sense that increased HSP70 levels in tumor cells do not inhibit the antitumoral activity of TNF and IFN-γ. These results open a link to the clinic where, as mentioned before, hyperthermia is a rational method to cure malignancies. Heat treatment is also applied in isolated limb perfusion, the first hyperthermic application dating back to 1958 (Creech et al., 1958). In hyperthermic isolated limb perfusion, TNF is combined with IFN-γ and melphalan under mild conditions from 39°C to 40°C for 1 hr (Minor et al., 1985; Olieman et al., 1999). One might speculate that these hyperthermic conditions induce HSP70, which in turn provides a protective mechanism against the toxicity accompanying treatments with TNF/IFN-γ/melphalan.

We conclude that whole-body HS of mice confers protection against TNF-induced lethality and toxicity and that this HS treatment coincides with a strong induction of HSP70 in the jejunum, which is a primary target of TNF toxicity. Based on our studies with *hsp70.1*$^{-/-}$ mice we believe that HSP70 itself is at least necessary for the conferred protection. We also show that HS treatment prevents TNF-induced IL-6 production in the serum and TNF-induced damage at the jejunum. Applying HS in an antitumor protocol did not inhibit the antitumor capacity of a combination of TNF and IFN-γ but prevented the subsequent lethality. This might open new perspectives for the application of HS treatment in the clinic.

Experimental Procedures

Animals

Female C57BL/6 mice were obtained from Iffa-Credo (Saint Germain-sur-l'Arbresle, France) and were used at the age of 8–10 weeks. *Hsp70.1*$^{-/-}$ mice were bred as homozygotes in our facilities. Mice were kept in a temperature-controlled, air-conditioned animal house with 14-10 hr light/dark cycles; they received food and water ad libitum.

Generation of *hsp70.1*$^{-/-}$ Mice

The mouse *hsp70.1* genomic clone was isolated from a 129 strain mouse genomic library (Stratagene, La Jolla, CA) by a standard plaque hybridization procedure using a 3′-noncoding sequence of the *hsp70.1* gene as specific probe. The targeting vector was constructed as follows. The *hsp70.1*-coding sequence was replaced with the *PMC1-NeoR* gene (Stratagene) fragment from which the promoter region was removed. When homologous recombination occurs in a correct way, the *NeoR* fusion gene is expressed in-frame starting from the *hsp70.1* promoter. E14 ES cells were transfected with 30 μg of the linear targeting vector DNA per 5×10^7 cells by electroporation (800V, 300 mF; Bio-Rad Laboratories, Richmond, CA). G418 selection (150 mg/ml) was initiated 24 hr after electroporation; G418-resistant colonies were obtained after 7–10 days of selection. The targeted disruption was confirmed by Southern blot analysis. Chimeric mice were produced by injection of ES cells into F1 (C57BL/6J x CBA/CaLac) blastocysts. Heterozygotes for the targeted allele were obtained by breeding of chimeras with C57BL/6J mice (CleaJapan, Tokyo, Japan). Finally, homozygote knockout mice were produced by interbreeding of heterozygotes.

Reagents

Recombinant murine TNF and murine IFN-γ were produced in *Escherichia coli* and purified to homogeneity in the Ghent laboratory. Two different batches of TNF were used. For antitumor experiments, TNF had a specific activity of 9.1×10^7 IU/mg, the endotoxin contamination being $<$100 U/mg. In all other experiments, TNF with a specific activity of 1.2×10^9 IU/mg and an endotoxin contamination of $\sim$6 U/mg was used. IFN-γ had a specific activity of 1.1×10^8 IU/mg. The endotoxin contamination was assessed in a chromogenic Limulus amebocyte lysate assay (Coatest; Chromogenix, Stockholm, Sweden). Paraffin was purchased from Sigma Chemical Co. (St.

Louis, MO). Histo-clear was obtained from National Diagnostics (Atlanta, GA).

HS Induction

An empty mouse cage and a tray filled with water were placed in a hybridization oven (Amersham Pharmacia Biotech, Rainham, UK) for at least 2 hr at 42°C. These conditions should provide a relative humidity of 75% (Dietrich et al., 2000), which favors efficient HS (Fujio et al., 1987; Nowak et al., 1990). Mice were placed in the cage for 20 min at 42°C, after which they were transferred to a clean cage at room temperature.

Injections and Blood Collections

I.v. injections were performed in 0.2 ml and s.c. injections in 0.1 ml. Prior to injection, cytokines and reagents were diluted in an LPS-free isotonic solution. Blood was taken by cardial puncture during avertin anesthesia. Blood was allowed to clot for 30 min at 37°C and for at least 1 hr at 4°C, followed by two centrifugations at 20,000× g for 3 min. Serum was stored at −20°C until use.

