The Role of Heat Shock Protein 70 in Mediating Age-Dependent Mortality in Sepsis

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Heat shock proteins (HSPs) are a highly evolutionarily conserved group of molecules that play an important role in the host response to a wide variety of stresses, including infection, injury, oxidative damage, hypoxia, and thermal stress. HSP70 is the most extensively studied member of the family (1, 2), and has been shown to have an important role in numerous diseases. HSP70 modifies the host response to injury by increasing resistance to numerous inflammatory mediators, including oxidants and TNF-α (3–6). HSP70 also inhibits NF-κB activation, altering prosurvival protein and cytokine expression (7, 8). In addition, immune cell receptors capture HSPs released from necrotic cells or HSP-containing exosomes (9, 10), and receptor engagement by HSP70 increases dendritic cell production of TNF-α, IL-6, and IL-1β, IL-6, and chemokines (11–13).

HSP70 has been implicated in the pathophysiology of sepsis, a disease that kills >200,000 people annually in the United States (14–17). HSP70 levels are decreased in both animal and human studies in sepsis. Cecal ligation and puncture (CLP), a model of fecal peritonitis, impairs pulmonary HSP70 levels (18), and expressing HSP70 in the lungs via adenoviral vector decreases pulmonary histologic abnormalities, prevents alveolar type II cell proliferation, and improves short-term survival following CLP (19, 20). Giving parenteral glutamine following CLP improves survival, an effect that is mediated through HSP70 because the survival benefit is not seen in HSP70−/− mice (21, 22). In addition, HSP70 induction is impaired in lymphocytes in septic mice (23). Parenteral glutamine supplementation increases serum HSP70 in critically ill surgical patients and is associated with a decreased intensive care unit length of stay (24). Genetic variants of HSP70 have also been associated with the development of septic shock in patients (25, 26).

Sepsis is a disease that primarily affects the aged. More than 60% of septic patients are older than 65 y of age, and 80% of deaths from the disease occur in aged patients (14, 27). Animal studies suggest that the pathophysiology of sepsis is different depending upon a host’s age, as aged mice have significantly increased mortality, an altered inflammatory response, and increased apoptosis when subjected to CLP, burn injury, or endotoxemia (28–36). Gut epithelial apoptosis is increased in both animal models and human autopsy studies of sepsis (37–39), and this is further disproportionately increased in aged septic mice (35). This may be significant because preventing sepsis-induced gut apoptosis improves survival in murine models of sepsis (40–42). The inflammatory response is also increased in aged septic mice (29, 30, 32–34, 43, 44).

The relationship between HSP70 and aging is complex. HSP70 is induced in response to both intrinsic and extrinsic stress. Because aging theory postulates that life span is causally linked to the ability of a host to resist these stresses, the ability of HSP70 to respond to and resist these stresses may lead to increased life span (45–47). Supporting this theory is evidence that lifelong systemic overexpression of HSP-encoding genes (including HSP70) increases...

Germline deletion of HSP70 has no impact on survival on young mice in three different mouse models of sepsis. Male 6-wk-old HSP70−/− mice and WT controls were subjected to CLP (n = 21–29/group) (A), P. aeruginosa pneumonia (n = 31–71/group) (B), or S. pneumoniae pneumonia (n = 25–44/group) (C). There were no differences in survival between HSP70−/− and WT mice in any model, although there was a nonsignificant trend toward improved survival in HSP70−/− mice after CLP.

Although there has been extensive research into HSP70 and sepsis, sepsis and aging, and HSP70 and aging, the complex interactions among HSP70, sepsis, and aging have not previously been studied. This study sought to determine whether there was an age-dependent role of HSP70 in sepsis. In light of the importance of gender on HSP70 expression (55, 56), outcomes from critical illness (57–59), and life expectancy with normal aging (60), the interaction of the three variables was studied in both male and female mice.

