Iran . J . Immunol
ISSN 1735-1383

Iran. J. Immunol. March 2008, 5 (1), 51-56

Abdollah Jafarzadeh, Ali Esmaeeli-Nadimi, Mehdi Shariat

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Article Type: Research

High Sensitivity C-Reactive Protein and Immunoglobulin G against *Chlamydia Pneumoniae* and Chlamydial Heat Shock Protein-60 in Ischemic Heart Disease

Abdollah Jafarzadeh*[1], Ali Esmaeeli-Nadimi[2], Mehdi Shariati[3]

[1]Department of Immunology, [2]Department of Cardiology, [3]Department of Histology, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

ABSTRACT

Background: Inflammation and infectious agents such as *Chlamydia pneumoniae* have been associated with cardiovascular disease. **Objective:** To evaluate the serum high sensitivity C - reactive protein (hs-CRP) and antibodies against *Chlamydia pneumoniae* and Chlamydial heat shock protein-60 (Cp-HSP60) in patients with ischemic heart disease (IHD). **Methods:** 62 patients with IHD having either acute myocardial infarction (AMI; n=31) or unstable angina (UA; n=31) and 31 sex- and age- matched healthy subjects as a control group were enrolled in this study. Serum samples of participants were tested for the presence of hs-CRP and antibodies against *C. pneumoniae* and Cp-HSP60 using ELISA method. **Results:** The seroprevalence of anti-C. pneumoniae antibody in AMI group (93.5%) or UA group (90.3%) was significantly higher than the control group (61.3%; $p<0.001$). The seroprevalence of anti-Cp-HSP60 IgG was 22.6% in healthy subjects with mean end titer of 43.1 ± 6.32. The seropositive rates of anti-Cp-HSP60 were 48.4%, 54.8% and 51.6% in AMI, UA and the overall IHD groups with mean end titers of 94 ± 22.86, 113.8 ± 24.25 and 103.9 ± 16.57, respectively. Both the seroprevalence and the mean titer of anti-Cp-HSP60 in patients groups were significantly higher than those observed in the control group ($p<0.04$ and $p<0.03$, respectively). Moreover, the mean serum hs-CRP levels was significantly higher in the IHD group as compared to the control group (21.6 µg/ml ± 3.73 vs 2.5 µg/ml ± 0.52; $p<0.00001$). The mean serum hs-CRP levels of AMI (30.3 µg/ml ± 6.07) or UA (12.9 µg/ml ± 3.85) groups were also significantly higher than those observed in the control group ($p<0.00001$ and $p<0.001$, respectively). Furthermore, the difference of the mean serum hs-CRP levels between AMI and UA groups was also significant ($p<0.02$). **Conclusions**: These results showed that the seroprevalence of antibodies against C. *pneumoniae* and Cp-HSP60 and the serum levels of hs-CRP and anti-Cp-HSP60 IgG were higher in patients with IHD.

Keywords: Ischemic Heart Disease, *Chlamydia pneumoniae*, HSP60, CRP

*Corresponding author: Dr Abdollah Jafarzadeh, Department of Immunology, Medical School, Rafsanjan University of Medical Sciences, Rafsanjan, Iran. Tel: (+) 98 391 5234003, Fax: (+) 98 391 5225209, e-mail: Jafarzadeh14@yahoo.com

INTRODUCTION

Traditional risk factors, such as hypercholesterolemia, hypertension, diabetes, genetic abnormalities, smoking and obesity, can explain only about half of the cases of coronary heart disease (1). It has been suggested that inflammatory reactions might also play an etiological role in cardiovascular disease. Moreover, an association between IHD and some infectious agents including *C. pneumoniae*, *Helicobacter pylori*, herpes simplex virus, cytomegalovirus, hepatitis A, respiratory tract and dental infections has been reported in some epidemiological studies (2). Of these pathogens, *C. pneumoniae* is the most strongly implicated in atherosclerotic diseases and the results from other pathogens have been reported less consistently.

