HSP60 in heart failure: abnormal distribution and role in cardiac myocyte apoptosis

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Submitted 26 June 2007; accepted in final form 3 August 2007

HSP60 is an important member of the heat shock protein family, thought to be primarily a mitochondrial protein, although it is encoded by the nuclear genome. Previously, our laboratory (8) reported that HSP60 is doubled in end-stage heart failure. HSP60 binds Bax and Bak in the cytosol of the myocyte, and reduction in HSP60 precipitates apoptosis (6). Simulated ischemia in cardiac myocytes resulted in translocation of HSP60 to the plasma membrane before reoxygenation (5). Redistribution of HSP60 to the plasma membrane was associated with movement of Bax to the mitochondria and apoptosis.

Observations of HSP60 translocation in isolated myocytes motivated studies on the redistribution of HSP60 in the failing heart, where apoptosis is thought to be a mechanism of myocyte death. To address this question, we developed a rat model of heart failure with ligation of the left anterior descending artery. Cardiac echo was used to follow changes in function and chamber size. Studies were conducted long after coronary ligation and thus reflected the progressive changes of the failing ventricle, rather than ischemia. Upregulation of HSP60 correlated with increased expression of proinflammatory cytokines, brain natriuretic peptide (BNP), and atrial natriuretic peptide (ANP) 9 and 12 wk postligation. In addition, we examined distribution of HSP60 in explanted human hearts with dilated (DCM) and ischemic cardiomyopathy (ICM). We report that HSP60 is present in the cell membrane as well as the plasma early on in the development of heart failure and that this abnormal distribution of HSP60 persists in advanced heart failure. This has implications for the progressive nature of heart failure, because antibodies to HSP60 are present in the plasma of many individuals and HSP60 is thought to be a ligand for Toll-like receptor 4 (TLR-4), part of the innate immune system (13). In fact, membrane HSP60 correlated with the presence of apoptosis. Thus the presence of HSP60 in the plasma membrane is not a passive event; rather, its translocation is a significant response that exacerbates the disease state.

METHODS

Heart Failure Model

A rat coronary ligation model of heart failure was developed using the approach of Tanonaka et al. (29), which results in changes in heat shock protein expression similar to those we have found in human heart failure. The animal protocol was approved by the University of California, Davis Animal Research Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Sixty-five 5- to 6-mo-old male Sprague-Dawley rats were anesthetized with a combination of ketamine, acepromazine, and xylazine. Following intubation and placement on a respirator, a left lateral thoracotomy was performed in the fourth intercostal space, and a ligature (6.0 nylon suture) was placed around the left anterior descending coronary artery (LAD) 2 mm below its origin. Ischemia was verified by visual inspection, the chest was closed, and the rat was extubated and returned to its cage. This large infarct, needed to generate heart failure, had a 24-h mortality of 35.9%. Thereafter, there were only four deaths in the remaining 41 rats, two at 6 wk and 2 at 11 wk, all from congestive heart failure (CHF). Fifteen of the rats underwent thoracotomy but no ligation (sham). Fourteen rats were studied at 8–9 wk (9 wk group). Six rats were followed until 12 wk. Sham rats were followed for 9 or 12 wk after sham surgery.

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Cardiac Echo

Rats were studied 9 and 12 wk after ligation to examine progression of changes over time. The rats were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylazine, their chests were shaved, and an echocardiogram was done (Acuson, Sequoia model CS12, 15-MHz probe). Two-dimensional imaging was used to identify the short-axis position. Three consecutive m-mode images were collected in the short-axis view and saved for analysis of chamber size and fractional shortening. In a small subset, while the rat was under anesthesia, a carotid artery catheter was passed into the left ventricle (LV) to measure LV end-diastolic pressure (LVEDP), confirming that the rats were in heart failure with elevated LVEDP (19.0 ± 0.6 vs. 1.7 ± 0.3 mmHg). Hearts were collected at 9 and 12 wk to track changes in HSP60. Heart-to-body weight ratio was recorded. Plasma samples were collected for assay and stored at ∼80°C until use.

Human Heart Samples

Human heart samples were collected at transplantation at Baylor College of Medicine as part of a program run by one of the authors (G. Torre-Amione). All explanted hearts are preserved by immediately freezing them in liquid nitrogen. Failing heart samples came from adults identified as having either DCM or ICM. This tissue bank of failing hearts has Institutional Review Board (IRB) approval at Baylor. Normal adult human heart samples were collected in a similar fashion from brain-dead donors by a private company (T-Cubed). Heart samples were provided to the authors without identifying information but with a brief medical history, sex, and age. Pre- and post-LV assist device (LVAD) samples were obtained at the time of procedure under an IRB-approved protocol at Baylor (G. Torre-Amione). All LVAD patients went to transplant, and a post-LVAD sample was collected at the time of transplant.

