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Heat shock protein 70 and antibodies to heat shock protein 60 are associated with cerebrovascular atherosclerosis

Ružica Galović [a,*], Zlata Flegar-Meštrić [b], Vinko Vidjak [c], Mirela Matokanović [d], Karmela Barišić [d]

[a] University Hospital Centre Zagreb, Clinical Department of Laboratory Diagnostics, Kišpatićeva 12, 10000 Zagreb, Croatia
[b] University Hospital Merkur, Institute of Clinical Chemistry and Laboratory Medicine, Zajceva 19, 10000 Zagreb, Croatia
[c] University Hospital Merkur, Institute of Radiology, Zajceva 19, 10000 Zagreb, Croatia
[d] University of Zagreb, Faculty of Pharmacy and Biochemistry, Dept. of Medical Biochemistry and Hematology, Domagojeva 2, 10000 Zagreb, Croatia

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ABSTRACT

Objectives: Heat shock proteins (Hsps) are produced by all cells, including vascular, to ensure stress protection. Damaged cells release Hsps in their local environment and systemic circulation. The aim of this study was to investigate the involvement and prognostic utility of serum Hsp60 and Hsp70, and the respective antibodies anti-Hsp60 and anti-Hsp70 in subjects with advanced atherosclerosis resulting in high degree of cerebrovascular stenosis.

Design and methods: Ultrasound Doppler examination of carotid arteries was used to discriminate between control and cerebrovascular atherosclerosis subjects. Twenty eight subjects without carotid obstruction were selected as controls. Fifty patients with obstruction of cerebrovascular blood flow were evaluated for the degree of stenosis of cerebral arteries by digital subtraction angiography. In parallel, serum concentrations of Hsp60, Hsp70, anti-Hsp60 and anti-Hsp70 were measured by ELISA kits.

Results: Anti-Hsp60 was significantly higher ($P = 0.003$) in the atherosclerosis group than in the control group (23.62 ng/L vs. 15.28 ng/L, respectively, expressed as median). Circulating Hsp70 was lower in the atherosclerosis than in the control group ($P = 0.048$), with respective median values of 0.00 μg/L vs. 0.22 μg/L. Concentrations of Hsp60 and anti-Hsp70 did not differ significantly between the control and atherosclerosis group.

Conclusions: Higher circulating anti-Hsp60 is associated with advanced cerebrovascular atherosclerosis as a consequence of the autoimmunity part of the inflammation and bursting of atherosclerosis. Higher levels of Hsp70 observed in the control group could be protective in the development of atherosclerotic changes.

1. Introduction

In the pathogenesis of atherosclerosis, both inflammatory and immune components seem to be involved [1,2]. The inflammatory component of atherosclerosis might involve immune reactivity to heat shock proteins [2]. As a part of atherogenesis, vascular cells and tissues are exposed to various unfavorable conditions such are mechanical injury, oxidized LDL, inflammation and cytokine stimulation. Under these conditions, heat shock proteins (Hsps) are produced by the affected cells to ensure their protection [3]. Members of Hsp70 family and Hsp60 family assist in correct folding of newly synthesized or stress denatured proteins, refolding of misfolded and aggregated proteins, membrane translocation of organellar and secretory proteins, and control of the activity of regulatory proteins [4,5]. Antibodies against microbial Hsp60 family were previously linked to atherosclerosis due to their cross-reacting abilities [6,7], but autoantibodies against Hsp60 developed by other, non-infectious inducers, are found to be even stronger contributors to endothelial cell damage [8,9].

Both Hsps and their antibodies are in certain concentrations normally present in peripheral circulation, but they are abundantly released during arterial cell damage and destruction. Several studies have indicated possible association of Hsp60, Hsp70, anti-Hsp60 and anti-Hsp70 with the pathogenesis, progression, severity and prognosis of atherosclerosis or cardiovascular diseases [8–14]. Literature data regarding serum levels of Hsp60, Hsp70 and respective antibodies are variable depending on type and stage (acute phase, chronicity) of vascular disease. Here, we tried to elucidate an additional segment of this disease by examining serum levels of Hsp60, Hsp70 and respective antibodies in atherosclerotic patients with established stenosis of cerebral arteries.