A specific autoimmune response to HSPs is also considered to play a key role in the pathogenesis of cardiovascular diseases. In experimental models, it has been shown that immunization with microbial HSP65 induced atherosclerotic lesions (3), suggesting a role of autoimmune processes, with the HSPs as autoantigens, in the development of cardiovascular disease. Several findings indicate that the chlamydial 60-kDa HSP (HSP60), may represent an antigenic stimulus in eliciting strong immune responses with immunopathological sequelae of chronic chlamydial infections (4). The relation of AMI with increased (5) or unchanged (6) levels of anti-human HSP60, or with increased (6) or decreased (7) anti-microbial HSP65 levels has also been reported.

There is also increasing evidence that CRP, a marker of inflammation, is an independent risk factor for cardiovascular disease and can be a valuable tool in assessing at risk populations. It has been also reported that measurement of the serum levels of CRP using a high sensitivity assay (hs-CRP) can demonstrate subclinical inflammatory states, which may reflect vascular inflammation (8). This study was conducted to evaluate the serum hs-CRP levels and antibody against *C. pneumoniae* and Chlamydial HSP60 in Iranian patients with IHD to clarify the possible relation.

SUBJECTS AND METHODS

Sixty two patients (aged 40-65 years) with IHD who were admitted to Ali-ebne-Abitaleb hospital of Rafsanjan (a city located in Kerman province in the south east of Iran) were enrolled in this study. Patients were classified into 2 groups according to well established criteria, as having AMI (n=31) or UA (n=31). AMI was diagnosed by the presence of two of the following three criteria: i) prolonged chest pain compatible with AMI, ii) typical ECG changes, and iii) raising of cardiac enzymes. Unstable angina patients were in class lllB according to Braunwald classification (9). Specific serum antibodies and CRP were measured in patients with AMI one week after admission. In patients with UA, measurements were done at admission time. A third sex- and age-matched group with similar geographic and socioeconomic backgrounds, consisted of 31 subjects without any IHD, was registered as a control group. The healthy control group was recruited among blood donors of Rafsanjani Blood Transfusion Center. Peripheral blood (2-4 milliliters) were collected from all the subjects after obtaining informed consent, and the sera were separated and stored at –20°C.

Determination of *Chlamydia Pneumoniae*-Specific Antibodies in Serum. The serum anti-C. pneumoniae immunoglobulin G was measured using the commercial enzyme-linked immunosorbent assay (Diagnostika, Germany). Serum levels of anti-Chlamydial

HSP60 IgG were also assayed by ELISA method using commercial kits (Diagnostika, Germany) and the end titers of anti-Chlamydial HSP60 IgG were calculated according to the manufacturer's guideline.

Determination of Serum hs-CRP Levels. Serum hs-CRP levels were measured in duplicate using commercial ELISA kits (Monobind, USA). Serum hs-CRP levels were expressed in µg/ml and the sensitivity of this assay was found to be 0.2 µg/ml.

Statistical Analysis. Differences in variables were analyzed using Kruskal-Wallis, Mann-Whitney U-test, Chi-square and Fisher exact tests as appropriate and P values of less than 0.05 were considered significant. All the available data were analyzed by a computer program (SPSS version 11.5, Chicago, IL, USA).

RESULTS

Baseline Characteristics of Subjects. Baseline characteristics of AMI, UA and healthy control groups have been shown in Table 1. Only patients with minimal traditional major risk factors or without any identified major risk factors of IHD such as hyperlipidemia, hypertension, obesity, diabetes, and smoking were enrolled in the study. There were no significant differences among the 3 groups for the age and gender ratio. Moreover no statistically significant differences were observed with respect to the presence of a traditional risk factor for IHD.

Table 1. Baseline characteristics of patients and control groups

Groups	AMI (n=31)	UA (n=31)	Control (n=31)
Age[y] (mean + SEM)	48.9 ± 7.1	49.6 ± 7.1	49.4 ± 7.3
Sex (Men/Women)	16/15	16/15	16/15
Hypertension [n]	3	3	0
Dyslipidemia [n]	3	2	0
Diabetes mellitus [n]	3	1	0
Current smoking [n]	1	2	0
Obesity	0	0	0

y and n represent the year and number, respectively.

No statistically significant differences were observed among groups with respect to the age, sex or presence of a particular risk factor.

