Protective Effect of Human Heat Shock Protein 60 Suggested by Its Association with Decreased Seropositivity to Pathogens

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The presence of heat shock protein 60 (Hsp60) in human plasma has been linked with cardiovascular disease (CVD). In this study, the examination of the relationship between Hsp60 in plasma and seropositivity for three microbial agents, which are thought to be risk factors for CVD, surprisingly revealed a negative association between Hsp60 and seropositivity, suggesting a protective effect of this circulating stress protein.

The many stressors in biological systems have led to the evolution of stress proteins (20), many of which are molecular chaperones (8) which fold client proteins. The prototypic chaperone is chaperonin, or heat shock protein 60 (Hsp60) (9), an oligomeric protein with a cavity for protein folding (8).

Stress proteins from infectious agents are generally potent immunogens (6, 29), and the Hsp60 proteins from mycobacteria and chlamydiae act as immunomodulatory proteins (25). This has led to the proposal that human Hsp60 is a cross-reactive antigen responsible for the pathogenesis of atherosclerosis (26). Infectious agents implicated in the pathogenesis of atherosclerosis include chlamydiae, Helicobacter pylori, cytomegalovirus (CMV), herpes simplex virus (HSV), and Epstein-Barr virus (EBV) (11).

In addition to being an immunogen, Hsp60 is a potent intercellular signaling molecule able to activate a range of immune and vascular endothelial cells (16). Such signaling would be of biological importance only if Hsp60 is excreted/secreted by cells. This idea has led to the examination of human blood for the presence of human Hsp60. Surprisingly, a large proportion of humans with normal health have Hsp60 in their circulatory systems (7, 15, 21). The range of levels of circulating Hsp60 is enormous, and these levels have been correlated with the development of atherosclerosis as assessed by carotid artery intima/media thickness (27) and borderline hypertension (22). Our own studies have revealed that levels of circulating Hsp60 in British civil servants correlate with measures of psychological distress (15), a well-established risk factor for cardiovascular disease (24). With a cohort of teenagers, we found that those individuals with Hsp60 in the circulatory system showed definite evidence of vascular dysfunction (7).

Thus, circulating Hsp60 may be both a direct proinflammatory and a potent immunomodulatory signal. A key question is how levels of this cell stress protein in circulation relate to immunity to infectious agents implicated in the pathogenesis of atherosclerosis.

Participants in this study were 392 healthy members of the Whitehall II epidemiological cohort (19). The sample population included 225 men and 137 women aged 51 to 72 years. Blood was collected from these individuals, and plasma was prepared.

Height, weight, and waist and hip circumferences were measured using standardized methods, and body mass indexes (BMI) and waist/hip ratios were calculated. A sample was drawn from fasting participants for lipid analyses, and levels of total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein (HDL) cholesterol were determined. Systolic and diastolic blood pressure was measured with the participant seated.

Human Hsp60 was specifically measured in triplicate by using a two-site enzyme-linked immunosorbent assay (ELISA) (23). The presence of anti-Hsp60 antibodies in the form of immune complexes was determined by immunoprecipitation of Hsp60 and by plasma spiking and recovery experiments with Hsp60.

CMV IgG ELISA. For the CMV immunoglobulin G (IgG) ELISA, the antigen was derived from CMV strain Ad 169 cultured in human fibroblasts. The antigen was extracted by sonication in 0.1 M glycine buffer at pH 9.5. A control antigen was included. The ELISA procedure was as described for CMV.

HSV IgG ELISA. For the HSV IgG ELISA, the antigen was a lysate of the infected cells prepared as reported previously (12). A control antigen was included. The ELISA procedure was as described for CMV.
TABLE 1. Characteristics of study participants

| Characteristic                        | No. of persons in sample population | Value for group<sup>a</sup> |
|---------------------------------------|-------------------------------------|----------------------------|
| Age (yr)                              | 392                                 | 60.3 ± 5.5                 |
| Body mass index (kg/m²)               | 390                                 | 26.5 ± 4.4                 |
| Waist/hip ratio                       | 392                                 | 0.930 ± 0.09               |
| No. of smokers (%)                    | 363                                 | 23 (6.3)                   |
| Total cholesterol level (mmol/liter)  | 392                                 | 5.64 ± 0.97                |
| Low-density lipoprotein cholesterol level (mmol/liter) | 390 | 3.47 ± 0.89               |
| HDL cholesterol level (mmol/liter)    | 392                                 | 1.60 ± 0.45                |
| Triglyceride level (mmol/liter)       | 392                                 | 1.24 ± 0.72                |
| Systolic blood pressure (mm Hg)       | 389                                 | 126.8 ± 16.6               |
| Diastolic blood pressure (mm Hg)      | 389                                 | 74.1 ± 10.6                |
| Median plasma Hsp60 level (ng/ml)     | 392                                 | 303.5                      |
| No. (% with detectable plasma Hsp60)  | 392                                 | 259 (66.1)                 |

<sup>a</sup> Values are means ± standard deviations unless otherwise indicated. Units of measure differ and are listed with the characteristics.