2. Subjects and methods

2.1. Subjects

The study group included 50 patients, 15 women and 35 men, with symptoms of cerebrovascular insufficiency and stenosis of carotid artery

* Corresponding author at: University Hospital Centre Zagreb, Department of Laboratory Diagnostics, Kišpatićeva 12, 10000 Zagreb, Croatia.
E-mail address: rgalovic@kbc-zagreb.hr (R. Galović).

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of more than 50% of the lumen. The presence of manifest coronary atherosclerosis was excluded by physical and electrocardiographic examination. After duplex ultrasound examination, the degree of carotid artery stenosis at angiography was measured according to standard method [15].

The control group consisted of 28 people, 15 women and 13 men, with normal Doppler examination of carotid arteries and excluded coronary atherosclerosis by physical and electrocardiographic examination. They did not have a history of diabetes mellitus and were not under therapy with statins.

All participants were interviewed, data on diabetes mellitus, drug consummation and smoking were recorded. Smokers were defined as those reporting daily smoking. Obesity was defined in terms of patient's body mass index (BMI) and the patients with a BMI ≥ 25 were considered overweight [16]. Informed consent was obtained from all subjects according to the guidelines of the Ethics Committee of the Merkur University Hospital.

2.2. Methods

Serum concentrations of Hsp60, Hsp70, anti-Hsp60 and anti-Hsp70 were determined from sera which were fresh frozen on −80 °C, using commercially available ELISA kits (Stressgen Assay Designs, USA: *Hsp60 ELISA Kit*, *Hsp70 High Sensitivity EIA Kit*, *Anti-Human Hsp60* (*total*) *ELISA Kit*, and *Anti-Human Hsp70* (*total*) *ELISA Kit*).

All measurements concerning metabolic status (total cholesterol, HDL and LDL cholesterol, triacylglycerol) and C-reactive protein (CRP) were performed using routine clinical laboratory methods accredited according to ISO 15189, using fresh sera. Subjects with CRP values above reference interval (>10 mg/L) were not included in any of the study groups because of the possible presence of an active inflammatory disease [16].

Data sets were described using non-parametric presentation. Significance of the differences between the groups was determined using Mann–Whitney U-test. The X^2 significance test was used to analyze qualitative variables. Correlations were analyzed by Spearman correlation coefficient. All P values presented are two-tailed, and P values below 0.050 were considered to reflect statistically significant differences. Analyses were performed using MedCalc Software 11.4.4 (Mariakerke, Belgium).

3. Results

The general characteristics of the study groups are presented in Table 1. The groups differ significantly regarding age, but could be considered as age relevant because the influence of age on the main tested parameters was excluded by correlation testing (Hsp60, Hsp70, anti-Hsp60, anti-Hsp70; $P > 0.050$ for all of the correlation coefficients, results presented in Table 2). As expected, the groups differ significantly regarding statin therapy, as well as diabetes mellitus, but those were included in the selection criteria for the groups. Statin therapy and diabetes mellitus did not influence main tested parameters (Table 2). The groups were not different regarding other general characteristics (gender, obesity, and smoking habits).

In the present study, Hsp60 levels were not different between the tested groups, but the difference was observed for anti-Hsp60, which was markedly higher in the cerebrovascular atherosclerosis group than in the control group (Table 3). Circulating Hsp70 was lower in the cerebrovascular atherosclerosis group than in the control group (Table 3). There was no significant difference in anti-Hsp70 levels. Circulating Hsp60 was detected in 68% of all subjects (74% in patients with cerebrovascular atherosclerosis and 54% of the control subjects), and Hsp70 in 40% of all subjects (32% in patients with cerebrovascular atherosclerosis and 60% of the control subjects). Anti-Hsp60 and anti-Hsp70 antibodies were detected in all of the subjects. Serum levels of Hsp60, Hsp70, anti-Hsp60 and anti-Hsp70 were independent from age, diabetes and statin therapy (Table 2). Distributions of proportions across the quartiles were examined for Hsps and respective antibodies. There was no difference in distribution of proportions for Hsp60. The proportions of subjects of the tested groups were differently distributed regarding anti-Hsp60 antibody ($X^2 = 26.5$, $P < 0.0001$). Higher proportions of subjects from the control group could be found below the median value, in the first and the second quartile, whereas the proportion of the cerebrovascular atherosclerosis patients dominates above the median value, within the third and the fourth quartile (Fig. 1). The proportions of subjects of the tested groups were also differently distributed across the quartiles of whole population regarding Hsp70 ($X^2 = 12.1$, $P = 0.0005$), concerning the third quartile of the whole subject population as a layout (Fig. 2). No differences in distribution of proportions across the quartiles were found for anti-Hsp70.