Materials and Methods

Animals

HSP70−/− mice (which have germline deletions of both HSP70.1 and 70.3) were generated by backcrossing 129/SvJ × C57BL/6 HSP70−/− mice (61) onto a 129/SvJ background. To determine the life span of both knockout and wild-type (WT) control animals, unmanipulated mice were aged to 25 mo. Based upon the life span tables generated (see Results), subsequent experiments on septic mice were performed on 6- to 12-wk-old mice (young) or 16- to 17-mo-old mice (aged). All mice were maintained on 12-h light/dark cycles with free access to food and water at all times. Experiments were performed in accordance with National Institutes of Health guidelines and were approved by both the Washington University and Emory University Animal Studies Committees.

Sepsis models

CLP was performed as a model of polymicrobial, intra-abdominal sepsis (62). Under isoflurane anesthesia, a midline abdominal incision was performed, and the cecum was exteriorized and then ligated ∼10−15% distal to the ileocecal valve. The cecum was then punctured once with a 30-gauge needle and gently squeezed to extrude some stool. The cecum was returned to the abdomen, and the abdominal wall was closed. Mice were given a s.c. injection of 1 ml 0.9% NaCl to compensate for third-space fluid losses. In addition, animals were given 50 mg/kg/day imipenem/cilastin for broad-spectrum antibiotic coverage. Animals were either sacrificed at 24 h or followed 7 d for survival. Note, aged WT and HSP70−/− male mice, aged female HSP70−/− mice, and young male HSP70−/− mice were operated on at the same time, but are broken down into three different comparisons in the manuscript based upon a prehoc determination (aged male HSP70−/− versus aged male WT, aged male HSP70−/− versus aged female HSP70−/−, and aged male HSP70−/− versus young male HSP70−/− m). To assess whether HSP70 had an effect on survival in an alternate model of sepsis, experiments were also performed using pneumonia as a model of monomicrobial, intrapulmonary sepsis. Pneumonia was induced via direct intratracheal injection of either Pseudomonas aeruginosa (ATCC 27853) or Streptococcus pneumoniae (strain 99.55, capsular subtype 6A) (63, 64). P. aeruginosa was placed in trypticase soy broth with constant shaking overnight. The resulting culture was centrifuged at 6000 × g, washed twice with 0.9% NaCl, and resuspended to a density of 0.2 A600nm. S. pneumoniae was placed on 5% blood agar plates overnight, washed, and resuspended to an absorbance of 1.0 A600nm. Under isoflurane anesthesia, a midline cervical incision was made, and either 20 μl P. aeruginosa (4 × 107 CFU) or 60 μl S. pneumoniae (2-4 × 107 CFU) diluted in 0.9% NaCl was introduced into the trachea with a 29-gauge syringe. Following incision closure, mice were given a s.c. injection of 1 ml 0.9% NaCl to compensate for third-space fluid losses. Mice receiving S. pneumoniae also received a single dose of ceftriaxone at a dose of 50 mg/kg. Both of these models were designed to have ~50% mortality in young mice.

Intestinal epithelial apoptosis

Apoptotic cells in the intestinal epithelium were quantified in 100 contiguous well-oriented crypt-villus units per animal using two complementary techniques: H&E staining and active caspase-3 staining (65). Apoptotic cells were identified on H&E−stained sections by morphologic criteria in which cells with characteristic nuclear condensation and fragmentation were considered to be apoptotic. For active caspase-3 staining, intestinal sections were deparaffinized, rehydrated, and incubated in 5% hydrogen peroxide for 10 min. Slides were then placed in Ag Decloaker (Biocare Medical, Concord, CA) and heated in a pressure cooker for 45 min to remove the paraffin. The sections were then immersed inzsodium citrate (pH 6.0) at 95°C for 20 min and then cooled on ice for 2 min. The tissue sections were then immersed in 1% hydrogen peroxide and incubated for 10 min at room temperature to inactivate endogenous peroxidase. Nonspecific binding was blocked by incubation in normal goat serum (Vectashield; Vector Laboratories, Burlingame, CA) for 15 min, and the primary antibody (caspase-3 antibody; Cell Signaling Technology, Beverly, MA) was applied to the sections at a 1:100 dilution overnight. Following several washes in TBS, the sections were incubated with a biotinylated goat anti-rabbit antibody for 30 min, followed by AEC reagent for 10 min. The sections were then counterstained with methyl green and mounted with permanent mounting medium.