Separation of Plasma Membrane, Cytosol, and Mitochondria

Plasma membrane, cytosol, and mitochondria were prepared as previously described (2, 6, 26). Citrate synthase activity, an index of mitochondrial integrity, was measured as previously reported (5, 24). Plasma membrane had no significant citrate synthase activity, and the difference between mitochondrial and plasma membrane citrate synthase activity was ∼20-fold. Alkaline phosphodiesterase I activity was measured as a marker of plasma membrane, and a 20-fold difference in activity was detected between plasma membrane and the trivial activity in the mitochondrial fraction (2). HSP60 in fractions was compared by Western blotting with equal amounts of protein loaded per lane. Percent HSP60 per fraction was calculated based on relative amounts by Western blotting corrected by total protein per fraction, using the following equation: [(density of HSP60 band for fraction A/sum of density bands for all 3 fractions) × (total protein in fraction A)/] (sum of protein in all 3 fractions).

Lipid Rafts

Lipid rafts were isolated using the approach of Oliferenko et al. (14) and Lafont et al. (9). The method is based on the insolubility of these structures in the nonionic detergent Triton X-100. Briefly, the heart tissue was homogenized in isolation medium containing 150 mM NaCl, 5 mM EDTA, and 25 mM Tris·HCl, pH 7.4, supplemented with a cocktail of protease inhibitors and 1% Triton X-100. Four volumes of OptiPrep were added to 2 volumes of homogenate. OptiPrep was diluted with the isolation medium to give 35, 30, 25, and 20% (wt/vol) iodixanol. Equal volumes of sample and the four gradients were added to a tube and centrifuged for 4 h at 160,000 g in the SW41 Ti rotor. Lipid rafts were collected from the top interface.

Western Blotting and ELISA

Western blotting and analysis was performed as previously described (6, 12). Anti-HSP60 was obtained from StressGen (Vancouver, Canada) and used at a concentration of 1:15,000. Caveolin-1 (polyclonal; Santa Cruz Biotechnology) and flotillin antibodies (monoclonal; BD Transduction) were used at 1:1,000. Affinity-purified secondary antibody-horseradish peroxidase (rabbit and mouse; Amersham) was used at 1:1,000. HSP60 was measured using ELISA (StressGen). Likewise, cytokines were measured using ELISA (Pierce, Rockford, IL).

In Vitro Transcription and Translation

In vitro transcription and translation was done using a kit (Promega) as previously described (7).

Immunocytochemistry

Immunocytochemistry was done as previously described (25). The monoclonal antibody for HSP60 was obtained from StressGen and used at a 1:500 concentration.

Flow Cytometry

To detect HSP60 on the surface of cardiac myocytes, cardiac myocytes were isolated from sham and CHF rats as previously described (27). Before calcium was reintroduced, the cells were adjusted to 200,000–300,000 cells/ml, fixed with formalin for 15 min at 4°C, incubated with 10 μg/ml FITC-conjugated mouse anti-HSP60 monoclonal antibody (BD Pharmingen) for 45 min at 37°C, washed, and analyzed using FACScan flow cytometry (Becton Dickinson, San Jose, CA). To detect intracellular HSP60, cells were permeabilized with 0.1 mg/ml lysophosphatidylcholine (Avanti Polar Lipids) in buffer formalin for 15 min at 4°C before incubation with the HSP60 antibody. In an alternative approach, cells were treated with propidium iodide (PI) immediately after isolation, washed, fixed as described above, and then incubated with anti-HSP60-FITC, as described above. After washing, flow cytometry was done to compare cells labeling with each or both of these fluorescent markers.

Flow cytometry was used to correlate the presence of HSP60 on the membrane with myocyte viability. Double-label flow cytometry was done to determine whether the presence of HSP60 on the plasma membrane increased apoptosis. Caspase-3/7 activation was quantified using a fluorescently labeled inhibitor of these caspases, which binds only to the activated form of the caspase (Vybrant FAM caspase-3 and -7 assay kit; Molecular Probes) (20). A similarly labeled inhibitor for caspase-8 (Vybrant FAM caspase-8; Molecular Probes) was used to quantify caspase-8 activation. Flow cytometry allowed the identification of the percentage of cardiac myocytes with HSP60 on the plasma membrane that had activation of caspase-3/7 and caspase-8 compared with cells that did not have HSP60 on the plasma membrane. For the caspase studies, a biotin-labeled anti-HSP60 antibody (Labvision, Fremont, CA) was used. Avidin-Texas red was used to attach a fluorescent tag.

