Int. J. Exp. Path. (2008), 89, 209–215
doi: 10.1111/j.1365-2613.2008.00586.x

ORIGINAL ARTICLE

Antibody titres to heat shock protein 27 are elevated in patients with acute coronary syndrome

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INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY

Summary

IgG antibody titres to heat shock protein 27 (anti-Hsp27) were measured to determine whether these titres were affected in patients admitted with acute coronary syndrome. Blood samples were taken from 94 patients admitted with acute coronary syndrome. Anti-Hsp27 IgG titres were determined using an in-house enzyme-linked immunosorbent assay (ELISA) in the first and second 12 h after the onset of symptoms and compared with values for 81 age- and sex-matched control subjects. Median antibody titres to Hsp27 in the first sample from patients whose diagnosis was a myocardial infarction ($n = 42$) was 0.41 absorbancy units (range 0.28–0.57) and for those with unstable angina ($n = 52$) was 0.31 (range 0.20–0.42), both being significantly higher than for controls ($n = 81$), which was 0.08 (range 0.05–0.15) ($P < 0.05$). However, titres fell in the second samples collected in the coronary syndrome patients and were then no longer significantly different from controls ($P > 0.05$). Myocardial infarction patients also had significantly higher anti-Hsp27 titres in the first 12 h than patients with unstable angina ($P < 0.05$), but again the difference in the second sample did not reach statistical significance ($P > 0.05$). Serum antibody titres to Hsp27 rise and fall rapidly after the onset of acute coronary syndrome, and may be an early marker of myocardial ischaemia as patients with myocardial infarction or unstable angina both had high titres.

Keywords
acute coronary syndrome, antibody titre, ELISA, heat shock protein 27, myocardial infarction, unstable angina

Received for publication: 4 October 2007
Accepted for publication: 11 February 2008

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Cells respond to a variety of environmental stresses by expressing a family of proteins called the heat shock proteins (Hsps) (Morimoto 1993; Udelsman *et al.* 1993). This response is highly conserved through evolution (Xu *et al.* 1994; Soltys & Gupta 1996). The primary physiological function of the Hsps is as molecular chaperones that protect

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proteins from damage by binding to immature or partially denatured proteins, helping them to fold into their native conformation and regain functional activity (Becker & Craig 1994; Soltys & Gupta 1996; Snoeckx *et al.* 2001). Potential risk factors for atherosclerosis such as infections, oxidized low-density lipoprotein (ox-LDL), oxidative stress, hypertension and biochemical stress, also stimulate Hsp expression by macrophages and smooth muscle cells (Morimoto 1993).

Hsp27 is a member of the small Hsp (sHsp) family of proteins and has a molecular weight of approximately 27 KDa, although it can form large aggregates of up to 800 KDa in the cytosol (Mehlen *et al.* 1997). In adults, Hsp27 is expressed at high levels in several normal tissues including breast, uterus, cervix, placenta, skin, lung, heart and platelets (Ciocca *et al.* 1993).

In addition to its role as a chaperone, Hsp27 also has several other potentially important roles. These include cell migration (Kaida *et al.* 1999; Hirade *et al.* 2002; Negre-Aminou *et al.* 2002; Akamatsu *et al.* 2004), apoptosis (Garrido *et al.* 1999; Arrigo 2000; Charette *et al.* 2000; Clermont *et al.* 2003), protection against oxidative stress (Arata *et al.* 1995; Loktionova & Kabakov 1998, 2001; Arrigo *et al.* 2005), endothelial barrier function (Loktionova *et al.* 1996; Loktionova & Kabakov 1998; Hirano *et al.* 2004) and modulation of inflammation (De *et al.* 2000; Chen *et al.* 2004). All of these functions may have an impact on the process of atherogenesis and it has been reported that Hsp27 expression is increased in the vessels adjacent to the plaque in atherosclerotic lesions (Park *et al.* 2006). Hsp27 is also a major constituent of myocardial tissue that may be released following myocardial injury (Tanonaka *et al.* 2001; Pantos *et al.* 2003). In the present study, we aimed to investigate changes in serum anti-Hsp27 concentrations associated with acute coronary syndrome, comparing levels with those of healthy subjects.