FIGURE 1. Germline deletion of HSP70 has no impact on survival on young mice in three different mouse models of sepsis. Male 6-wk-old HSP70−/− mice and WT controls were subjected to CLP (n = 21–29/group) (A), P. aeruginosa pneumonia (n = 31–71/group) (B), or S. pneumoniae pneumonia (n = 25–44/group) (C). There were no differences in survival between HSP70−/− and WT mice in any model, although there was a nonsignificant trend toward improved survival in HSP70−/− mice after CLP.

FIGURE 2. HSP70−/− (n = 10) and WT (n = 28) animals were followed daily until they died or were determined to be moribund and were sacrificed. Approximately 65−75% of unmanipulated HSP70−/− and WT mice survived to 16–17 mo. Based upon this, experiments in aged animals were performed on 16- to 17-mo-old mice.

FIGURE 3. Mortality is higher in aged septic HSP70−/− mice than aged septic WT mice. Aged male HSP70−/− and WT mice were subjected to 1 × 30 CLP, given fluids and antibiotics, and followed for survival 7 d after their septic insult. Survival was increased in WT mice compared with HSP70−/− mice (n = 9–13/group; p = 0.01).

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min to facilitate Ag retrieval. Sections were then blocked with 20% goat serum (Vector Laboratories, Burlingame, CA) and incubated with rabbit polyclonal anti–active caspase-3 (1:100; Cell Signaling Technology, Beverly, MA) overnight at 4˚C. Sections were then incubated with goat anti-rabbit biotinylated secondary Ab (1:200; Vector Laboratories) for 30 min at room temperature, followed by Vectastain Elite ABC reagent (Vector Laboratories) for 30 min at room temperature. Slides were developed with diaminobenzidine and counterstained with hematoxylin.

Cytokine analysis
Peritoneal fluid was obtained by lavaging the peritoneum with 5 ml sterile 0.9% NaCl and then centrifuged to obtain a supernatant used for cytokine analysis. Whole blood was collected retro-orbitally and centrifuged at 5000 rpm for 5 min in serum separator tubes. Both peritoneal and plasma cytokine concentrations were evaluated using a Bio-Plex Pro Mouse Cytokine Standard Group I Kit (Bio-Rad, Hercules, CA) per the manufacturer’s protocol. All samples were run in duplicate.

Western blots
Left lung tissue was removed, snap frozen, and stored at −80˚C. Protein lysates were prepared from frozen tissue by homogenizing in a 5× vol of ice-cold homogenization buffer (66). Lysates were centrifuged at 10,000 rpm at 4˚C for 5 min, and the supernatant was collected. The Bradford protein assay was used to determine total protein concentration, and protein samples (40 μg) were then heated at 95˚C for 5 min after being added to an equal volume of 2× Laemmli buffer. Samples were run on polyacrylamide gels (Bio-Rad) for 45 min at 180 V and then transferred to Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad) for 2 h at 80 V. Membranes were blocked in 5% nonfat milk in TBS with 0.1% Tween 20 (Sigma-Aldrich) at room temperature for 30 min. Membranes were then incubated overnight at 4˚C with rabbit anti-HSP90 polyclonal Ab (1:4000; StressGen, Victoria, BC, Canada), rabbit anti-HSP25 polyclonal Ab (1:4000; StressGen), or rabbit anti–β-actin Ab (1:2000; Cell Signaling Technology). Membranes were washed and incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit IgG (1:1000; Cell Signaling Technology) and developed via a chemiluminescent system (Pierce, Rockford, IL). Proteins were detected following exposure to x-ray film.

Myeloperoxidase assay
The pulmonary vasculature was perfused with 1 ml PBS, and lungs were snap frozen and stored at −80˚C. Right lower lobe sections were thawed, weighed, and homogenized in 4 ml 20 mM potassium phosphate buffer with 0.5 g/dl hexadecyltrimethyl ammonium bromide. This was sonicated for 90 s and then incubated for 2 h at 60˚C. Samples were then centrifuged, and 100 μl supernatant was placed into 2.9 ml 50 mM potassium phosphate buffer (pH 6.0) with 0.167 mg/ml O-dianisidine and 0.0005% hydrogen peroxide. Absorbance was measured for 3 min at 460 nm. Myeloperoxidase activity per gram of protein was calculated using the rate of change in absorbance over 3 min and the protein content of the sample.