Tissue Embedding, Tissue Sectioning, and Staining

Tissues were removed after cervical dislocation and immediately fixed in a 4% paraformaldehyde solution at room temperature. After passing through baths of 50%, 70%, 95%, and 100% ethanol and 100% Histo-clear, tissues were embedded in paraffin. Four micrometer sections were prepared with a microtome, followed by hematoxylin/eosin staining. TUNEL staining was performed with the Deadend colorimetric apoptosis detection system (Promega Biotec, Madison, WI). Tissue sections were treated according to the manufacturer's instructions. In brief, samples were treated for 10 min with a proteinase K solution and then incubated with TdT enzyme and biotinylated dUTPs for 60 min at 37°C. After blocking the endogenous peroxidase activity, sections were incubated with horseradish peroxidase conjugated to streptavidin and developed with diaminobenzidine, yielding dark brown staining of nuclei.

For immunohistochemistry, paraffin sections were incubated overnight at room temperature with a goat anti-mouse HSP70 antibody (1/50; Santa Cruz Biotechnology, Santa Cruz, CA). A rabbit anti-goat biotin-coupled antibody was used for 45 min at room temperature (1/400) as secondary antibody. The signal was amplified by tyramide signal amplification (Du Pont, Wilmington, DE); visualization was obtained with an AEC+ chromogen substrate (Dako, Carpinteria, CA).

Tumor Cell Culture and Inoculation

B16Bl6 melanoma cells were kept in culture in DMEM. Cells were harvested by treatment for 5 min with cell dissociation buffer (Life Technologies, Paisley, UK). After three washes in an LPS-free isotonic solution, cells were counted and brought at a concentration of 6×10^6/ml. They were injected s.c. in 100 μl into the back right limb.

TSI, Body Weight, and Body Temperature

TSI was obtained by multiplying the smaller and larger diameters of the tumor. Body weight and rectal body temperature were recorded with an electronic balance (Mettler Toledo, Prague, Czech Republic) and an electronic thermometer (model 2001; Comark Electronics, Littlehampton, UK), respectively.

Tissue Isolation and Homogenization

Tissues were homogenized with a homogenizer (model RZR 2020 from Heidolph-Instruments, Kelheim, Germany) in 2 ml ice-cold glycerol buffer (10% glycerol, 5 mM EDTA, 10 mM Tris/HCl [pH 7.4], and 200 mM NaCl), supplemented with 1 mM PMSF. The homogenates were centrifuged at 13,000× g for 20 min at 4°C; the supernatant was stored at −20°C until further analysis.

Assays

Serum IL-6 was determined with a 7TD1 bioassay (Van Snick et al., 1986). Nitrate and nitrite levels (NO_x^-) in the serum were assessed as previously described (Van Molle et al., 1997). This procedure, slightly adapted by us, is based on the detection of nitrite by a complex reaction resulting in a dark purple color (Griess, 1879).

Western Blot

Fifty micrograms of total protein of the different tissue homogenates was loaded on a 7.5% polyacrylamide gel and run at 150V. After electrophoretic separation, proteins were blotted on a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) for 2 hr at 120 mA. Blots were blocked with 1% BSA and 0.1% Triton X-100 overnight at 4°C. They were incubated for 2 hr at room temperature with a biotinylated mouse anti-HSP70 antibody (SPA-810B; Stressgen Biotechnologies Corporation, Victoria, Canada) and diluted 1/3000 in BSA/Triton X-100. After three washes, blots were incubated with streptavidin-AP (1/1500; BioSource International, Camarillo, CA) and developed with NBT/BCIP (Roche Molecular Biochemicals, Basel, Switzerland).

DNA Fragmentation ELISA

Twenty percent homogenates of jejunum samples were made in glycerol buffer supplemented with 1 mM PMSF, 0.3 mM aprotenin, 1 mM leupeptin, and 1 mM oxidized glutathione and centrifuged for 30 min at 13,000× g. The supernatant was stored at −20°C. DNA fragmentation was quantified by immunochemical determination of histone-complexed DNA fragments in a microtiter plate as previously described (Salgame et al., 1997).

Fluorogenic Substrate Assay for Caspase Activity

Caspase-like activities were determined by incubation of jejunum homogenate (containing 50 μg of total protein) with 50 μM of the fluorogenic substrate Ac-DEVD-amc (Peptide Institute, Osaka, Japan) in 150 μl cell-free system buffer containing 10 mM HEPES [pH 7.4], 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM DTT. Release of fluorescent amc was measured for 1 hr at 2 min intervals by fluorometry (Cytofluor; PerSeptive Biosystems, Cambridge, MA). Data are expressed as units of cleavable ac-DEVD-amc activity after application of a serial dilution of free amc as a standard, where one unit is the activity releasing 1 pmol/amc in 1 min at 30°C.

Statistical Analysis

Survival curves (Kaplan-Meyer plots) were compared using a log-rank test. Final lethality was compared with a chi^2 test. Means ± SD were compared with a Student's t test. *, **, and *** represent $p = 0.01–0.05$, $p = 0.001–0.01$, and $p < 0.001$, respectively.

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