Materials and methods

Subjects

A total of 94 patients (62 males, 32 females, mean age: 58.67 ± 1.49) with acute coronary syndrome were recruited. They attended the Accident and Emergency Centre with chest pain within the preceding 12 h, and were subsequently admitted to the Coronary Care Unit of Qaem Hospital, Mashad, Iran. They were recruited between April 2006 and January 2007. The presence of a myocardial infarction or unstable angina was determined by a cardiologist, according to the World Health Organization (WHO) criteria using ECG, echocardiography and serum troponin I positivity. Blood samples were taken from each patient for analysis; on admission and 12 h from the onset of chest pain. These 94 patients were subsequently divided into two groups based on their final diagnosis: group 1 comprised 42 patients who were troponin I positive and had a final diagnosis of myocardial infarction (29 males, 13 females, mean age: 56.21 ± 2.28) and group 2, comprising 52 patients who were troponin I negative and with a final diagnosis of unstable angina (32 males, 20 females, mean age: 60.83 ± 1.93). Eighty-one age- and sex-matched healthy subjects without a history of coronary heart disease, and who were referred to the Mashhad Central Laboratory for a routine check-up, (64 males, 17 females, mean age: 54.86 ± 0.99), were recruited as the control group.

Each subject gave written consent to participate in the study, which had previously been given approval by the Mashhad University of Medical Sciences' research ethics committee.

Anthropometric and other measurements

For all subjects, anthropometric parameters including weight, height and waist circumference were measured using a standard protocol. Waist circumference was measured at the level of the umbilicus (at the level midway between the lower rib margin and the iliac crest). Subjects were asked to breathe normally, and to breathe out gently at the time of the measurement. The hip circumference measurement was taken at the point yielding the maximum circumference over the buttocks. Height, body weight, waist and hip circumference were measured with the subjects dressed in light clothing after an overnight fast. The body weight of each subject was measured with a standard scale to an accuracy of ±0.1 kg, and height was measured to an accuracy of ±0.1 cm. Blood pressure was measured twice while the patients were seated and rested for 15 min, using a standard mercury sphygmomanometer calibrated by the Iranian Institute of Standards and Industrial Research. The interval between each blood pressure measurement was at least 30 min, and the average of the two measurements was taken as the blood pressure. The systolic blood pressure was defined as the appearance of the first sound (Korotkoff phase 1) and the diastolic blood pressure was defined as the disappearance of the sound (Korotkoff phase 5) during deflating of the cuff. The body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2).

Blood sampling

Venous blood samples were collected from each patient on hospital admission and approximately 12 h later, and for

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the control group one blood sample was taken on the day of laboratory sampling after a 12-h fast. Following venepuncture of an antecubital vein, blood samples were collected into Vacutainer® tubes and centrifuged at 10,000 *g* for 15 min at 4 °C. After separation, aliquots of serum were frozen at −80 °C until the day of analysis.

Biochemical analysis and troponin I test

All chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, UK) unless stated otherwise.

Serum cardiac troponin I (cTnI) concentrations were measured using a specific two-sided immunoassay (Dimension Flex; Dade Behring, Marburg, Germany). The detection range for cTnI was 0.04–40 ng/ml, requiring further dilutions if necessary. cTnI values > 0.1 ng/ml were considered positive.

Lipid profiles and blood glucose

A full 12 h fasted lipid profile comprising total cholesterol, triglycerides and high-density lipoprotein cholesterol (HDL-C) was determined for each patient. Serum lipid and fasting blood glucose concentrations were measured by routine enzymatic methods.

