Elevated Heat Shock Protein 60 Levels Are Associated With Higher Risk of Coronary Heart Disease in Chinese

Xiaomin Zhang, MD, PhD; Mei’an He, MD, PhD; Longxian Cheng, MD, PhD; Ying Chen, MD; Li Zhou, MD; Hesong Zeng, MD, PhD; A. Graham Pockley, PhD; Frank B. Hu, MD, PhD; Tangchun Wu, MD, PhD

Background—Although heat shock protein 60 (Hsp60) is implicated in the pathogenesis of atherosclerosis, its role in coronary heart disease (CHD) is uncertain. This study explored the influence of circulating Hsp60 on CHD in a large case-control study, as well as the impact of acute myocardial infarction on Hsp60 levels in a prospective study.

Methods and Results—Plasma Hsp60 and anti-Hsp60 antibody levels were determined by immunoassay. In the case-control study (1003 patients with CHD, 1003 matched control subjects), Hsp60 levels were higher in patients with CHD and were related to CHD (OR comparing extreme quartiles = 4.14, \( P < 0.0001 \)). This association remained after adjustment for traditional risk factors (\( P \) for trend < 0.0001). Individuals having high levels of Hsp60 (greater than the median of 160.24 ng/mL) and anti-Hsp60 antibody (greater than the median of 38.42 U/mL) were at a greater risk of CHD than those with low levels (OR 2.30, \( P < 0.0001 \)). Stronger additive effects (OR 14.04, \( P < 0.0001 \)) were apparent at higher Hsp60 and anti-Hsp60 antibody levels (>1000 ng/mL and greater than the median of 38.42 U/mL, respectively). The simultaneous presence of high Hsp60 and anti-Hsp60 antibody levels, current smoking, hypertension, and diabetes were cumulatively associated with CHD. Individuals who had any 4 or more of these 5 factors had an OR of 38.61 for CHD (\( P < 0.0001 \)) compared with individuals who had none of these factors. For the prospective study, blood was drawn from 20 patients immediately after admission for acute myocardial infarction and 2, 3, and 7 days thereafter. Hsp60 levels were significantly higher on the day of and the day after arrival than 7 days after an acute myocardial infarction (\( P = 0.011 \) and \( P = 0.026 \), respectively).

Conclusions—Elevated Hsp60 levels are associated with an increased risk for CHD, and Hsp60 and anti-Hsp60 antibody levels combine to increase this risk. In addition, acute myocardial infarction induces Hsp60 release. (Circulation. 2008; 118:2687-2693.)

Key Words: heat-shock protein 60 ■ antibody ■ heart diseases ■ coronary disease

It has been suggested that autoimmunity contributes to the initiation and progression of atherosclerosis,1–3 and the heat shock (stress) protein 60 (Hsp60) has been identified as a possible autoantigenic determinant.4–5 Work from our laboratory and others has shown that high levels of antibody to human Hsp60 (anti-Hsp60) in the circulation are associated with the presence and severity of coronary heart disease (CHD).6–7 Pockley et al5 have reported that Hsp60 levels are associated with early cardiovascular disease in individuals with borderline hypertension, and Xu et al6 have reported that Hsp60 levels are significantly elevated in subjects with prevalent/incident carotid atherosclerosis in a population-based study involving 826 subjects. Pockley and colleagues have also reported that Hsp60 levels in individuals with established hypertension are similar to those in normotensive control subjects10 and that Hsp60 levels in patients with peripheral and renal vascular disease are similar to those in corresponding control subjects.11 The literature concerning the relationship between circulating Hsp60 and atherosclerosis is therefore varied and in need of clarification.