Statistics
Data were analyzed using the statistical software program Prism 4.0 (GraphPad, San Diego, CA) and are presented as mean ± SEM. Survival studies were analyzed using the log rank test. All other data were tested for Gaussian distribution using the Shapiro–Wilk normality test. If data were found to have Gaussian distributions, comparisons were performed using the Student’s t test. If not, comparisons were performed using the Mann–Whitney U test. A p value <0.05 was considered to be statistically significant.

Results
HSP70 has no effect on mortality in young septic mice
Young male HSP70−/− mice and WT controls were subjected to three different models of sepsis (CLP, P. aeruginosa pneumonia, and S. pneumoniae pneumonia) and followed 7 d for survival (Fig. 1). There were no statistically significant differences in survival between HSP70−/− and WT mice in any model.

FIGURE 4. Gut epithelial apoptosis is increased in aged septic HSP70−/− mice compared with aged septic WT mice. Apoptosis was quantitated in 100 contiguous crypts by both active caspase-3 (A) and H&E staining (B) (n = 14–18/group; p = 0.001 and 0.004, respectively).

FIGURE 5. Systemic inflammatory response is increased in aged septic HSP70−/− mice compared with aged septic WT mice. Systemic levels of TNF-α, IL-6, IL-1β, and IL-10 were all increased in HSP70−/− mice (n = 13–15/group; p = 0.008, 0.01, 0.0008, 0.03, respectively).
HSP70 prevents mortality in aged septic mice

Unmanipulated male and female HSP70−/− and WT mice were first followed until they died or became moribund and were sacrificed to determine their life spans. Approximately 65–75% of unmanipulated HSP70−/− and WT mice survived to 16–17 mo, a survival percentage similar to that expected for 70- to 75-y-old humans (Fig. 2) (67). As such, 16- to 17-mo mice were used in subsequent experiments examining the role of HSP70 in aging and sepsis.

Aged male HSP70−/− and WT mice were then subjected to CLP and followed for survival. Survival was increased in WT mice compared with HSP70−/− mice (Fig. 3).

HSP70 prevents sepsis-induced gut epithelial apoptosis and systemic inflammation in aged male mice

A different cohort of aged male HSP70−/− and WT mice was sacrificed 24 h after CLP. Gut epithelial apoptosis was increased in HSP70−/− mice compared with WT mice whether assayed by active caspase-3 or H&E staining (Fig. 4). The systemic inflammatory response was markedly increased in HSP70−/− mice with elevated TNF-α, IL-6, IL-10, and IL-1β compared with WT mice (Fig. 5). Although there were differences in local peritoneal cytokines between the mice, these were less pronounced than the differences seen in plasma cytokines (Fig. 6).

HSP70 decreases sepsis-induced pulmonary inflammation

In light of data demonstrating that pulmonary administration of HSP70 improves pulmonary dysfunction in CLP-induced adult respiratory distress syndrome (19), pulmonary pathology and neutrophil infiltration were examined in HSP70−/− and WT mice. Pulmonary inflammation was more severe in HSP70−/− mice compared with WT mice, as judged by a pathologist blinded to sample identity (Fig. 7). In contrast, no significant differences were noted between animals in the myeloperoxidase assay (data not shown). To determine whether knocking out HSP70 caused compensatory changes in other HSPs, HSP25 and HSP90 protein levels were examined in aged male HSP70−/− and WT mice. No differences in HSP25 or HSP90 were noted, regardless of whether an animal expressed HSP70 (data not shown).

Gender alters mortality in aged, but not young HSP70−/− mice

Both young and aged male and female HSP70−/− were subjected to CLP and followed for survival. Survival was markedly increased in aged female HSP70−/− mice compared with aged male HSP70−/− mice (Fig. 8A). In contrast, there were no differences in mortality between young male and female HSP70−/− mice (Fig. 8B).