Serum anti-Hsp27 titres

Serum Hsp27 antibody titres were measured using an in-house ELISA assay. Microtitre plates (Nunc Maxisorp, Nottingham, UK) were coated with 100 ng per well recombinant human Hsp27 dissolved in 50 μl carbonate buffer pH, 9.6 incubated for 18 h at 4 °C under humidified conditions. The wells were washed three times in wash buffer (PBS containing 0.05% Tween-20). Non-specific binding was reduced by blocking each well with 2% goat serum in PBS and 250 μl added to each well and incubated for 30 min in 37 °C and 30 min at room temperature. Wells were washed three times with PBS. Serum was diluted 1:100 with 2% goat serum in PBS and 100 μl added to the each wells in duplicated and incubated for 30 min at room temperature. After washing (four times in wash buffer and two times in PBS), 100 μl peroxidase conjugated-goat anti-human IgG (Sigma-Aldrich, Poole, UK) diluted 1:500 with 2% goat serum in PBS, was added to each wells, and incubated for 30 min at room temperature. After washing (four times in wash buffer and two times in PBS), 100 μl of TMB substrate (100 μl of 6 mg/ml TMB in DMSO was added to 10 ml of 50 mM acetate buffer, pH 4.5, containing 3 μl H_2O_2) was added per well and plate incubated for 15 min in the dark at room temperature.

The reaction was terminated by adding 50 μl 2 M HCl per well. Optical density at 450 nm was measured using a Labsystems iEMS Reader MF Microtitre plate reader with a reference wavelength of 620 or 570 nm. The within-assay and between-assay precision was 3.5% and 5.2% respectively. After correction for the non-specific background absorbance (subtracting the absorbance of uncoated wells from the antigen-coated wells for each sample), the results were expressed in optical density units.

Statistical analysis

All statistical analyses were performed with MINI-TAB (release 13, Minitab Inc, 2000, State College, PA, USA). Values were expressed as mean ± SEM or, in the case of non-normally distributed data, as median and inter-quartile range. Data that were normally distributed were analysed using student *t*-test (for two groups) or one-way analysis of variance (ANOVA) (for ≥3 groups). Data found to be non-normally distributed were analysed using the non-parametric Mann–Whitney test (for two groups) or Kruskal–Wallis test (for ≥3 groups). For comparison between two related samples, the Wilcoxon signed ranks test was used. A two-sided *P*-value of <0.05 was considered statistically significant. To analyse the relationship between anti-Hsp27 and individual coronary risk factors, Spearman's correlation was used due to the non-normally distribution of anti-Hsp27.

Results

Demographic data

There was a high frequency of a previous history of coronary heart disease (44%), hyperlipidaemia (27%), hypertension (47%), diabetes mellitus (25%) and smoking habit (39%) in the patient group, and this was significantly higher than for the control group ($P < 0.05$). Serum triglycerides, total and LDL-C, fasting blood glucose, body mass index (BMI), waist:hip ratio, systolic and diastolic blood pressure were also significantly higher in patients compared with the controls, as may be expected. The mean age and the proportion of females in the groups (34% of patients compared with 17% of healthy subjects) did not differ significantly ($P > 0.05$). There were no significant differences in any of these parameters between the troponin I positive (myocardial infarction) and the negative (unstable angina) subgroups ($P > 0.05$). The demographic data for the patients and controls are presented in Table 1.

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Table 1 Comparison of clinical and biochemical characteristics of patients and controls

