Joint Effects of Antibody to Heat Shock Protein 60, Hypertension, and Diabetes on Risk of Coronary Heart Disease in Chinese

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BACKGROUND: Several studies have suggested an association between antibody to human heat shock protein 60 (anti-Hsp60) and coronary atherosclerosis, but the results have been inconsistent. The aim of this study was to investigate the association between anti-Hsp60 and coronary heart disease (CHD) and to determine whether anti-Hsp60, hypertension, and diabetes have joint effects on CHD risk.

METHODS: We measured the concentrations of anti-Hsp60 in 1003 CHD patients and 1003 age- and sex-matched control subjects without CHD events.

RESULTS: Concentrations of anti-Hsp60 were significantly higher in CHD patients than in controls. Increasing concentrations of anti-Hsp60 were significantly associated with higher risk of CHD (P for trend <0.0001) and with increasing severity of CHD as assessed by number of diseased vessels detected with angiography [odds ratio (OR) 3.67, 95% CI 1.56–8.64, P = 0.003] after multivariate adjustment for traditional CHD risk factors. There were strong joint effects of high concentrations of anti-Hsp60 and hypertension (OR 5.17, 95% CI 3.95–6.75, P < 0.0001) and diabetes (OR 6.49, 95% CI 4.52–9.33, P < 0.0001) on CHD risk; simultaneous occurrence of high anti-Hsp60 concentrations, hypertension, and diabetes conferred a dramatically higher risk of CHD (OR 20.99, 95% CI 12.50–35.24, P < 0.0001) in multivariate analyses.

CONCLUSIONS: Anti-Hsp60 is independently associated with CHD risk, and a combination of high anti-Hsp60, hypertension, and diabetes is particularly detrimental for CHD risk.

Coronary heart disease (CHD)5 is a multifactorial disease, and autoimmunity is considered to be one of its most important mechanisms (1). Heat shock protein (Hsp) is a possible autoantigenic determinant that may play a significant role in the development of atherosclerosis. High concentrations of antibody to human Hsp60 (anti-Hsp60) have been shown to be associated with CHD in small case-control studies (276 cases/129 controls and 424 cases/321 controls, respectively) (2, 3). In a cross-sectional study, a strong association was detected between concentrations of anti-Hsp60 and both the presence and the severity of CHD in 391 patients (4). However, no association between IgG anti-Hsp60 and levels of coronary calcification was found in 201 healthy, asymptomatic subjects (5). Huitinen et al. (6) found no association between coronary risk and IgG anti-Hsp60 in dyslipidemic middle-aged males (239 case-control pairs). Kocsis et al. (7) reported that anti-Hsp60 concentration did not predict the development of new cardiovascular events. These inconsistent results may be due to differences in study populations and small sample sizes. A recent study (8) indicated that there may be ethnic differences in the circulating concentrations of Hsps. Our previous studies (9) found a significant association between anti-Hsp60 and increased risk of electrocardiographic abnormalities characteristic of chronic myocardial isch-
emia, sinus arrhythmia, and ectopic rhythm. Therefore, we further tested whether there was a dose–response relationship between anti-Hsp60 and the risk of CHD in a Chinese Han population. In addition, we examined joint effects of anti-Hsp60, hypertension, and diabetes on risk of CHD because previous studies have suggested that anti-Hsp60 may also be involved in hypertension (10) and diabetes (11–13).

Materials and Methods

STUDY POPULATION

The case-control study was composed of 1003 CHD patients and 1003 age-matched (±5 years) and sex-matched healthy controls. Patients between 40 and 79 years old who were hospitalized were consecutively recruited from 3 hospitals (Tongji Hospital, Union Hospital, and Wugang Hospital) in Wuhan (Hubei, China) between May 2004 and October 2006. The criteria for inclusion of patients in the study were stenoses ≥50% in at least 1 major coronary artery by coronary angiography and a diagnosis of CHD based on WHO criteria (14). Patients with congenital heart disease and vascular disease were excluded. Of the 1003 CHD cases, 492 had myocardial infarction, 224 had unstable angina, and 287 had stable angina. We obtained fasting blood samples from the patients on the morning after admission. Controls were selected randomly from healthy subjects matched by area of residence in the same city. Medical histories and physical examinations were performed on all controls; none had clinical or diagnostic evidence for CHD or had received intervention therapy for CHD. We recruited 1078 patients and 1065 controls, and the participation rate was 93.0% among the patients and 94.2% among the controls. After exclusions, 1003 pairs of cases and controls were included in this study.