Gut epithelial apoptosis was increased in aged male HSP70−/− mice compared with aged female HSP70−/− mice whether assayed by active caspase-3 or H&E staining (Fig. 9). However, this was not accompanied by statistically significant changes in either the systemic or local inflammatory response (Figs. 10, 11). Despite the differences in mortality, the only statistically significant cytokine differences between aged male and female HSP70−/− mice were elevated plasma IL-6 and diminished peritoneal IL-13 in male mice.

Aged male HSP70−/− mice have higher mortality than young male HSP70−/− mice

Young and aged male HSP70−/− were subjected to CLP and followed for survival. Survival was higher in young male HSP70−/− mice compared with aged male HSP70−/− mice (Fig. 12A). The increased mortality seen in aged septic male HSP70−/− mice was...
associated with increased gut epithelial apoptosis whether assayed by active caspase-3 or H&E staining (Fig. 12B, 12C). However, this was not accompanied by statistically significant changes in either the systemic or local inflammatory response, with the exception of decreased plasma G-CSF in aged mice (data not shown).

**Discussion**

This study shows that HSP70 mediates mortality in aged mice subjected to sepsis, but not in young mice given the same insult. This suggests that alterations in HSP70 may be one of the reasons that survival is lower in aged patients who become septic compared with younger patients with the same disease. Possible mechanisms for how HSP70 modulates mortality in aged septic hosts include preventing gut epithelial apoptosis and preventing both pulmonary and systemic inflammation.

This study gives new insights into the interactions among HSP70, aging, and gender in survival from sepsis. A major finding is that survival is worse in aged septic HSP70−/− mice compared with WT mice, but no significant differences were found between young septic HSP70−/− mice and WT mice in three models of sepsis. This suggests HSP70 plays a role in mediating mortality in aged, but not young mice. These findings are complementary to findings that tissue-specific expression of HSP70 in young mice in the lungs improves 48-h survival following CLP (no longer-term survival experiments have been published) (19, 20) or that giving parenteral glutamine (which upregulates HSP70) improves survival after CLP in young mice (21, 22). Taken together, the results suggest that whereas a basal amount of HSP70 is not necessary for an appropriate host response to infection in a young animal, a supranormal amount may be adaptive. In contrast, a basal amount of HSP70 appears to be required for an aged host to respond to a septic insult, possibly due to the accumulation of life stresses that occur prior to injury onset.

The fact that there was greater pulmonary inflammation in aged septic HSP70−/− mice than WT is consistent with work by Weiss and Deutschman (19) showing that lung-specific delivery of HSP70 prevents adult respiratory distress syndrome following lethal CLP, as well as studies demonstrating glutamine administration increases pulmonary HSP70 and prevents lung injury. However, this study also yields possible new mechanisms through which HSP70 might modulate mortality in aging and sepsis. Aged septic HSP70−/− mice had increased gut epithelial apoptosis and increased systemic inflammation compared with aged septic WT mice. Because prevention of gut apoptosis improves survival in both CLP and pneumonia (40, 41), it is plausible that the increase in gut apoptosis seen in aged septic HSP70−/− mice is responsible for the increased mortality seen in these animals. Similarly, an altered inflammatory response has been repeatedly implicated as one of the central causes of mortality from sepsis (68, 69). The inflammatory response is also increased in aged septic mice (29, 30, 32–34, 43, 44). It is therefore possible that when the inflammatory changes of HSP deletion are superimposed upon the already abnormal host milieu in aging and sepsis, this plays a role in mediating the increased mortality seen in aged septic HSP70−/− mice.

Our finding that mortality is similar between young septic HSP70−/− and WT mice differs from published findings by Singleton and Wischmeyer (70), which found increased mortality in HSP70−/− mice following CLP. Possibilities for these seemingly contradictory results include multiple differences in study design, including whether antibiotics were used (none versus imipenem), the type of anesthesia used (ketamine and xylazine versus inhaled isoflurane), and the size of injury (2 × 22 CLP versus 1 × 30 CLP). Whereas we do not rule out the possibility that HSP70 plays an important role in modulating mortality under differential experimental conditions, the fact that we saw no statistically significant differences in survival between young HSP70−/− and WT mice in three distinct models of sepsis strengthens the results presented in this work.