Anti-*C. Pneumoniae* IgG Seropositivity. The overall seroprevalence of anti-C. pneumoniae IgG among patients with IHD (91.9%) was significantly ($p<0.005$) higher than that observed among control group (61.3%). The seropositivity rate of anti-*C. pneumoniae* antibody was 93.5% in AMI group and 90.3% in UA group. The seroprevalence of anti-*C. pneumoniae* IgG in either AMI or UA groups was significantly higher than the control group ($p<0.001$). The seroprevalence of anti-*C. pneumoniae* was similar in patients with either AMI or UA (Table 2).

Table 2. Comparison of the hs-CRP and IgG against *C. pneumoniae* and Chlamydial HSP60 in patients with ischemic heart disease and control group

Group	Number	Anti-CP	Anti-C-HSP60	Mean titer of Anti-cHSP60	Means levels of hs-CRP (µg/ml)
AMI	31	29 (93.5%)	15 (48.4%)	94 ± 22.86*	30.3 ± 6.07*
UA	31	28 (90.3%)	17 (54.8%)	113.8 ± 24.25	12.9 ± 3.85
Total	62	57 (91.9%)	32 (51.6%)	103.9 ± 16.57	21.6 ± 3.73

Control	31	19 (61.3%)	7 (22.6%)	43.1 ± 6.32	2.5 ± 0.52

*Results expressed as mean ± SEM

Anti-Chlamydial HSP60 Seropositivity. The overall seroprevalences of anti-Cp-HSP60 IgG were 51.6% and 22.6% in patients with IHD and the healthy subjects with mean end titers of 103.9 ± 16.57 and 43.1 ± 6.32, respectively (Table 2). The seropositive rates of anti-Cp-HSP60 IgG were 48.4% and 54.8% in AMI and UA groups with mean end titers of 94 ± 22.86 and 113.8 ± 24.25, respectively. The seroprevalences of anti-Cp-HSP60 IgG in AMI group, UA groups or the overall IHD group were significantly higher than the control group ($p<0.04$). The mean titer of anti-Cp-HSP60 IgG in patients groups was also significantly higher than that observed in the control group ($p<0.03$). However, the difference of mean titers of anti-Cp-HSP60 IgG between AMI and UA groups was not statistically significant.

Serum Levels of hs-CRP. Hs-CRP values ranged from 0.36 µg/ml to 15.49 µg/ml in control group, from 1.31 µg/ml to 98.83 µg/ml in AMI group and from 0.26 to 78.47 µg/ml in UA group. The mean serum hs-CRP levels were 21.6 µg/ml ± 3.73 in IHD group and 2.5 µg/ml ± 0.52 in control group (Table 2). The mean serum hs-CRP levels were significantly higher in IHD group than the control group ($p<0.00001$). Moreover, the mean serum hs-CRP levels were 30.3 µg/ml ± 6.07 and 12.9 µg/ml ± 3.85 in AMI and UA groups, respectively (Table 1). Statistical analyses showed that the mean serum hs-CRP levels of AMI or UA groups were significantly higher than that of control group ($p<0.00001$ or $p<0.001$, respectively). Furthermore the difference of the mean serum hs-CRP level between AMI or UA groups was also statistically significant ($p<0.02$).

DISCUSSION

The results of the present study showed that the seroprevalence of anti-*C. pneumoniae* IgG was significantly higher in AMI or UA groups (and also in the overall IHD group) as compared to the control. The association of of *C. pneumoniae* infection with cardiovascular disease was originally observed by Saikku et al. (10), who determined *C. pneumoniae*-specific IgG in patients with coronary artery disease. However, some investigators also have assessed the relationship between *C. pneumoniae* and IHD, reporting a strong positive (11), a mild (12) and even a negative association (13). These inconsistencies may be attributed largely to differences in study design or in the population backgrounds, such as the prevalence of other risk factors. Moreover, both host and bacterial factors should be considered in order to understand the pathogenesis of *C. pneunoniae*-associated diseases. However, several possible mechanisms have been proposed by which *C. pneumoniae* infection could increase the risk of IHD. In patients, detection of *C. pneunoniae* antigens and DNA have been observed in atheromatous lesions (14), and viable organisms have been also cultivated from atherosclerotic lesions (15). Moreover, infection of vascular endothelial cells with *C. pneumoniae* may also stimulate the secretion of soluble factors that elicit proliferation of smooth muscle cells (16). Furthermore, macrophages infected with *C. pneumoniae* show accelerated uptake of LDL and transformation into foam cells (17).