The study was approved by the Ethics Committee of Tongji Medical College, and informed written consent was obtained from each subject.

We used a structured questionnaire to collect information. All subjects underwent standardized interviews conducted by trained interviewers. Participants were asked about their demographic data, medical history, history of diseases, family history of cardiovascular disease, and lifestyle habits (including smoking and alcohol consumption).

ASSESSMENT OF DEMOGRAPHIC DATA, LIFESTYLE, AND MEDICAL HISTORY

Hypertension was defined as blood pressure ≥140/90 mm Hg or having received treatment for hypertension. Diabetes was defined as fasting glucose concentrations ≥7.0 mmol/L or taking insulin or oral hypoglycemic agents. Family history was positive if first-degree relatives (parents, siblings) had CHD or stroke. Subjects who had smoked fewer than 100 cigarettes over the course of their lives were defined as nonsmokers. Those who stopped smoking ≥1 year previously were considered past smokers, and the rest were defined as current smokers. Body mass index (BMI) was calculated as weight (kg)/height (m)². Through the questionnaire process, we documented use of medications such as aspirin and/or statins.

ASSAY OF PLASMA ANTI-Hsp60

We used indirect ELISA to measure the concentrations of anti-Hsp60 in plasma, as described (15–17). Briefly, 96-well microtiter plates (Corning 2592) were coated with 0.5 μg/mL recombinant human Hsp60 (NSP-540; StressGen) in 100 μL PBS (pH 7.2) per well overnight at 4 °C. After washing with wash buffer (0.5 mol/L NaCl, 2.5 mmol/L NaH₂PO₄, 7.5 mmol/L Na₂HPO₄, and 0.1% Tween 20) 3 times, plates were blocked with 1% BSA (Sigma) in wash buffer for 2 h at 37 °C. Plates were washed 3 times and incubated for 1 h at 37 °C with a 1:50 dilution of plasma in wash buffer containing 1% BSA. After washing 3 times, plates were incubated with horseradish peroxidase–conjugated goat antihuman IgG (14-16-06; KPL) diluted 1:3000 with wash buffer for 1 h at 37 °C. Finally, plates were washed 4 times, and 100 μL substrate solution containing tetramethylbenzidine (Sigma) was added to wells and incubated for 15 min at 37 °C. The reaction was stopped with 1 mol/L H₂SO₄, and absorbance (A) was read at 405 nm using a Bio-Tek plate reader. We used serial dilutions of anti-Hsp60 rabbit polyclonal antiserum (AB3497; Chemicon) as standards. We analyzed each standard or sample in duplicate wells, averaged the A values, and converted A values to concentrations according to the standard curve.

OTHER CLINICAL ASSAYS

Fasting glucose, total cholesterol, and triglycerides were assayed using standard laboratory procedures in the Department of Clinical Laboratory at Union Hospital, Tong Medical College.

STATISTICAL ANALYSIS

Baseline characteristics are presented as mean (SD) for continuous variables and as percentages for categorical data. Because anti-Hsp60 concentrations were not normally distributed, they are expressed as medians and interquartile ranges. For anti-Hsp60, the median was used as a cutoff value for division of data into high/low concentration categories. Continuous variables were
analyzed with 2-tailed t tests for normal distributions and the Mann-Whitney U test for nonparametric distributions. Categorical data were analyzed with \( \chi^2 \) tests. We used logistic regression analysis to evaluate the association between anti-Hsp60 and CHD, adjusting for covariates (including age, sex, smoking status, BMI, hypertension, and diabetes). All \( P \) values presented are 2-tailed, and \( P \) values <0.05 are considered statistically significant. Analyses were performed using SPSS12.0 software (SPSS Inc.).