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Only 1 study has undertaken a prospective analysis of the association of Hsp60 with the severity of CHD (\( n = 329 \)), and that study reported that Hsp60 levels were significantly correlated with both the extent index (longitudinal severity) and stenoses (transverse severity) of CHD.12 Herein, we investigated the association between circulating levels of

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Analysis of Plasma Hsp60 and Anti-Hsp60 Antibody Levels

Plasma Hsp60 levels were analyzed with a slightly modified version of a previously described sandwich ELISA.16,17 Briefly, 96-well microtiter plates (Corning, No. 2592, New York, NY) were coated with 2 μg/mL mouse anti-Hsp60 monoclonal antibody (SPA-805, Stressgen Bioreagents; now Assay Designs, Ann Arbor, Mich) in 100 μL of PBS buffer (pH 7.2) per well overnight at 4°C. This monoclonal antibody does not recognize 60-kDa stress proteins from prokaryotic organisms (manufacturer’s technical specifications), and hence, the findings of the present study specifically relate to the relationship between circulating levels of human Hsp60 and CHD. Plates were washed and blocked by incubation with 1% wt/vol bovine serum albumin (Sigma-Aldrich, St. Louis, Mo) in wash buffer for 2 hours at 37°C. After washing, recombinant human Hsp60 (NSP-540, Stressgen Bioreagents; 0 to 2500 ng/mL) or plasma samples (1:10 dilution) were added, and plates were incubated for 2 hours at 37°C. Bound Hsp60 was detected with a rabbit polyclonal anti-Hsp60 antiserum (SPA-805, Stressgen Bioreagents; 1/1000) for 2 hours at 37°C. Plates were washed and incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG (4050-05, Southern Biotech, Birmingham, Ala; 1/3000) for 30 minutes at 37°C. Binding of the enzyme-conjugated antibody was detected with tetramethylbenzidine substrate (Sigma-Aldrich). The reaction was stopped with 1 mol/L H2SO4, and the resultant absorbance was read at 405 nm with a BioTek plate reader (BioTek, Winooski, Vt). Hsp60 concentrations were calculated by reference to the standard dose–response curve. The sensitivity of the assay was 3.66 ng/mL.

Results

Characteristics of Study Population

The general characteristics of the case-control study population have been described previously.7 Briefly, systolic blood pressure and fasting glucose in the CHD group were significantly higher than in the control subjects. Total cholesterol levels were significantly lower in CHD case subjects than in control subjects, and this was probably due to the higher incidence of cholesterol-lowering medication use. As expected, CHD case subjects were more likely to have a history of hypertension or diabetes than control subjects. Smoking was more common in case subjects than control subjects. As indicated in Table 1, with the exception of age, sex, and body mass index, multivariate analysis revealed that the major and traditional CHD risk factors, such as hypertension, diabetes mellitus, family history of CHD, and current smoking, were significantly associated with the risk of CHD. Circulating Hsp60 levels were also significantly associated with CHD (adjusted OR 4.14, 95% CI 2.88 to 5.95). Of the 20 patients with AMI (17 men, 3 women; average age 67.1 ± 8.6 years) in the prospective study, 13 had a history of hypertension, 5 had diabetes mellitus, family history of CHD, and current smoking, were significantly associated with the risk of CHD. Circulating Hsp60 levels were also significantly associated with CHD (adjusted OR 4.14, 95% CI 2.88 to 5.95). Of the 20 patients with AMI (17 men, 3 women; average age 67.1 ± 8.6 years) in the prospective study, 13 had a history of hypertension, 5 had diabetes mellitus, and 14 had a history of smoking. Seven patients had been treated with nitrates before admission, and...
Plasma Hsp60 Levels
Plasma Hsp60 levels in the control and CHD groups are indicated in Figure 1. Circulating Hsp60 was detectable in 407 (40.6%) of the 1003 control individuals and 479 (47.8%) of the 1003 CHD patients. Although no significant difference in the prevalence of Hsp60 seropositivity between control subjects and CHD patients was found (P>0.05), median Hsp60 levels were markedly higher in subjects with CHD (171.4 ng/mL, quartile range 92.8 to 319.7 ng/mL versus 128.7 ng/mL, quartile range 60.0 to 250.2 ng/mL; P<0.0001). Further analysis revealed that the proportion of subjects in the control and CHD groups with Hsp60 levels >1000 ng/mL was 1.2% and 7.0%, respectively (P<0.0001). The levels of Hsp60 in patients with myocardial infarction, unstable angina, and stable angina were similar (P>0.05), and no associations between Hsp60 levels and the medication taken were apparent.