Confocal Microscopy

Cardiac myocytes were seeded onto laminin-coated coverslips and incubated at 37°C for 2 h to allow adherence. To identify the localization of HSP60 on plasma membrane, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, blocked in 2% BSA for 60 min at room temperature, incubated with 1 μg/ml mouse anti-HSP60 monoclonal antibody (StressGen) overnight at 4°C, and developed with 4 μg/ml Texas red-conjugated goat anti-mouse antibody (Abcam). Confocal microscopy at ×600 magnification was carried out using a Zeiss confocal laser scanning microscope.

Statistical Analysis

Results are means ± SE. Data were analyzed using a one-way ANOVA followed by a Holm-Sidak test or an ANOVA on ranks followed by Dunn’s test or a Student-Newman-Keuls test. LVAD data were provided to the authors without identifying information but with an IRB-approved protocol at Baylor (G. Torre-Amione). All LVAD patients went to transplant, and a post-LVAD sample was collected at the time of transplant.
were analyzed using a paired t-test. Antibody data were compared using a χ² test. A P < 0.05 was considered significant.

RESULTS

Heart Failure Model

Coronary ligation was chosen as a model of heart failure to model ischemic cardiomyopathy. By 9 wk post-coronary ligation, a fibrous scar had replaced the region of infarct and no ischemia was present. As shown in Fig. 1A, this model of heart failure resulted in a significant increase in heart-to-body weight ratio at 9 and 12 wk. Cardiac echo was used to measure LV dimensions in the short-axis view and to calculate fractional shortening (Fig. 1B). LV diastolic dimensions (LVDD) did not significantly increase until 12 wk (Fig. 1C), whereas fractional shortening was markedly decreased at both 9 and 12 wk (Fig. 1D). Along with these functional differences, significant changes in ANP, BNP, and TNF-α did not occur until 12 wk (Fig. 2, A–C). Thus, over the course of 12 wk, rats with coronary ligation showed progressive CHF.

HSP60 in Heart Failure

Previously, our group (8) reported that HSP60 is doubled in the end-stage failing human heart. Total LV HSP60 levels increased ~40–50% by 9–12 wk compared with controls (Fig. 2D) with a mean value of 0.37 μg/mg soluble protein. Assay of plasma samples showed that by 9 wk, levels of HSP60 were 12.3 ± 2.1 ng/ml plasma compared with 0 for controls (P < 0.001).

HSP60 Distribution in Heart Failure

Previously, our group (5) has observed that HSP60 moves to the plasma membrane of isolated adult cardiac myocytes following hypoxia and reoxygenation. To determine whether the same abnormal distribution of HSP60 occurs in the chronic injury state of heart failure, LV samples were fractionated into plasma membrane, cytosol, and mitochondrial fractions. As shown in Fig. 3A, a significant amount of HSP60 (3.0 ± 0.8%, P < 0.05) was detected in the plasma membrane fraction at 9 wk, and this increased to 8.2 ± 1.0% at 12 wk (P < 0.05 vs. 9 wk and controls). Virtually no HSP60 was detected in control plasma membrane fractions.

Fig. 1. A: heart-to-body weight ratio in sham vs. 9- and 12-wk congestive heart failure (CHF) rats. B: representative cardiac echo showing normal wall motion in sham and hypokinesis in CHF heart. Arrows point to septum in both echoes, with reduced wall motion in the CHF heart. C: left ventricular diastolic dimensions (LVDD; cm) in sham vs. 9- and 12-wk CHF rats. D: fractional shortening in sham vs. 9- and 12-wk CHF rats. In A–D, n = 6–14/group. *P < 0.05 vs. sham.
Because total HSP60 is increasing at the same time as cellular distribution is changing in CHF, we were interested in how absolute levels of HSP60 changed in various fractions. As shown in Fig. 3B, the overall pattern of change was similar, but the increase in mitochondrial HSP60 at 9 wk was more marked, as was the concomitant decrease in cytosolic HSP60. Although mitochondrial HSP60 increased, overall protein levels in the mitochondrial fraction were unchanged. Total mito-

Fig. 2. A: plasma atrial natriuretic peptide (ANP) content. B: plasma brain natriuretic peptide (BNP) content. C: plasma tumor necrosis factor (TNF)-α content. D: rat LV heat shock protein (HSP) 60 level. Cytokine and HSP60 levels were determined using ELISA (n = 5–19/group). *P < 0.05 vs. sham.