### Results

#### Characteristics of Study Population

The general characteristics of the study population are displayed in Table 1. Systolic blood pressure and fasting glucose were found to be significantly higher in the CHD group than in the control group. Total cholesterol was significantly lower in CHD cases than in controls, probably owing to more frequent use of cholesterol-lowering medication in the CHD cases. As expected, CHD cases were more likely to have a history of hypertension or diabetes than controls. Smoking was more common among cases than controls. Plasma concentrations of anti-Hsp60 were significantly higher in CHD cases than in controls \( (P < 0.0001) \).

#### Anti-Hsp60 and Cardiovascular Risk Factors and Medication Use

We found significantly higher plasma anti-Hsp60 concentrations in controls with hypertension than in controls without hypertension \( (P = 0.0047) \). Similar results were found in controls with and without diabetes \( (P = 0.017) \). Anti-Hsp60 concentrations were significantly lower in CHD cases who reported using medications (aspirin and/or statins) \( (n = 388) \) than in those CHD patients who did not use these medications \( (n = 615) \) \( (P = 0.043) \). However, anti-Hsp60 concentrations were not found to differ significantly among myocardial infarction, unstable angina, and stable angina patients \( (P > 0.05) \). Anti-Hsp60 concentrations were not found to be associated with smoking or obesity.

#### Anti-Hsp60 and CHD Risk

As shown in Fig. 1, anti-Hsp60 was significantly associated with risk of CHD [odds ratio (OR) comparing the highest quartile of anti-Hsp60 with the lowest quartile = 2.34, 95% CI 1.76–2.98]. Increasing concentra-
tions of anti-Hsp60 were significantly associated, in a dose-responsive manner, with increased risk of CHD after multivariate adjustment for traditional CHD risk factors such as age, sex, smoking status, diabetes, hypertension, and BMI ($P$ for trend $<0.0001$).

**ANTI-Hsp60 AND CHD SEVERITY**

Of the 201 CHD patients having positive coronary angiography, the OR for patients having multivessel disease ($\geq 2$ diseased vessels) increased with anti-Hsp60 concentrations. The adjusted OR of having multivessel disease for patients in highest quartile of anti-Hsp60 concentrations was 3.67 (95% CI 1.56–8.64, $P$ for trend $=0.003$) compared with the lowest quartile, after controlling for age, sex, smoking status, BMI, hypertension, and diabetes (Fig. 2). High concentrations of anti-Hsp60 were related to greater CHD severity in a dose-responsive manner ($P$ for trend $=5 \times 10^{-4}$).

**JOINT EFFECTS OF ANTI-Hsp60 AND HYPERTENSION AND DIABETES ON CHD RISK**

As shown in Table 2, high concentrations of anti-Hsp60 combined with hypertension were associated with a >4-fold (OR 5.17, 95% CI 3.95–6.75, $P < 0.0001$) higher risk of CHD than normotensive subjects with low concentrations anti-Hsp60. Adjustment for other potential confounders, including age, sex, smoking status, and BMI, did not substantially affect this risk. A similar additive effect was obtained when high concentrations of anti-Hsp60 and diabetes were considered together (Table 3). The adjusted OR for diabetic subjects with high concentrations of anti-Hsp60 was 6.49 (95% CI 4.52–9.33, $P < 0.0001$) compared with diabetic subjects with low concentrations of anti-Hsp60. Compared with normotensive, nondiabetic subjects with low concentrations of anti-Hsp60, the risk of CHD for hypertensive and diabetic subjects with high concentrations of anti-Hsp60 was increased more than 20-fold (OR 20.99, 95% CI 12.50–35.24, $P < 0.0001$) after adjustment for age, sex, smoking status, and BMI (Table 4).

**Discussion**

There is increasing evidence that immune mechanisms are involved in the pathogenesis of atherosclerosis (1, 18). It is well established that cardiovascular risk factors, such as infections, biomechanical stress, oxidized LDL, and free radicals, directly stimulate cells of the arterial wall and/or other tissues to express high concentrations of Hsps (19). Hsps have been found to be highly expressed in cardiovascular tissues and atherosclerotic plaques (20, 21), but very little is known about the precise mechanism for the role of Hsp60 in the pathogenesis of CHD. Wick et al. (20) first hypothesized that autoimmune reactions to Hsps could be crucial in contributing to atherosclerosis. Xu (19) also hypothesized that induction of antibodies directed against Hsps by macrophages presenting antigens to T and B cells produces an immune response. Associations between Hsp antibodies and risk of CHD, myocardial infarction, stroke, hypertension, and restenosis...
Table 2. OR for risk of CHD using the combination of anti-Hsp60 concentrations and hypertension.