Relationship Between Hsp60 Levels and Risk Factors for CHD
Plasma Hsp60 levels were not associated with risk factors for CHD such as sex, smoking, body mass index, hypercholesterolemia, hypertension, or diabetes in the healthy-subject group (all P>0.05); however, Hsp60 levels were significantly associated with age. The OR of elevated Hsp60 levels (75th quartile 250.17 ng/mL) in a subpopulation ≥60 years of age was 1.71 (95% CI 1.04 to 2.82) compared with subjects <60 years old. There was no statistically significant correlation between Hsp60 and anti-Hsp60 antibody levels.

Relationship Between Hsp60 Levels and CHD
The relationship between plasma Hsp60 levels and the presence of CHD is presented in Table 2. Increasing Hsp60 levels were significantly associated with an elevated risk of CHD (P for trend ≤0.0001). Subjects in the highest quartile for Hsp60 levels (>75th) were almost 5 times more likely to have CHD than those in the lowest quartile (OR 4.87, 95% CI 3.06 to 7.76). This relationship was maintained after adjustments for traditional CHD risk factors, including age, sex, smoking status, body mass index, hypertension, diabetes, and family history of CHD. However, there was no association between plasma Hsp60 levels and the severity of CHD as assessed by the number of diseased vessels. As described elsewhere, the adjusted OR comparing the highest with the lowest quartile of anti-Hsp60 antibody levels was 2.34 (95% CI 1.76 to 2.98).

Joint Effects of Hsp60 and Anti-Hsp60 Antibody on CHD Risk
High levels of Hsp60 (greater than the median, 160.24 ng/mL) and anti-Hsp60 antibody (greater than the median, 38.42 U/mL) were associated with a more than 2-fold greater risk of CHD compared with subjects with low levels of Hsp60 and anti-Hsp60 antibody (OR 2.30, 95% CI 1.44 to 3.67, P<0.0001; Table 3). Multivariate adjustment for other potential confounders had no impact on this relationship. When detectable, Hsp60 levels were stratified into 2 ranges: ≤1000 ng/mL (“low” Hsp60) and >1000 ng/mL (“high” Hsp60), and a more additive effect was obtained when high levels of Hsp60 and anti-Hsp60 antibody were considered together. The adjusted OR for subjects with high Hsp60 and anti-Hsp60 antibody levels was 14.04 (95% CI 3.11 to 63.40, P<0.0001) compared with those subjects with low levels of Hsp60 and anti-Hsp60 antibody, after adjustment for other potential factors (Table 4).

Cumulative Effect of Hsp60, Anti-Hsp60 Antibody, and Other CHD Risk Factors on Risk of CHD
When current smoking, hypertension, and diabetes were included as other risk factors (coded as 0 or 1) for a total of 5 possible CHD associated factors, the cumulative effect after adjustment for age, sex, body mass index, and family history of CHD was stronger. Compared with individuals

Figure 1. Distribution of plasma Hsp60 levels. Each bar shows the number of individuals with plasma Hsp60 levels that exceed the detectable range.

Table 2. ORs for CHD by Levels of Hsp60 (Quartiles)
Table 3. Combined Effects of Hsp60 and Anti-Hsp60 Antibody Levels on the OR for the Risk of CHD

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control, n</th>
<th>CHD, n</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Hsp60 + low anti-Hsp60 antibody</td>
<td>117</td>
<td>68</td>
<td>1†</td>
<td>1†</td>
</tr>
<tr>
<td>Low Hsp60 + high anti-Hsp60 antibody</td>
<td>90</td>
<td>110</td>
<td>2.10 (1.40–3.16)</td>
<td>1.37 (0.84–2.22)</td>
</tr>
<tr>
<td>High Hsp60 + low anti-Hsp60 antibody</td>
<td>119</td>
<td>144</td>
<td>2.08 (1.42–3.06)</td>
<td>1.94 (1.24–3.05)</td>
</tr>
<tr>
<td>High Hsp60 + high anti-Hsp60 antibody</td>
<td>81</td>
<td>157</td>
<td>3.34 (2.23–4.98)</td>
<td>2.30 (1.44–3.67)</td>
</tr>
</tbody>
</table>