Chlamydia microimmunofluorescence assay. A chlamydia IgG microimmunofluorescence assay (Focus Technologies, Cypress, CA) was used according to the manufacturer’s instructions. Each slide was read in a fluorescence microscope (Zeiss Axiosplan) by two independent investigators, with congruent results.

The associations among the statuses of antibodies to the three infectious agents, demographic factors, and biological risk factors were analyzed using product-moment correlations. We created a total seropositivity index by summing up the number of microbes to which individuals had antibodies, and this value could range from 0 to 3. The distribution of Hsp60 in plasma was highly skewed, as we have previously described (23), so we compared individuals with and without detectable Hsp60. The association between detectable Hsp60 and total seropositivity was analyzed using \( \chi^2 \) statistics. Multivariate analysis was carried out to determine whether the association between the Hsp60 level and serostatus was independent of other factors. Multiple logistic regression was performed with parameters including age, sex, BMI, and systolic and diastolic blood pressure in the model. Results are presented as odds ratios with 95% confidence intervals, with participants with zero seropositivity as the reference group.

The characteristic of the population are shown in Table 1. Two-thirds of the participants had Hsp60 in their plasma. The 392 blood samples were measured for the presence of antibodies to *Chlamydia pneumoniae*, CMV, and HSV (Table 2). Between 52.3% and 63.2% of the population showed an immune response to each infectious agent. Of the participants, 23.7% were positive for all three microbes while 46 (11.7%) were negative for all microbes. Antibodies to *C. pneumoniae* were more common in older participants (*P* = 0.031) and in men rather than women (*P* < 0.001) and were positively correlated with systolic blood pressure (*P* = 0.002) and negatively related to HDL cholesterol levels (*P* = 0.007). Women were more likely to be positive for CMV (*P* = 0.014). HSV positivity correlated with BMI (*P* = 0.002) and with diastolic blood pressure (*P* = 0.031). Total seropositivity correlated with age (*P* = 0.035), BMI (*P* < 0.001), and systolic and diastolic blood pressure (*P* = 0.030). Table 3 shows the total seropositivity-Hsp60 relationship. The presence or absence of Hsp60 in the circulatory system was examined. Of those participants with zero seropositivity, 76.1% had detectable Hsp60, compared with 54.8% of those who were positive for all three antibodies. The trend across these categories is significant (*P* = 0.007). In multiple logistic regression, the odds of having Hsp60 in the plasma were significantly reduced for those with multiple serropositivities after adjusting for age, sex, BMI, and systolic and diastolic blood pressure (*P* = 0.019).

Substantial evidence supports the surprising hypothesis that

TABLE 2. Antibody statuses of participants

| Participant group and antibodies<sup>a</sup> | No. (%) of participants |
|---------------------------------------------|-------------------------|
|                                            | Positive | Negative |
| All participants                           |          |          |
| *C. pneumoniae* antibodies (n = 380)        | 240 (63.2) | 140 (36.8) |
| CMV antibodies (n = 386)                    | 202 (52.3) | 184 (47.7) |
| HSV antibodies (n = 390)                    | 232 (59.5) | 158 (40.5) |
| Participants with positive or negative serology results (n = 392) |          |          |
| No antibodies                              | 46 (11.7)  |          |
| Antibodies to one agent                    | 111 (28.3) |          |
| Antibodies to two agents                   | 142 (36.2) |          |
| Antibodies to three agents                 | 93 (23.7)  |          |
| Participants with single seropositivity (n = 111) |          |          |
| *C. pneumoniae* antibodies without CMV or HSV antibodies | 18 (16.2)  |          |
| CMV antibodies without *C. pneumoniae* or HSV antibodies | 56 (50.5)  |          |
| HSV antibodies without *C. pneumoniae* or CMV antibodies | 37 (33.3)  |          |
| Participants with double seropositivity (n = 142) |          |          |
| *C. pneumoniae* antibodies plus CMV antibodies | 40 (28.2)  |          |
| *C. pneumoniae* antibodies plus HSV antibodies | 51 (35.9)  |          |
| CMV antibodies plus HSV antibodies          | 51 (35.9)  |          |

<sup>a</sup> n, number of participants.
chaperones such as Hsp60 are potent immunogens (6, 13, 29) and powerful immunomodulators (25, 26), and a recent study (3) suggests that immunization with Hsp60 may protect against type I diabetes. The paradoxical immunogenicity of Hsp60 may be explained by the recent finding that this protein has potent intercellular signaling actions with both pro- and anti-inflammatory responses (10, 17). The finding that a large proportion of the human population has Hsp60 in circulation brings the bioactivity of Hsp60 into sharp relief. Levels can range from small nanograms to amounts of several micrograms per milliliter of plasma (7, 15, 21, 22, 23, 27). At levels greater than 1 μg/ml, Hsp60 will have a cellular effect (17). This raises the obvious question of the consequences of having biologically active Hsp60 in circulation.