<table>
<thead>
<tr>
<th>Anti-Hsp60</th>
<th>Hypertension</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 anti-Hsp60 (&lt; median)</td>
<td>No</td>
<td>386</td>
<td>164</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Yes</td>
<td>171</td>
<td>282</td>
<td>3.88 (2.98–5.05)</td>
<td>4.32 (3.26–5.72)</td>
</tr>
<tr>
<td>High anti-Hsp60 (&gt; median)</td>
<td>No</td>
<td>269</td>
<td>167</td>
<td>1.46 (1.12–1.91)</td>
<td>1.46 (1.11–1.93)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Yes</td>
<td>177</td>
<td>390</td>
<td>5.19 (4.02–6.69)</td>
<td>5.17 (3.95–6.75)</td>
</tr>
</tbody>
</table>

* Adjusted for age, sex, smoking status, and BMI.

Table 3. OR for risk of CHD using the combination of anti-Hsp60 concentrations and diabetes.

<table>
<thead>
<tr>
<th>Anti-Hsp60</th>
<th>Diabetes</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 anti-Hsp60 (&lt; median)</td>
<td>No</td>
<td>514</td>
<td>320</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Yes</td>
<td>43</td>
<td>126</td>
<td>4.71 (3.24–6.84)</td>
<td>4.86 (3.24–7.27)</td>
</tr>
<tr>
<td>High anti-Hsp60 (&gt; median)</td>
<td>No</td>
<td>399</td>
<td>358</td>
<td>1.44 (1.18–1.76)</td>
<td>1.39 (1.13–1.71)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Yes</td>
<td>47</td>
<td>199</td>
<td>6.80 (4.81–9.62)</td>
<td>6.49 (4.52–9.33)</td>
</tr>
</tbody>
</table>

* Adjusted for age, sex, smoking status, and BMI.

Table 4. OR for risk of CHD using the combination of anti-Hsp60, hypertension and diabetes.

<table>
<thead>
<tr>
<th>Anti-Hsp60</th>
<th>Hypertension</th>
<th>Diabetes</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 anti-Hsp60 (&lt; median)</td>
<td>No</td>
<td>No</td>
<td>361</td>
<td>115</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Yes</td>
<td>No</td>
<td>153</td>
<td>205</td>
<td>4.21 (3.13–5.66)</td>
<td>4.77 (3.48–6.52)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>No</td>
<td>Yes</td>
<td>25</td>
<td>49</td>
<td>6.15 (3.64–10.41)</td>
<td>6.36 (3.64–11.12)</td>
</tr>
<tr>
<td>Low</td>
<td>Yes</td>
<td>Yes</td>
<td>18</td>
<td>77</td>
<td>13.43 (7.71–23.38)</td>
<td>14.78 (7.90–23.88)</td>
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<tr>
<td>Hypertension</td>
<td>No</td>
<td>No</td>
<td>246</td>
<td>109</td>
<td>1.39 (1.02–1.90)</td>
<td>1.38 (1.00–1.90)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Yes</td>
<td>Yes</td>
<td>153</td>
<td>249</td>
<td>5.11 (3.82–6.83)</td>
<td>5.50 (4.04–7.48)</td>
</tr>
<tr>
<td>High anti-Hsp60 (&gt; median)</td>
<td>No</td>
<td>No</td>
<td>23</td>
<td>58</td>
<td>7.92 (4.67–13.40)</td>
<td>7.69 (4.46–13.25)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Yes</td>
<td>Yes</td>
<td>24</td>
<td>141</td>
<td>18.44 (11.40–29.83)</td>
<td>20.99 (12.50–35.24)</td>
</tr>
</tbody>
</table>

* Adjusted for age, sex, smoking status, and BMI.
after angioplasty have been documented in several studies (5, 15, 22–24), but the results are inconsistent (2, 4–7). Our study is the largest case control study so far on the relationship between anti-Hsp60 and risk of CHD, providing evidence that increasing anti-Hsp60 concentrations are associated with CHD risk in a dose-responsive manner.