Low Hsp60: Hsp60 level = median (160.24 ng/mL). High Hsp60: Hsp60 level > median (160.24 ng/mL). Low anti-Hsp60 antibody level: anti-Hsp60 antibody level = median (38.42 U/mL). High anti-Hsp60 antibody level: anti-Hsp60 antibody level > median (38.42 U/mL).

$P_{test}<0.0001$.

*Adjusted for age, sex, smoking status, body mass index, hypertension, diabetes mellitus, and family history of CHD.

† Reference group.

Table 4. Combined Effects of Hsp60 and Anti-Hsp60 Antibody Levels on the OR for the Risk of CHD

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control, n</th>
<th>CHD, n</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Hsp60 + low anti-Hsp60 antibody</td>
<td>226</td>
<td>172</td>
<td>1†</td>
<td>1†</td>
</tr>
<tr>
<td>Low Hsp60 + high anti-Hsp60 antibody</td>
<td>169</td>
<td>237</td>
<td>1.84 (1.39–2.44)</td>
<td>1.34 (0.96–1.88)</td>
</tr>
<tr>
<td>High Hsp60 + low anti-Hsp60 antibody</td>
<td>10</td>
<td>40</td>
<td>5.26 (2.56–10.81)</td>
<td>8.49 (3.60–20.03)</td>
</tr>
<tr>
<td>High Hsp60 + high anti-Hsp60 antibody</td>
<td>2</td>
<td>30</td>
<td>19.71 (4.65–83.61)</td>
<td>14.04 (3.11–63.40)</td>
</tr>
</tbody>
</table>

Low Hsp60: Hsp60 level ≤1000 ng/mL. High Hsp60: Hsp60 level >1000 ng/mL. Low anti-Hsp60 antibody level: anti-Hsp60 antibody level ≤ median (38.42 U/mL). High anti-Hsp60 antibody level: anti-Hsp60 antibody level > median (38.42 U/mL).

$P_{test}<0.0001$.

*Adjusted for age, sex, smoking status, body mass index, hypertension, diabetes mellitus, and family history of CHD.

† Reference group.

who had none of these 5 factors, those who had >4 exhibited an OR of 38.61 for CHD (95% CI 14.85 to 100.41, $P_{test}<0.0001$; Table 5).

Dynamic Changes of Hsp60 in Patients With AMI

Plasma Hsp60 was detectable in 15 of the 20 patients with AMI on their arrival, in 13 on the following day, in 10 on day 3, and in 8 on day 7. Hsp60 levels on the day of arrival (117.47 ng/mL) and the following day (124.41 ng/mL) were significantly elevated compared with those on day 7 after the onset of AMI (0.00 ng/mL; $P=0.011$ and $P=0.026$, respectively). Hsp60 reached a relative peak value on day 2 (in 3 of the 20 patients, Hsp60 levels were >1000 ng/mL), after which they gradually declined (Figure 2).

Discussion

The stress protein Hsp60 is a nuclear-encoded protein that is found primarily in mitochondria. Although more commonly considered to be an intracellular molecule, it is now known that Hsp60 can be released from cells and that it is present in the peripheral blood of normal individuals. Furthermore, seroepidemiological studies suggest a possible pathogenic role of circulating Hsp60 in the development and progression of atherosclerosis.

Previous studies have shown that high levels of Hsp60 (>1000 ng/mL) are able to maximally stimulate myeloid cells to produce proinflammatory mediators and endothelial cells to express vascular adhesion proteins, which suggests that this protein and the immune reactivity to it might contribute directly to the pathogenesis of atherosclerosis. The present study, therefore, compared the risk of developing CHD in individuals with very high levels of Hsp60 (>1000 ng/mL) to that in individuals having lower levels.