Circulating Hsp60 and certain infectious agents are now recognized to be risk factors for cardiovascular disease. A range of organisms, principally the bacteria C. pneumoniae and H. pylori and the viruses CMV, HSV, and Epstein-Barr virus, have been implicated (11). With chlamydial infection, attention has focused on the Hsp60 protein of C. pneumoniae (17). There is evidence that this protein can contribute to the pathology of experimental atherosclerotic lesions in mice infected with C. pneumoniae (5). Furthermore, it has been reported that high levels of antibodies to human Hsp60 and C. pneumoniae are independent risk factors for coronary atherosclerosis, but their simultaneous presence substantially increases the risk for disease development (2). Viruses do not produce stress proteins. However, an internal peptide of human Hsp60, which is recognized by circulating antibodies to Hsp60 in atherosclerotic patients, shares homology with the CMV proteins UL122 and US28 and these proteins are recognized by patient antibodies. Of interest, purified IgGs against Hsp60 and the viral peptides bound nonstressed human endothelial cells and induced endothelial cell apoptosis. It was concluded that such a mechanism for inducing endothelial cell apoptosis could act as an initiating event in atherogenesis (1).

This study is part of a set of larger prospective studies of healthy British civil servants (Whitehall I and II studies) designed to identify risk factors for cardiovascular disease. These studies have identified social gradients and psychological distress as major risk factors (18). Studies of a small cohort of Whitehall II participants revealed that the majority have circulating Hsp60 and that levels of this protein correlate with measures of psychological distress in women (15). A larger, as-yet-unpublished study has confirmed this preliminary finding and has related levels of Hsp60 to measures of psychological distress and social deprivation in both males and females (A. Shamaei-Toussi, A. Steptoe, A. R. Coates, and B. Henderson, unpublished results). In the Whitehall population under study, between 52.3% and 63.2% of the population showed an immune response to each infectious agent. Of the participants, 23.7% were positive for all three microbes, while 46 (11.7%) were negative for all microbes. Antibodies to C. pneumoniae were more common in older participants, with more men than women showing seropositivity. Of interest, C. pneumoniae antibody levels were positively correlated with systolic blood pressure but were negatively related to HDL cholesterol levels. Women were more likely to be positive for CMV than men. HSV positivity correlated with BMI and with diastolic blood pressure (P = 0.031). Total seropositivity correlated with age, BMI, and systolic and diastolic blood pressure (P = 0.030).

As discussed, a growing number of risk factors for cardiovascular disease appear to relate to the immune responsiveness to bacterial or human Hsp60 (or related peptides) and/or to the presence of Hsp60 in the circulation and to seropositivity for certain infectious agents. Is there any relation between these different risk factors? The obvious assumption would be that the levels of Hsp60 in the circulation reflect underlying cell stress levels, potentially caused by cryptic infectious agents such as those implicated in the pathogenesis of atherosclerosis. Therefore, one would expect high levels of Hsp60 to correlate with high levels of seropositivity for infectious agents. To our surprise, the levels of Hsp60 in the blood of the Whitehall cohort were inversely correlated with the seropositivity for the three infectious agents under study. In the case of the immune responsiveness to C. pneumoniae, if the response was principally to the Hsp60 proteins of this organism (it produces three distinct Hsp60 proteins) (14), then high levels of human Hsp60 in the blood could act to remove cross-reactive antibodies, lowering the antibody titer. However, the method used to measure seropositivity used whole organisms, making this a less likely explanation. Indeed, there was no evidence for the presence of antibodies to human Hsp60 in the plasma tested. The same explanation is unlikely to apply to the viruses. This situation, therefore, raises the possibility that one consequence of Hsp60's being in circulation is that it protects against infection with agents such as C. pneumoniae and viruses. Recent data from Irun Cohen's laboratory has revealed that human, but not bacterial, Hsp60 proteins can interact with both T cells (28) and B cells (4) to regulate their behavior. With both cell types, Hsp60 shows the ability to downregulate responsiveness. For example, human Hsp60 stimulates murine B cells to produce interleukin 10 which has anti-inflammatory and immunomodifying activity (4). Could this lead to our finding of decreased seropositivity? If so, this may be providing a link between psychological factors and immune responsiveness. Further studies are obviously required to determine the relationship between the release of host Hsp60 and the immunological responsiveness to infectious agents such as bacteria or viruses.

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**TABLE 3. Seropositivity and plasma Hsp60 levels**

| No. of agents for which participants were seropositive (n) | % of participants with Hsp60 detectable in plasma (%) | Odds of having Hsp60 detectable in plasma (95% CI)* |
|-----------------------------------------------------------|-----------------------------------------------------|-----------------------------------------------------|
| 0 (46)                                                    | 76.1 (35)                                           | 1                                                  |
| 1 (111)                                                   | 70.3 (78)                                           | 0.78 (0.35–1.73)                                    |
| 2 (142)                                                   | 66.9 (95)                                           | 0.67 (0.31–1.45)                                    |
| 3 (93)                                                    | 54.8 (51)                                           | 0.43 (0.19–0.95)                                    |

*P value

Notes: CI, confidence interval; n, number of participants.
Data were adjusted for age, sex, BMI, and systolic and diastolic blood pressure.
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