Fig. 3. A: cellular distribution of HSP60 in CHF vs. sham hearts (n = 4–9/group). B: absolute levels of HSP60 in cellular fractions. C: representative fractions for sham and 2 CHF hearts (both 9 wk). Two micrograms of total protein were loaded in each lane: P or PM, plasma membrane; M or MIT, mitochondria; C or CYT, cytosol. Although the amount of plasma membrane HSP60 appears to be approximately one-third that in mitochondria, the plasma membrane only has 10–20% as much protein as the mitochondria. Therefore, total HSP60 in the plasma membrane fraction is much less than that in the mitochondria. D: non-denaturing Western blot showing cytosolic HSP60 running at 60 and ~90 kDa. Separation of the protein is nonlinear. *P < 0.05 vs. sham. +P < 0.05 vs. sham and 12 wk.
Fig. 4. A: cellular distribution of HSP60 in human cardiomyopathy by Western blot analysis. *Hearts 1–3 have ischemic cardiomyopathy (ICM), and hearts 4–6 have dilated cardiomyopathy (DCM). See Table 1 for summary of data. Although the amount of plasma membrane HSP60 appears to be about one-half that in mitochondria in failing heart samples, the plasma membrane only has 10–20% as much protein as the mitochondria. Therefore, total HSP60 in plasma membrane fraction is much less than that in mitochondria. B: summary time course (left) of changes in cellular distribution in failing rat heart and comparison with end-stage human heart (bars at right). C: immunocytochemistry of normal vs. failing human heart showing distribution of HSP60 (brown). D: comparison of plasma membrane vs. mitochondrial HSP60 in human hearts pre- and post-LV assist device (LVAD) implantation. Data labeled A–D represent 4 different patients pre- and post-LVAD. Normal heart represents small biopsies from 5 normal donor hearts combined. Note HSP60 sometimes runs as a doublet on gel, but this is not consistent. Doublet may represent posttranslational modification. *P < 0.05 vs. sham. +P < 0.05 vs. sham and 12 wk.

Mitochondrial protein at 9 wk was 1.27 ± 0.05 mg/g tissue for the CHF group compared with 1.28 ± 0.03 mg/g tissue for the controls. Thus there was a preferential increase in HSP60 alone, rather than an increase in total mitochondrial protein. HSP60 is thought to be synthesized in the cytosol from a nuclear transcript as a pre-HSP60 with a 26-amino acid mitochondrial transport sequence (MTS) at the amino terminus (19). After transport to the mitochondria, the MTS is cleaved and some of the HSP60 returns to the cytosol (19). The presence of this 26-amino acid (molecular mass 3.1 kDa) MTS was clearly distinguished from cleaved HSP60 by 10% SDS-PAGE (19). As shown in Fig. 3C, Western blot analysis of mitochondrial HSP60 at 9 wk did not demonstrate the doublet that would be expected if uncleaved HSP60 accumulated in the mitochondria (Fig. 3C). Careful analysis of both total and mitochondrial HSP60 by Western blotting with a 12% SDS-PAGE showed no evidence of the preprotein form (P1), although after in vitro transcription and translation, this could be easily detected (data not shown).

In the mitochondria, HSP60 with HSP10 forms a heptamerous barrel within which folding of imported proteins occurs. We next examined whether in the cytosol, the tertiary structure of HSP60 was different. As shown in Fig. 3D, a nondenaturing gel transferred to a membrane and developed for HSP60 demonstrated two bands: one at 60 kDa and one at 90 kDa. Thus HSP60 in the cytosol is a monomer and also binds to one or more small proteins, as has been reported in the literature (6, 18).

**HSP60 Localization in End-Stage Human Hearts**

Cell fractionation studies were done on samples of end-stage left ventricles, removed at the time of transplant, to determine whether the cellular distribution of HSP60 was altered, as in the rat heart failure model. Seven DCM (5 male, 56.8 ± 6.4 yr old) and six ICM hearts (6 male, 66.5 ± 1.0 yr old) were studied. As shown in Fig. 4A, both DCM and ICM hearts had HSP60 in the plasma membrane fraction compared with none in normal hearts (see Table 1). Based on the relative amounts and total protein in the mitochondria and plasma membrane, we estimated that 7.7% of HSP60 was associated with the plasma membrane in heart failure compared with 92.3% with the mitochondria.