Group	Controls	Patients MI	UA	Combined ACS
n	81	42	52	94
Female%	21	30	38	34
Smoker%	14	46	33	39**
CHD%	0	32	55	44***
Diabetic%	6	16	33	25*
Hyperlipidaemic%	11	16	38	27*
Hypertensive%	10	35	57	47***
ST elevation in ECG (%)	0	60	0	27
Age (years)	54.86 ± 0.99	56.21 ± 2.28	60.83 ± 1.93	58.67 ± 1.49
BMI (kg/m^2)	26.98 ± 0.47	26.71 ± 0.75	26.70 ± 0.59	26.70 ± 0.47
Waist: hip ratio	0.95 ± 0.01	1.02 ± 0.02	1.09 ± 0.03	1.06 ± 0.02***
Fasting blood sugar (mmol/l)	5.59 ± 0.16	8.55 ± 0.99	8.20 ± 0.91	8.37 ± 0.67**
Total cholesterol (mmol/l)	4.57 ± 0.17	5.78 ± 0.19	5.83 ± 0.26	5.79 ± 0.12***
Triglycerides (mmol/l)	1.10 (0.76–1.72)	0.88 (0.76–1.46)	1.28 (0.86–1.87)	1.63 (1.05–2.00)**
HDL-C (mmol/l)	1.32 ± 0.03	1.17 ± 0.03	1.20 ± 0.04	1.19 ± 0.02***
LDL-C (mmol/l)	2.72 ± 0.13	3.57 ± 0.06	3.58 ± 0.16	3.57 ± 0.09***
Systolic blood pressure (mmHg)	122.77 ± 1.24	123.45 ± 5.36	138.37 ± 4.07	131.63 ± 3.38***
Diastolic blood pressure (mmHg)	77.00 ± 1.05	83.57 ± 4.40	83.87 ± 2.19	83.73 ± 2.30***

MI, myocardial infarction; UA, unstable angina; ACS, acute coronary syndrome. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Values are expressed as mean ± SEM, or medial and interquartile range. Between-group comparisons were assessed using the unpaired *t*-test for normally distributed data and Mann–Whitney test for non-parametric data (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Hsp27 antibody titres in patients and controls

Overall, patients with acute coronary syndrome had significantly higher antibody titre to Hsp27 in the blood sample taken in the first 12 h compared with controls, and this was also true for both subgroups of patients with myocardial infarction or unstable angina ($P < 0.001$, Table 2).

Hsp27 antibody titres were also significantly higher in the patients diagnosed with myocardial infarction compared with patients with unstable angina in the first samples taken ($P < 0.05$, Table 2). Anti-Hsp27 titres were also significantly higher in the first 12-h sample compared with second 12-h sample for either the entire patient group, or in the individual subgroups of myocardial infarction and unstable angina ($P < 0.001$, Table 2). However, Hsp27 antibody titres in the second 12-h samples did not differ significantly between healthy controls and the combined patient group with chest pain, nor for the individual subgroups of patients ($P > 0.05$, Table 2).

Table 2 Comparison of serum anti-Hsp27 titres (absorbance units) in patients and controls

Group		Controls	Patients MI	UA	Combined ACS
Anti- Hsp27 Antibody titres	First 12 h	0.08 (0.05–0.15)	0.41 (0.28–0.57)*,***	0.31 (0.20–0.42)***	0.35 (0.26–0.48)***
	Second 12 h		0.09 (0.04–0.17)†	0.07 (0.03–0.15)†	0.09 (0.04–0.16)†

MI, myocardial infarction; UA, unstable angina; ACS, acute coronary syndrome. Values are expressed as median and interquartile range. Between-group comparisons were assessed by Mann–Whitney test (for two independent groups), Wilcoxon test (for two related groups) and Kruskal–Wallis test (for≥3 groups) as data were not normally distributed.
***$P < 0.001$ comparison between patients and controls; *$P < 0.05$ comparison between MI and UA subgroups of patients with ACS; † $P < 0.001$ comparison between patients' first and second 12 h samples.

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Table 3 Correlations (*r*) between serum anti-Hsp27 and individual coronary risk factors

Group	Patients	Controls
Age	0.024	−0.034
BMI	0.140	−0.265**
Waist/hip ratio	0.154	0.001
Systolic blood pressure	−0.027	0.024
Diastolic blood pressure	−0.029	0.036
Fasting blood sugar	−0.021	−0.202
Total cholesterol	0.102	−0.058
Triglycerides	0.094	0.211*
HDL-C	−0.051	−0.167
LDL-C	0.039	−0.073

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Correlations were assessed using Spearman correlation coefficients (* $P < 0.05$, ** $P < 0.01$).

