Heat shock proteins in cardiosurgery patients

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Abstract

Objective: Cytoplasmic members of the heat shock protein HSP70, family, inducible HSP72 and constitutive HSC73, are known to protect cells and organisms against harmful factors including ischemia, trauma, etc. The up-regulation of HSP70 was shown to greatly increase resistance of myocardial cells in vitro as well as in transgenic animals. It seems reasonable to expect that in patients undergoing open heart surgery cytoplasmic HSP70 should play a protective role, reducing the risk of the myocardial cell injury.

Methods: Using Western blotting, we determined levels of HSP72 and HSC73 in myocardium and peripheral blood lymphocytes of 51 patients with coronary and valvular diseases. In all the cases, HSP70 was detected in samples of the right atria before and after cardiopulmonary bypass.

Results: Induction of HSP72 was observed in 40% of all patients and correlated with the endurance of cardiopulmonary bypass and with disease duration in 33 patients with coronary artery disease. The cardioprotective effect of the elevated pre-operational level of HSP72 was shown to correlate with the lower activity of cardiospecific enzymes in the coronary disease patients. The HSC73 level in the right atria did not depend on conditions of the open heart surgery, while in some cases, it was increased after bypass. No correlation has been found between pre-operational content of HSP72/HSC73 in lymphocytes and its pre- or post-bypass content in myocardium.

Conclusion: HSP72 is implicated in cardioprotection in combination with some other factors, and its pre-operational level, among other parameters, might be of prognostic value.

Keywords: Heat shock proteins; Myocardial ischemia; Myocardial reperfusion injury; Cardiopulmonary bypass

1. Introduction

The search for appropriate factors providing protection of myocardium during open heart surgery remains one of the fundamental problems for establishment of the pre-operative and intraoperative cure strategy [1]. It is well known that the major stress-protein HSP70 plays an important role in cytoprotection of many types of cells against different stresses by preventing denaturation, or by enhancing proper assembly, of cellular proteins and structures [2,3]. The mammalian HSP70 family consists of four proteins: mitochondrial HSP75, endoplasmic HSP78, and two cytoplasmic proteins: constitutive HSC73 and inducible HSP72. The latter two have similar structures and properties, and both of them bind misfolded, newly synthesized polypeptides in the ATP-dependent manner, which reflects their chaperonic activity [4]. It is suggested that HSP72 and HSC73 can substitute each other in normal cells, whereas HSP72 expression is necessary for a cell response to some cytotoxic factor(s). The physiological role of HSP70 family proteins is well documented. In most cases these proteins were found to protect cells against factors inducing cell injury and leading to necrosis or apoptosis. Recent reports have demonstrated that in experimental animals the HSP70 expression can be stimulated by ischemia [5]. On the other hand, up-regulation of HSP70 in cells by gene transfer was shown to greatly increase resistance of myocardial cells in vitro and in transgenic animals [6,7]. Therefore, in the coronary disease patients subjected to open heart surgery, cytoplasmic HSP70 may be expected to play a protective role, reducing the risk of the myocardial cell injury.

The recent review [8] supports the suggestion that induction of the HSP70 expression in myocardium provides the ‘second window’ of protection in the heart pre-conditioning. Although this problem is of great importance, only a few papers deal with this objective, most of them reporting rather contradictory results [9–11].

We determined the HSC73 and HSP72 contents in the
right atria of patients undergoing open heart surgery and analyzed correlation between HSP70 levels in myocardium and the activity of cardiospecific enzymes in blood; the latter are widely used as markers of the cardiomyocyte alteration.

Although the HSP70 content in the heart tissue might be of a prognostic value, this approach hardly is realistic for the use in future. We supposed that changes in the HSP70 content could be associated with genetic variations in a given human population [12]. Therefore, we expected that the analysis of the protein amount in lymphocytes taken from the patients would allow us to define the level of HSP70 expression in the whole organism and possibly to correlate this level with that in myocardial cells. In the present paper we report results of estimation of HSP72 and HSC73 levels in lymphocytes in comparison to those in myocardium biopsies.

2. Methods

2.1. Patients

Using a protocol approved by the Scientific Forum at the Military Medical Academy, St. Petersburg, Russia, we had obtained informed consent from all patients before they were included in the study. Examined were 51 patients that underwent open heart surgery with cardiopulmonary bypass for the congenital, acquired or coronary disease from February 1997 to May 1997. Epidemiological data are presented in Table 1. Anesthesia was the same for each patient. Cardiopulmonary bypass was established with 2:1 blood/crystalloid ratio at a flow rate from 2 to 4 l/min. All patients were cooled to 28±29°C (rectal). In addition, there was aortic crossclamping, with a myocardial arrest induced by antegrade infusion of a cold, high potassium, cardioplegic solution. Subsequent infusions of the cardioplegic solution with lower levels of potassium were repeated every 20 min.