To determine the full distribution of HSP60, cell fractionation was performed on additional samples to compare cytosol, mitochondria, and plasma membrane distribution of HSP60. As shown in Table 1, 1.7% of HSP60 was present in the plasma

**Table 1. Distribution of cardiac HSP60 among mitochondria, plasma membrane, and cytosol in human and rat hearts**

<table>
<thead>
<tr>
<th>Human</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal LV</td>
</tr>
<tr>
<td>PM</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>67.7±5.9</td>
</tr>
<tr>
<td>C</td>
<td>32.3±5.9</td>
</tr>
</tbody>
</table>

Values indicate distribution of cardiac heat shock protein 60 (HSP60) among plasma membrane (PM), mitochondria (M), and cytosol (C) in human and rat hearts as determined by cell fractionation followed by Western blotting (n = 3–8/group, except for cardiac myocytes, where n = 2). DCM, dilated cardiomyopathy; ICM, ischemic cardiomyopathy; CHF, congestive heart failure; LV, left ventricle.
membrane of DCM hearts and 2.4% in ICM hearts. Roughly 40% of HSP60 was in the cytosol in these failing hearts. For comparison, cell fractionation was done on normal human heart samples (all male, 54.0 ± 2.1 yr old). None had a cardiac history, although one was found to have atrial fibrillation at the time of presentation. Normal human ventricle had no HSP60 in the plasma membrane and 32.3% HSP60 in the cytosol. Figure 4B shows a comparison of HSP60 distribution during development of heart failure in the rat compared with that in advanced human failure. Both groups show similar plasma membrane distribution of HSP60. As shown in Fig. 4C, HSP60 expression was increased throughout the myocytes by immunocytochemistry, although the intensity varied across different fields.

LVAD implantation represents an intervention in advanced heart failure that is associated with hemodynamic improvement, even though most of these patients go on to transplant. We analyzed paired pre- and post-LVAD samples and correlated this analysis with outcome: transplant, death, or clinical improvement. The seven LVAD patients (2 male, 56.3 ± 7.8 yr old) had significant amounts of HSP60 in the plasma membrane (Fig. 4D). Comparing just mitochondrial and plasma membrane distribution showed that at implantation, 12.1 ± 3.9% of HSP60 was in the plasma membrane, and at the time of removal this value was 10.2 ± 3.3% (Table 2, P = NS).

Lipid Rafts

Lipid rafts are regions rich in signaling receptors and cytodomains and are hot zones of membrane activity, such as protein transport. As shown in Fig. 5, HSP60 was found in lipid rafts prepared from explanted human hearts. Both caveolin-1 and flotillin, lipid raft markers, were found in association with HSP60 (Fig. 5, A and B).

Is HSP60 Detectable on the Cell Surface?

If HSP60 is exposed on the cell surface, it can be recognized by antibodies or released, which could activate the innate immune system. Two different approaches were taken to address whether HSP60 was accessible to immunodetection by a monoclonal antibody. Isolated rat cardiac myocytes were prepared from failing and sham hearts at 9 wk. The myocytes were immediately fixed, developed with FITC-labeled anti-HSP60, and detected using flow cytometry. As shown in Fig. 6B,

![Diagram](image)

**Table 2. Effect of LVAD implantation on HSP60 distribution in plasma membrane relative to mitochondria**

<table>
<thead>
<tr>
<th>Device</th>
<th>Membrane HSP60, % Pre-LVAD</th>
<th>Membrane HSP60, % Post-LVAD</th>
<th>Outcome</th>
</tr>
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<tbody>
<tr>
<td>TVAD</td>
<td>30.4</td>
<td>2.1</td>
<td>Transplant</td>
</tr>
<tr>
<td>TVAD</td>
<td>7.1</td>
<td>6.2</td>
<td>Transect, poor prognosis</td>
</tr>
<tr>
<td>NVAD</td>
<td>0.02</td>
<td>13.9</td>
<td>Transplant</td>
</tr>
<tr>
<td>DVAD</td>
<td>21.8</td>
<td>28.4</td>
<td>Transplant</td>
</tr>
<tr>
<td>DVAD</td>
<td>9.7</td>
<td>6.2</td>
<td>Transplant</td>
</tr>
<tr>
<td>DVAD</td>
<td>6.6</td>
<td>7.2</td>
<td>Transplant</td>
</tr>
<tr>
<td>DVAD</td>
<td>8.2</td>
<td>7.6</td>
<td>Transplant</td>
</tr>
</tbody>
</table>