Correlation between CHD risk factors and anti-Hsp27 titres

Univariate analysis between serum anti-Hsp27 titres and individual coronary risk factors showed that serum Hsp27 antibody titres were significantly related to BMI ($P < 0.01$, Table 3) and serum triglycerides ($P < 0.05$, Table 3) in the control group. In the patient group, antibody titres to Hsp27 did not associate with any of the conventional risk factors on univariate analysis ($P > 0.05$, Table 3).

Discussion

Several studies have shown that Hsp27 is over expressed in cardiac myocytes following ischaemia–reperfusion, and may thereby be playing a cardioprotective role (Bluhm *et al.* 1998; Yamboliev *et al.* 2000; Vander Heide 2002). The concentration of Hsp27 in serum is reported to be decreased in patients with atherosclerosis, and it has been suggested that Hsp27 may be a potential biomarker of atherosclerosis (Martin-Ventura *et al.* 2004, 2006).

The present study was undertaken to determine whether serum Hsp27 antibody titres are different in patients with acute coronary syndrome (myocardial infarction and unstable angina) and whether they change following an acute clinical event. Titres were found to be raised in the first 12 h after the onset of acute coronary syndrome but decreased to normal in the second 12 h. There has been one previous report on the changes in serum Hsp27 antigen following acute coronary syndrome, in which it was found that there was an increase in serum Hsp27 ($P < 0.01$) and Hsp70 ($P < 0.01$) in patients with acute coronary syndrome (Park *et al.* 2006). There was no significant difference between serum Hsp27 level in subjects with coronary artery disease risk factors and healthy individuals ($P > 0.05$), although the number of patients recruited to their study was small. Serum Hsp70 has also been reported to increase after myocardial infarction ($P < 0.01$) and to remain elevated for about 24 h. This again was a study in a small population and furthermore the patients probably incurred relatively small myocardial infarcts because of the early institution of intensive intervention (all patients had undergone primary percutaneous coronary intervention, with thrombolysis, or conservative treatment) (Dybdahl *et al.* 2005). The time course of Hsp release has also been studied in a rat heart model of myocardial infarction that has shown that serum Hsp60 concentrations begin to increase after 6 h after the induction of a myocardial infarction, reaching a peak at 24 h and decreasing thereafter (Schett *et al.* 1999). These changes in Hsp60 concentrations were accompanied by a decrease in serum anti-Hsp65, which is consistent with other reports (Birnie *et al.* 1994; Hoppichler *et al.* 1996).

We have found that serum Hsp27 antibody titres are high in patients with chest pain who have suffered an acute myocardial infarction or have unstable angina, being higher in the former, and possibly related to the extent of myocardial ischaemia or necrosis. Hsp27 antibody titres then appear to fall to near normal levels after about 12 h post-event, whilst other markers of myocardial injury (cardiac Troponin I and T, and CK-MB) remain elevated for longer periods (Achar *et al.* 2005). The fall in Hsp27 antibody titres beyond 12 h post event may be due to the formation of immune complexes between anti-Hsp27 and Hsp27 antigen that may be released from necrotic myocardial tissue. These complexes are then probably cleared rapidly via Fc-receptor mediated uptake by cells of the reticuloendothelial system as has been previously proposed for Hsp65 antibodies following a myocardial infarction (Schett *et al.* 1999).

The high Hsp27 antibody titres in patients with unstable angina with acute chest pain, could be attributed to myocardial ischaemia, in the absence of frank necrosis (Wu & Clive 1997; Tanonaka *et al.* 2001).

In conclusion we have established an assay for measuring serum Hsp27 antibody titres and have found these to be high in the first 12 h after the onset of an acute coronary syndrome, whether myocardial infarction or unstable angina. The rapid fall in serum Hsp27 antibody titres is unlike some of the other established markers of cardiac ischaemia (e.g. cardiac troponin I and CK-MB). Whilst Hsp27 is not specific to the myocardium, changes in Hsp27 antibody titres may an indicator of acute myocardial ischaemia and may be

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useful in the evaluation of recurrent acute cardiac events (e.g. reinfarctions).

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