We found a strong positive association between elevated levels of Hsp60 and the risk of CHD. The association was independent of conventional CHD risk factors such as age, sex, smoking status, body mass index, hypertension, diabetes, and family history of CHD. Although median levels of Hsp60 in patients with CHD were markedly higher than those in their corresponding control subjects, there was no difference in the prevalence of Hsp60 seropositivity. The lack of difference in plasma Hsp60 levels in patients with myocardial infarction, unstable angina, and stable angina despite different degrees of immunoreactivity or severity of myocardial damage among the 3 types of CHD is currently unexplained; however, this might reflect the fact that the release of Hsp60 into the peripheral circulation or its clearance from the circulation is influenced by factors that are common to all conditions.

Atherosclerosis is considered to be an inflammatory condition, and numerous studies have reported a significant association between markers of inflammation (eg, CRP) or infection (Chlamydia pneumoniae, cytomegalovirus, or Helicobacter pylori antibodies) and atherosclerosis. In a subsample, we found no correlation between CRP and Hsp60 levels. These findings are in accordance with those of Xiao et al, who have reported little correlation between CRP and human Hsp60 (hHsp60) levels. These data suggest that CRP and hHsp60 antibody and/or hHsp60 might influence or reflect the risk of atherosclerosis via different mechanisms.

Human and microbial Hsp60 plays a key role in chronic bacterial and viral infections, and 1 study has reported that C pneumoniae–derived Hsp60 is colocalized with human Hsp60 in human atherosclerotic plaques. A large number of studies have reported on the presence of antibodies to...
multiple pathogens (C. pneumoniae, H. pylori, and cytomegalovirus) and human/microbial Hsp60 or Hsp60 antibody in subjects with cardiovascular disease.\textsuperscript{25,26} Biasucci et al\textsuperscript{27} have indicated that C. pneumoniae-Hsp60 antibody is a very sensitive and specific marker of acute coronary syndrome. However, Burian et al\textsuperscript{28} have reported that high levels of antibodies to hHsp60 and C. pneumoniae are independent risk factors for CHD. Zhu et al\textsuperscript{29} and Veres et al\textsuperscript{29} also found no association between hHsp60 antibody and antibodies against different infectious agents (C. pneumoniae, H. pylori, and cytomegalovirus, etc) in cardiovascular events.

Given the association of anti-Hsp60 antibody levels with CHD,\textsuperscript{6,7} it might be that high Hsp60 and anti-Hsp60 antibody levels reflect a heightened state of immunity that is associated with or drives atherogenesis.\textsuperscript{2,30} We hypothesized that Hsp60 levels reflect a heightened state of immunity that is associated with CHD, and this combined effect is particularly strong when very high levels of Hsp60 and anti-Hsp60 antibody are considered together. Moreover, although current smoking, hypertension, and diabetes are major and well-established risk factors for CHD, the inclusion of Hsp60 and anti-Hsp60 antibody levels as risk factors strengthened the cumulative association with the disease. It might therefore be possible to use the combined information of these known risk factors to better estimate an individual's risk for developing CHD; however, this needs to be confirmed in larger prospective studies.

Although the precise mechanisms underlying the relationship between circulating Hsp60, circulating anti-Hsp60 antibody, and CHD have yet to be defined, an autoimmune component might account for the joint effect of Hsp60 and its corresponding antibody. Hsp60 is highly immunogenic and can be processed by antigen-presenting cells such as macrophages and presented to T and B lymphocytes.\textsuperscript{31} This results in the generation of autoantibodies and the clonal expansion of autoreactive T cells and B cells, thereby inducing an autoimmune reaction directly against vascular tissues.\textsuperscript{32} Simultaneously, the innate immune system can be activated directly by Hsp60, and this results in the secretion of proinflammatory cytokines such as tumor necrosis factor-\textalpha{}, interleukin-1, interleukin-6, and interleukin-12, the promotion of monocyte adhesion to endothelial cells, and the recruitment of inflammatory cells into vascular tissues.\textsuperscript{33–35} Both cellular and humoral responses directed against Hsp60 could therefore contribute to atherogenesis.\textsuperscript{32}