Values are percentages of HSP60 in plasma membrane relative to that in mitochondria following left ventricular assist device (LVAD) implantation. DVAD, DeBakey LVAD; TVAD, Thoratec LVAD; NVAD, Norvasc LVAD. All patients were transplanted.

myocytes from failing hearts expressed HSP60 on their surface. Control myocytes had only the baseline fluorescence seen with a nonspecific antibody and with autofluorescence (Fig. 6A). Mean events were 129.0 ± 13.6 in the failing myocytes compared with controls at 58.4 ± 13.6 (P < 0.05), and the signal intensity was far greater in the failing myocytes as shown in Fig. 6, A and B. Signal from the normal myocytes incubated with anti-HSP60 antibody did not differ from controls treated with a nonspecific antibody (Fig. 6A). Permeabilization with lysophosphatidylcholine exposed intracellular HSP60 to antibody as shown in Fig. 6, C and D. Positive events increased in both groups, and there was no difference between them after permeabilization, indicating that the 1.2-fold increase was due to detection of the upregulated plasma membrane HSP60.

Spatial distribution of HSP60 was also imaged by confocal microscopy. As shown in Fig. 6E, HSP60 is clearly evident on the surface of a fixed, but not permeabilized, failing myocyte treated with anti-HSP60 compared with a normal myocyte (Fig. 6F). Patterns varied, with some cells showing only HSP60 around the edge of the cell, whereas others had more extensive labeling along the plasma membrane. Normal myocytes showed no significant labeling on the cell surface. Permeabilization increased intracellular detection, since HSP60 fluorescence was clearly present on sarcomeric bands in the control myocytes (Fig. 6H). In failing myocytes, the pattern of HSP60 expression varied after permeabilization. Some cells showed the typical sarcomeric pattern of expression, but others had very abnormal patterns (Fig. 6G). These data clearly show surface upregulation of HSP60 in myocytes from failing hearts.

**Plasma Membrane HSP60 and Apoptosis**

Cardiac myocytes were isolated from failing hearts at 9 wk. Cells were examined for uptake and binding of FAM caspase-8, which is a cell-permeable compound that detects activated caspase-8, and this was compared with the presence of HSP60 on the cell surface. As shown in Fig. 7, A and B, myocytes with HSP60 on the cell surface were far more likely to have activation of caspase-8. For comparison, 20–25% of normal myocytes immediately after isolation are positive for caspase-8 activation (Kim SC and Knowlton AA, unpublished.
observed). Figure 7B shows FAM caspase-8 distributed throughout the cell, whereas HSP60 is localized in distinct clusters on the surface. Similarly, as shown in Fig. 7, C and D, HSP60 on the cell surface was associated with higher activation of caspase-3/7, using myocytes from the same hearts. Figure 8D shows localization of FAM caspase-3/7 throughout the myocyte with surface localization of HSP60 evident on the edge of the myocyte. Thus surface HSP60 was associated with higher activation of caspases and confirmed entry into the apoptotic cascade, supporting the hypothesis that cell surface HSP60 adversely affects myocyte viability.

To confirm that HSP60 on the cell surface did not correlate with increased permeability, we studied cardiac myocytes freshly isolated from failing hearts at 9 wk. Cells were not plated, to minimize changes from isolation and culture, but were immediately incubated with PI. After washing, the cells were fixed and incubated with anti-HSP60-FITC. Flow cytometry showed that there was no difference in PI uptake between cells that had HSP60 on their surface and those that did not, as summarized in Fig. 7E. The high uptake of PI in both groups reflects that the hearts were diseased, making cell isolation more damaging to the myocytes, and the cells had not undergone the initial plating step, which removes dead cells.