In addition to the main finding of this manuscript that HSP70 modulates mortality in sepsis in aged, but not young animals, we also examined the importance of both gender and age in isolation by comparing the following: 1) aged male and female septic HSP70−/− mice and 2) aged and young septic HSP70−/− mice. Although to our knowledge this is the first report of aged HSP70−/− mice, the findings that both male mice and aged mice had higher mortalities are consistent with existing literature demonstrating both are risk factors in the development and outcome from sepsis (58, 71). Interestingly, despite the
associated increase in gut apoptosis in septic male aged mice compared with either septic female aged mice or septic male young mice, there were minimal differences in either systemic or peritoneal cytokines. Thus, gut apoptosis is increased in mice with higher mortality in all comparisons studied (aged male HSP70−/− versus aged male WT, aged male HSP70−/− versus aged female HSP70−/−, aged male HSP70−/− versus young male HSP70−/−). In contrast, the systemic inflammatory response is significantly different only in aged male HSP70−/− versus aged male WT mice. This suggests that the differences in the inflammatory response are specifically due to the presence or absence of HSP, whereas differences in gut apoptosis may more generally reflect increased mortality rather than a specific response to alterations in HSP levels, despite the known antiapoptotic effect of HSP in the gastrointestinal tract (72).

This study has several limitations. First, we cannot conclude that unmanipulated HSP70−/− and WT mice have similar life spans, and thus cannot know if there is an impact of HSP70 deletion on survival independent of sepsis. Although there was no statistically significant difference detected between animals followed for life span (p = 0.2 in Fig. 2), this could be an artifact of the relatively small number of knockout mice (n = 10). Next, although septic aged male HSP70−/− have increased mortality and both increased gut apoptosis and inflammation, we cannot conclude that this correlation is causative without further experiments. Consistent with this, the results do not provide a central mechanism to explain our findings. Because the NF-κB pathway is closely linked to sepsis, apoptosis, and inflammation, future experiments examining both aging HSP70 expression and NF-κB activation would be important to perform. Furthermore, over the 5 y it took to...
The estrous cycle, potentially altering hormonal effects on survival. Animals. Additionally, female mice were operated on throughout contiguous crypts by both active caspase-3 (B) and H&E staining (C) (n = 8–14/group; p = 0.002 and 0.001, respectively).

FIGURE 12. Mortality is higher in aged male HSP70−/− mice than young male HSP70−/− mice subjected to CLP (A, n = 9–21; p = 0.002). Gut epithelial apoptosis is increased in aged male HSP70−/− mice compared with young male HSP70−/− mice subjected to CLP. Apoptosis was quantitated in 100 contiguous crypts by both active caspase-3 (B) and H&E staining (C) (n = 8–14/group; p = 0.002 and 0.001, respectively).

perform the study, two different surgeons performed the CLP experiments (K.W.M., A.C.F.). Whereas all experiments were performed in a blinded, paired fashion (such that an equal number of experimental mice and control mice were operated on and followed for survival), we cannot rule out that differences in surgical technique affected our results, such as the higher than would be expected mortality in young septic HSP70−/− and WT animals. Additionally, female mice were operated on throughout the estrous cycle, potentially altering hormonal effects on survival. Also, pulmonary pathology was not assessed in female or young mice. Bacterial load was also not evaluated in either the blood or peritoneal fluid in young or aged HSP70−/− and WT mice. Mice were assayed at only a single time point after the onset of sepsis, and it is impossible to know what differences exist between mice at times other than 24 h following the onset of sepsis. Finally, although survival studies were performed in three different models of sepsis in young mice, studies in aged mice were performed using only a single model (CLP), so it is unclear whether the results are generalizable to other models of sepsis.

Despite these limitations, our data indicate that HSP70 is an important mediator in survival in aged septic mice. Because the increased mortality seen in aged septic HSP70−/− mice compared with matched WT mice was not seen in young knockout mice compared with WT mice in three models of sepsis, the effect of HSP70 appears to be age specific. Whether the difference in mortality is mediated by increased gut apoptosis, increased pulmonary or systemic inflammation, or a mechanism not identified in this work should be the focus of future study.

Disclosures

The authors have no financial conflicts of interest.

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