The results of the present study showed that the seroprevalence and the mean titer of anti-Cp-HSP60 IgG were significantly higher in AMI or UA groups (and in the overall IHD group) as compared to the control. In rabbit experiments, HSP60 was first identified as the responsible autoantigen for the initiation of atherosclerosis (3). It has been demonstrated that upon depletion of HSP60-immunized rabbits from peripheral blood

mononuclear cells, this effect could be prevented. Later, similar results were obtained in LDL–receptor-deficient mice (4). In an experimental model, it has been demonstrated that the induction of tolerance to HSP65 reduced lesion size (18). Because HSPs are highly conserved during evolution, this process may lead to a cross-reaction between microbial and human HSP60. As long as human endothelial cells are not stressed, they do not express a significant amount of HSP60 on their surface. However, it has been shown that different forms of stress (e.g. heat, tumor necrosis factor, turbulent biomechanical stress at arterial branching points, and oxidized LDL) can lead to the expression of HSP60 on the arterial cell surface and in turn lead to an autoimmune reaction and local inflammation of the artery (4). Adhesion molecules are simultaneously expressed, and mononuclear cells infiltrate the wall, predisposing it to more severe lesions (19). Accordingly, it seems that a humoral and cellular cross-reaction occur between human and bacterial HSP60. We have observed that the titers of anti-Cp-HSP60 were higher in patient groups. These finding are compatible with the results of other studies (4, 20). However, no difference was observed in anti-Cp-HSP60 titers between UA and AMI patients, suggesting that the process is not likely to be related to myocardial necrosis.

The results of the present study also showed that the mean serum CRP levels of both AMI and UA groups were significantly higher than that observed in the control group. It has been reported that some risk factors such as increased blood pressure, increased body mass index, diabetes mellitus, low HDL levels and infections are associated with increased levels of CRP (8). Recent investigations have shown that CRP may directly contribute to the atherosclerotic process. In human atherosclerotic plaques, CRP has been found to colocalize with complement component near the areas of extracellular lipid deposition (21). In vitro studies suggest that CRP binds to modified LDL, especially to non-esterified cholesterol in LDL (22). CRP also may deposit in the intima of early atherosclerotic lesions and is chemotactic for monocytes which express a CRP receptor (23), suggesting that CRP may play a role in early atherogenesis via early monocyte recruitment. It is also shown that CRP induces the upregulation of cell adhesion molecules on cultured endothelial cells (24). Moreover, upregulation of lectin-like oxidized LDL receptor-1 (LOX-1) has been shown on human aortic endothelial cells cultured with CRP. Oxidized LDL binds to LOX-1 on endothelial cells, and this is a major step in atherogenesis. LOX-1 also increases monocyte binding to endothelial cells (8). Recently, it has been shown that transgenic mice for human CRP have increased thrombosis after arterial injury (25). These findings suggest that CRP may play a direct role in the initiation of cardiovascular disease.

However, MI results in the necrosis of cardiac muscle, which is a stimulus for CRP production. Accordingly, it has been shown that the hs-CRP levels rise in parallel to the amount of muscle necrosis, peaking at around day 2 post MI and then falling. Persistent elevations of hs-CRP after AMI, suggesting ongoing inflammation, predict recurrent events (26). However, all patients with MI can be considered “high risk” and should be treated aggressively. In UA, however, hs-CRP levels presented as a guide for classification of patients into higher and lower risk groups. Elevated hs-CRP levels associate with higher risk of recurrent events (27).

In conclusion, the results of the present study demonstrate higher seroprevalences of anti-*C. pneumoniae* and anti-Cp-HSP60 IgG and elevated serum levels of hs-CRP and anti-Cp-HSP60 IgG in patients with IHD.

ACKNOWLEDGEMENT

The authors wish to thank Mr Mohammad Taghi Rezayati and Mrs Maryam Nemati the technicians of Immunology Department for their invaluable help. This work was financially supported by a grant (No.85-9.20.2480) from Rafsanjan University of Medical Sciences, Rafsanjan, Iran.

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