HSP60 is quite immunogenic, and antibodies to HSP60 are found in the general population, possibly from prior bacterial infections (there is high homology between bacterial and human HSP60). Therefore, to determine whether heart failure with both surface localization and release of HSP60 into the plasma is associated with increased antibodies to HSP60, rat plasma samples from sham controls and failing hearts were compared for the presence of anti-HSP60 antibodies binding to purified recombinant human HSP60 (StressGen). This was assessed using a special apparatus to load 20 different antibody samples, and these were incubated with a nitrocellulose membrane containing a single band of HSP60 across the width of the membrane. Sixty percent of sham controls had antibodies to HSP60 at a 1:2,500 plasma dilution along with 60% of 9-wk CHF rats and 100% of 12-wk CHF rats (P = 0.045). At 1:10,000 dilution, none of the control samples were positive for anti-HSP60 antibody, but 20% of 9-wk CHF rats and 55% of 12-wk CHF rats were positive (Fig. 8A, P = 0.004). A representative slot blot is shown in Fig. 8B.

**DISCUSSION**

Coronary ligation in rats induced heart failure, which progressed over the time of the study. At 9 wk, decreased fractional shortening and increased HSP60 were present, and HSP60 was already in the plasma. By 12 wk, BNP, ANP, TNF-α, and LVDD were all increased. Studies demonstrated that not only was HSP60 localized in the plasma membrane fraction but also, importantly, it was detectable on the exofacial surface, where it is a potential antibody target or innate immune system ligand. Cell surface HSP60 was associated with apoptosis. A fundamental issue is HSP60 localization. When HSP60 is in the cytosol or mitochondria, it is antiapoptotic and protective. When HSP60 is in the plasma membrane or extracellular, it becomes a danger to the cell. There is precedent for this; cytochrome c is critical for redox when within the mitochondria, but when in the cytosol it is a key participant in the apoptotic cascade.

HSP60 is primarily thought a mitochondrial protein, but as we have shown, a significant amount of HSP60 is present in the cytosol in the normal heart, as has been observed in some other...
settings (6, 21, 23). Unexpectedly, at 9 wk, cytosolic HSP60 dropped significantly and mitochondrial HSP60 was greatly increased without any increase in overall mitochondrial protein. By 12 wk, the percentage and absolute amounts of mitochondrial HSP60 were similar to those for the control, but HSP60 persisted in the plasma membrane fraction. Analysis of end-stage human hearts, removed at the time of transplant, showed similar distribution of HSP60 to the failing rat hearts at 12 wk. Total HSP60 in the end-stage human heart is doubled compared with that in the failing rat hearts, which had a 38–52% increase in total HSP60 (8). However, the failing human heart, sustained by medications and other therapies, persists in a state of heart failure much longer than the experimental model.

Key questions are why is HPS60 in the plasma membrane fraction, and what is the consequence? In the current study, surface localization of HSP60 was associated with apoptosis. There are several reports that in mammalian cells, HSP60 translocates to the plasma membrane (11). Localization of HSP60 to the plasma membrane has been observed in several different types of cultured cells with a possible role in membrane transport or signaling (3, 22). However, more often, HSP60 has been found in association with the plasma membrane under conditions of stress or injury, as in the current study. HSP60 localized to the cell surface in stressed aortic endothelial cells has been postulated as a possible mechanism of atherosclerosis mediated through an immune response (1, 32). Spontaneous apoptosis and cell lysis have been associated

Fig. 7. A: summary of flow cytometry experiments showing that surface localization of HSP60 by flow correlates with activation of caspase-8. In normal myocytes immediately after isolation, 20–25% are positive for caspase-8 activation. For the failing heart cardiac myocytes, overall 56.7 ± 5.6% were positive for caspase-8 activation. B: confocal image of cardiac myocyte stained for activated caspase-8 (green, FITC, showing diffuse staining throughout cell) and for HSP60 (red). Merged image is shown at right. C: summary results of 4 experiments showing increased activation of caspase-3/7 when HSP60 is present on the cell surface. Overall, for the failing heart cardiac myocytes, 59.3 ± 3.6% were positive for caspase-3/7 activation. D: confocal image of cardiac myocyte stained for activated caspase-3/7 (green) and for HSP60 localization to cell surface (red). Caspase-3/7 localized throughout the cell. HSP60 is shown as a fine red rim at the edge of the cell. Merged image is shown at right. In A–D, n = 6/group. E: uptake of propidium iodide (PI) after isolation. No correlation was present between HSP60 on the surface and PI uptake (n = 10/group).

Fig. 8. A: percentage of heart failure vs. sham control rats with plasma anti-HSP60 antibody at 1:10,000 dilution. B: example of a slot blot. Only samples with a band lining up with HSP60 marker (shown at left) were scored as positive (n = 11–15/group).
with expression of HSP60 and HSP70 on the cell surface in lymphocytes (17, 30). Previous work, as well as our own data, suggest that membrane expression of HSP60 can target cells for destruction and may mediate the induction of antibodies against the cells themselves (4). In the current study, as heart failure progressed there was an increase in antibodies to HSP60.