Our data suggest that CHD patients who took aspirin and/or statins tended to have lower concentrations of anti-Hsp60 than CHD patients who were not taking aspirin and/or statins. Similar results have been found for anti-Hsp60 and anti-Hsp65 in acute cardiac chest pain patients (25). This may be due to anti-inflammatory effects of aspirin and statins. It is known that the statins have antiinflammatory, antioxidant, and immunoregulatory properties (26, 27). Ghayour-Mobarhan et al. (28) first reported that statin treatment was associated with a significant reduction in median antibody concentrations to Hsp60, Hsp65, and Hsp70 in dyslipidemic subjects.

High shear stress or increased blood pressure has been demonstrated to induce Hsp expression in endothelial cells or vascular smooth muscle cells (29, 30). Enhanced expression of Hsp70 has been detected in hypertensive experimental animals (31, 32). Pockley et al. (15) reported that Hsp65 and Hsp70 antibody titers were increased in subjects with hypertension, whereas Hsp60 antibody titers were similar to those in normotensive controls. Frostegard et al. (33) demonstrated that serum antibody titers to Hsp65 were increased in individuals with borderline hypertension. In this study, we found that anti-Hsp60 concentrations were significantly increased in hypertensive patients and that the combination of hypertension and high concentrations of anti-Hsp60 further increased CHD risk. Likewise, we found that the combination of diabetes and high concentrations of anti-Hsp60 was associated with substantially increased CHD risk. In animal experiments, nonobese diabetic mice developed high concentrations of anti-Hsp60 (13). Diabetic subjects have heightened T-cell responses to Hsp60 (11). These lines of evidence indicate that Hsp60 may trigger an attack from the immune system’s T cells in diabetes. In addition, it has been reported that patients with diabetes had significantly higher anti-Hsp70 and anti-Hsp90 concentrations than nondiabetic controls (34). Our results are consistent with these data and suggest that the combination of diabetes and increased anti-Hsp60 concentrations was associated with accelerated atherosclerosis, although the precise mechanisms remain to be investigated.

A major limitation of the present study is its case-control design, which limits the causal interpretation of the relationship between anti-Hsp60 and CHD risk because the blood samples were collected after the occurrence of CHD events. Thus, our results need to be confirmed in large prospective studies. Another limitation is that some patients took multiple medications, which limits our ability to tease out independent effects of individual drugs on anti-Hsp60 concentrations. Because we did not measure known biomarkers of chronic inflammation, such as C-reactive protein (CRP), it is possible that the association for anti-Hsp60 reflects the consequence of increased concentrations of CRP. This hypothesis needs to be tested in future studies. Nevertheless, our study was the largest conducted so far, and we were able to examine the effects of aspirin and/or statins on anti-Hsp60 concentrations. More importantly, the large sample size allowed us to examine the combined effects of increased anti-Hsp60 concentrations and the presence of hypertension and diabetes on CHD risk.

Our data showed that autoimmunity to IgG anti-Hsp60 was independently associated with CHD risk in a Chinese population, high anti-Hsp60 concentrations were positively associated with CHD severity, as assessed by the number of diseased vessels, and cardiovascular risk factors such as hypertension and diabetes were associated with increased concentrations of anti-Hsp60. The combination of these risk factors with increased anti-Hsp60 concentrations conferred a >20-fold increase in the risk of CHD. Pharmacological therapies that lower anti-Hsp60 may be beneficial for prevention and treatment of CHD.

**Grant/Funding Support:** This study was supported by research funds from the National Natural Science Foundation (30430590 and 30128021).

**Financial Disclosures:** None of the authors has any conflict of interest.

**Acknowledgments:** We are particularly grateful to all CHD patients and volunteers for participating in the present study and to the medical personnel of Tongji Hospital, Union Hospital, Wugang Hospital, and WT’s laboratory in Wuhan, Hubei Province, China for their kind assistance in collecting the data and samples.

**References**