A number of factors induce overexpression of Hsp60 in the vascular endothelium, as well as the expression of Hsp60 on their surface. For example, high shear stress induces Hsp60 expression in and on the surface of endothelial cells and vascular smooth muscle cells.\textsuperscript{36,37} The 60-kDa family of stress proteins is highly conserved, and antibodies to pathogen-derived Hsp60 react with human Hsp60. The induction of Hsp60 expression might therefore promote the cytotoxicity and injury of endothelial cells by anti-Hsp60 that are present in the peripheral circulation.\textsuperscript{38,39}

With respect to the prospective study, Hsp60 appears to be transiently released after AMI. The elevation of circulating Hsp60 levels is likely to result from the necrosis of cardiomyocytes and concomitant endothelial dysfunction.\textsuperscript{40–42} In this respect, Schett and colleagues\textsuperscript{43} have demonstrated that myocardial ischemia and subsequent myocardocyte injury induced by AMI can lead to the release of considerable amounts of Hsp60 into the circulation in an experimental rat model. Another crucial contributor to elevated Hsp60 levels might be AMI-related shear stress.\textsuperscript{36,37} Hoppichler et al\textsuperscript{44} reported that anti-Hsp65 antibody titers decline significantly.
after AMI and suggested that stress proteins released as a consequence of AMI are removed from the circulation by complex formation with preexisting anti-Hsp65 antibodies. Although the present data are consistent with this hypothesis, further studies aimed at better defining the mechanisms that regulate circulating Hsp60 levels are required.

A major limitation of the case-control study is that it does not allow the causal contribution of Hsp60 and immunity to Hsp60 to CHD to be defined. In addition, the number of patients with CHD who had positive coronary angiography was relatively small in case subjects in the present study (n=201). We cannot exclude the possibility that false-positive diagnoses of CHD might have reduced the power of the study to detect the effects of Hsp60. The fact that detectable Hsp60 was present in only 47.8% of CHD patients limits our ability to verify the association between Hsp60 and the severity of CHD, as assessed by the number of diseased vessels. Furthermore, the sample size in the prospective study was too small to enable us to draw any definitive conclusions. Nevertheless, these important findings are worthy of further investigation.

Conclusions

In summary, the present study identified a strong positive relationship between circulating levels of Hsp60 and the risk of CHD and provides the first evidence that Hsp60 and anti-Hsp60 antibody levels combined to increase this risk. Furthermore, we found that Hsp60 was released rapidly after AMI.

Acknowledgments

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Disclosures

Dr Pockley serves as a consultant/advisory board member of the Scott & White Clinic in Temple, Tex. The remaining authors report no conflicts.

References


31. Zhang et al. Hsp60 and Coronary Heart Disease

CLINICAL PERSPECTIVE

Although previous studies have suggested the involvement of heat shock protein 60 (Hsp60) in the pathogenesis of atherosclerosis, the relationship between circulating Hsp60 and coronary heart disease (CHD) remains uncertain. This study measured plasma Hsp60 levels in 1003 CHD patients and 1003 control subjects and plasma Hsp60 in 20 patients at multiple time points after the onset of acute myocardial infarction. Higher Hsp60 levels were significantly associated with an increased risk for CHD after multivariate adjustment for traditional risk factors. Furthermore, high levels of Hsp60 and anti-Hsp60 antibodies combined to increase the risk of CHD. This risk was further heightened with the presence of smoking, hypertension, and diabetes. With respect to acute myocardial infarction, Hsp60 levels were transiently increased in the immediate postevent period. This study provides substantial evidence for an important role for Hsp60 in CHD and acute myocardial infarction, and the data suggest that plasma Hsp60 levels might serve as a diagnostic and prognostic marker for CHD, especially when these are combined with other more established risk factors of CHD. These findings also suggest that pharmacological therapies that lower Hsp60 and anti-Hsp60 antibody levels might aid the management of CHD.
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<table>
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BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; WBC: white blood cell; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; Lp(α): lipoprotein(α); Apo: apolipoprotein.