**HSP60**

HSP60 is primarily a mitochondrial protein, but in mammalian cells from 20 to 40% of cellular HSP60 has been reported to be extramitochondrial (5, 23). Although HSP60 is thought to form a heptameric barrel with HSP10 forming the barrel ends, there are also data indicating that it exists at times in a monomeric form, as we saw in the cytosol, and it is able to refold proteins in this form (28). Other data suggest that HSP60 is in equilibrium between the monomeric and heptameric forms (11).

HSP60 in synthesized in the cytosol with a mitochondrial localization leader sequence (19). After translocation to the mitochondria, a 26-amino acid peptide (3.1 kDa) containing the mitochondrial localization sequence is cleaved from the amino terminus, and some HSP60 returns to the cytosol. The mechanism regulating distribution of HSP60 between the cytosol and mitochondria is unknown. We found an increase in mitochondrial HSP60 at 9 wk, but there was no evidence of failure to cleave HSP60. Little is known about the function of HSP60 in the cytosol, although it is known to complex with Bax, Bak, and Bcl-xL and is thought to have an antiapoptotic role (6, 18).

**HSP60: Paradoxically Injurious?**

**HSP60 as antigen.** A number of pathological states have been reported to be associated with antibodies to HSP60. In patients with cardiomyopathy, 85% of those with DCM and 42% with ICM had antibodies to HSP60 when a 1:200 dilution of serum was examined (10). Portig et al. (16) found far fewer heart failure patients with antibodies to HSP60 (only 10% of DCM and 1% of ICM) using a different approach. Nonetheless, the surface presentation of HSP60 on the myocyte combined with serum antibodies to this protein could target the myocyte for destruction via macrophage or neutrophil Fc recognition; this may be one mechanism fueling the downward spiral in heart failure. In the current study, we found an increase in antibodies to HSP60 as heart failure progressed with a clear increase in antibody titer over time.

With the limited availability of donor hearts, the LVAD provides a bridge to transplant or recovery. We did not see a reduction in membrane localized HSP60 with LVAD treatment, but the number of paired samples available was insufficient to exclude that a reduction in HSP60 occurs with LVAD treatment.

**HSP60 and innate immune system.** HSP60 could also activate the innate immune system. As we demonstrated, HSP60 is released into the plasma by the failing heart. Plasma HSP60 could interact with monocytes or bind to anti-HSP60 antibody and activate complement (15, 31, 33). This released protein could also act in a paracrine or autocrine manner to activate TLR-4, one of the receptors for the innate immune system, for which HSP60 has been reported to be a ligand (13). Activation of TLR-4 leads to the production of cytokines, including TNF-α, which in itself is toxic to the myocyte. HSP60 is present in the plasma TNF-α and the plasma at 9 wk. The increase in plasma TNF-α was not seen until 12 wk, supporting the suggestion that HSP60 helps drive the increase in TNF-α. Thus, by several different mechanisms, membrane and extracellular HSP60 can potentially target the cardiac myocyte for destruction.

Further work is needed to elucidate the mechanisms controlling cellular localization of HSP60 and the function of HSP60 in the plasma membrane. The increase in HSP60 and its redistribution likely have negative effects on the heart and may tag the myocyte for destruction, much in the way Fas operates. There are several key issues revealed in this study, including what drives the increase in HSP60. The transient drop in cytosolic HSP60 at 9 wk is puzzling; this is associated with an increase in mitochondrial HSP60. By 12 wk, although total HSP60 is elevated and is still present in the plasma membrane, there is a relatively normal distribution of HSP60 between the mitochondrial and cytosolic fractions. As discussed, the presence of HSP60 in the plasma membrane fraction on the cell surface and in the plasma provides several possible pathways to myocyte destruction. Understanding the function and regulation of HSP60 may provide a valuable early prognostic marker in heart failure and further our understanding of the mechanisms of disease progression.

**ACKNOWLEDGMENTS**

We thank Paul Munch for surgical assistance.

**GRANTS**

This work was supported by HL077281 (to A. A. Knowlton), HL079071 (to A. A. Knowlton), the Department of Veterans Affairs (to A. A. Knowlton), and AI47294 (to S. I. Simon).

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