Comparing the results obtained for the metabolic risk factors (total cholesterol, HDL and LDL cholesterol, triacylglycerol) between the group of patients with cerebrovascular atherosclerosis and the control group, significant differences were found for total cholesterol, HDL cholesterol and LDL cholesterol; all were lower in the group of patients

Table 1
Anthropometric and biochemical parameters for the control and cerebrovascular atherosclerosis group of patients (>50% stenosis of cerebral arteries).

	Control group ($n = 28$)	Cerebrovascular atherosclerosis group ($n = 50$)	P
Age (years; median, range)	58.0 (50–65)	66.5 (55–83)	<0.001*
Gender, male (n; %)	13 (46%)	35 (70%)	0.073
Obesity, BMI >25 kg/m^2 (n; %)	17 (61%)	32 (64%)	0.965
Statin therapy (n; %)	0	37 (74%)	<0.001*
Diabetes mellitus (n; %)	0	19 (38%)	<0.001*
Smoking (n; %)	5 (18%)	11 (22%)	0.887
Triacylglycerol (mmol/L; median, IQR[a])	1.4 (1.1–1.7)	1.5 (1.1–2.0)	0.300
Total cholesterol (mmol/L; median, IQR[a])	6.3 (5.9–6.8)	4.8 (3.8–5.9)	<0.001*
HDL-cholesterol (mmol/L; median, IQR[a])	1.5 (1.4–2.0)	1.3 (1.1–1.5)	<0.001*
LDL-cholesterol (mmol/L; median, IQR[a])	4.0 (3.4–4.4)	2.7 (2.0–3.7)	<0.001*
CRP (mg/L; median, IQR[a])	1.9 (1.2–2.7)	2.6 (1.4–4.6)	0.085

[a] IQR = interquartile range.
* Statistically significant difference between the control group and the cerebrovascular atherosclerosis group ($P < 0.050$).

Table 2
Results of Spearman correlation testing for relationships between age, statin therapy or diabetes mellitus and tested parameters in serum of all subjects.

Test	Age		Statin therapy		Diabetes mellitus	
	r	P	R	P	r	P
Hsp60	0.069	0.546	−0.088	0.447	−0.158	0.169
Hsp70	−0.125	0.273	−0.082	0.473	−0.148	0.195
Anti-Hsp60	0.150	0.210	0.209	0.098	0.174	0.126
Anti-Hsp70	0.187	0.100	0.050	0.664	−0.047	0.679

Significant correlations are considered with $P < 0.050$.

Table 3
Values of Hsp60, Hsp70, anti-Hsp60 and anti-Hsp70 in sera of control examinees and patients with cerebrovascular atherosclerosis.

	Control group ($n = 28$)	Cerebrovascular atherosclerosis group ($n = 50$)	
Tested parameter	Median (IQR[a])	Median (IQR[a])	P
Hsp60 (μg/L)	14.24 (0.00–31.81)	13.78 (0.00–31.84)	0.673
Hsp70 (μg/L)	0.22 (0.00–1.27)	0.00 (0.00–0.25)	0.048*
Anti-Hsp60 (ng/L)	15.28 (10.05–22.48)	23.62 (17.84–34.93)	0.003*
Anti-Hsp70 (ng/L)	83.49 (61.84–114.10)	83.42 (64.91–123.99)	1.000

[a] IQR = interquartile range.
* Statistically significant difference between the control group and the cerebrovascular atherosclerosis group ($P < 0.050$).