The first biopsy of the right atria was obtained after pericardium had been opened, before any surgical manipulation of the heart. The second biopsy specimen was taken from the same place of the right atria after the end of the cardiopulmonary bypass. The specimens were frozen in liquid nitrogen and stored at −70°C until analyzed.

Lymphocytes were isolated from the blood sample before the operation by centrifugation in a Ficoll-Verografin gradient for 30 min at 1500 rev./min in a clinical centrifuge. Then the lymphocytic fraction was collected and washed in a phosphate-buffered saline solution. Lymphocytes also were frozen in liquid nitrogen and stored at −70°C until analyzed.

The frozen myocardium samples and lymphocytes were homogenized in 10 volumes of lysing buffer (sodium chloride, 20 mmol/l; Tris-(hydroxymethyl)-aminomethane, 20 mmol/l, pH 7.5; 0.1% Triton X-100; protease inhibitors).

### Table 1

<table>
<thead>
<tr>
<th>Pre-operative data</th>
<th>Coronary disease</th>
<th>Valvular disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>33</td>
<td>18</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>52 ± 8</td>
<td>38 ± 14</td>
</tr>
<tr>
<td>Range of age (years)</td>
<td>39–70</td>
<td>7–63</td>
</tr>
<tr>
<td>Men/women</td>
<td>33</td>
<td>10/8</td>
</tr>
<tr>
<td>NYHA class (mean)</td>
<td>3.2 ± 0.5</td>
<td>7/0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intraoperative data</th>
<th>Coronary disease</th>
<th>Valvular disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cardiopulmonary bypass time (min)</td>
<td>122 ± 32</td>
<td>97 ± 28</td>
</tr>
<tr>
<td>Mean crossclamp time (min)</td>
<td>60 ± 15</td>
<td>71 ± 27</td>
</tr>
</tbody>
</table>

| a Values are expressed as mean ± SD. NYHA, New York Heart Association. |
| b Additionally two patients with atrial and ventricular septal defects were included in this group. Valvular disease etiology varied in these patients (congenital defect, rheumatic disease, endocarditis and prosthesis dysfunction). |

Protein concentrations in the samples were determined by a technique described by Bradford [13], with bovine serum albumin used as a standard.

2.2. HSP72 and HSC73 detection

One-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli [14], with 10% polyacrylamide concentration in the running gel. The first electrophoresis was performed to check the equal amount of protein per electrophoresis lane to confirm the protein concentration after staining with the solution: 0.5% Coomassie Blue R; 40% methanol; 10% acetic acid. After the second electrophoretic run, proteins were transferred to nitrocellulose membranes (0.22 μm pore size; Amersham, UK) for 2 h (0.5 mA) as described by Towbin et al. [15], using an LKB protein transfer system (Pharmacia-LKB, Uppsala, Sweden) (transfer buffer: glycine, 192 mmol/l; Tris–HCl, 25 mmol/l, pH 8.3; 0.1% SDS; 20% methanol).

After the protein transfer, the nitrocellulose membrane was blocked with 2% bovine serum albumin solution (ICN Pharmaceuticals, Inc., USA) in the phosphate-buffered saline solution. Then the blots were incubated with monoclonal antibodies: N69 for HSC73 and 2H9 for HSP72 [16] in the phosphate-buffered saline solution with 0.01% Tween-20 (PBTS) and 2% bovine serum albumin for 2 h. After three washes (for 5 min each) in PBTS, the blots were placed in a solution of secondary antibody (rabbit anti-mouse IgG conjugated with peroxidase (Sigma, USA) at 1:4000 dilution in PBTS with 2% bovine serum albumin for 1 h. The blots were washed twice in PBTS and immersed in the buffer: 0.01% dianisobenzidine; Tris–HCl, 100 mmol/l, pH 7.5; imidazole, 0.02 mmol/l, oxygen peroxide, 10 mmol/l). After development, the blots were washed in...
water and dried. A pure HSC73/HSP72 probe from the skeletal bovine muscle purified as described by Welch and Feramisco [17] and used earlier for raising monoclonal antibodies to HSP70 was applied in three different loadings as a calibration protein in blots and for comparison of levels of HSP70 in human tissues. Bands from immunoblots were scanned with a Plug-n-Scan 600 II SP scanner (Mustek, USA) and analyzed by a computer program for blot and gel analysis created by A. Yu. Lyangusov. Levels of HSC73 and HSP72 were defined as optional units (OU) which reflect intensity and area of the band.

2.3. Cardiospecific enzyme detection

The levels of aspartate aminotransferase, lactate dehydrogenase, creatine phosphokinase and its MB fraction were determined in blood samples taken at the 1st, 2nd, 3rd, 5th, and 7th days after operation, using an Abbott spectrum biochemical analyzer (Abbott Laboratories, USA).

2.4. Statistical analysis

Values are presented as means ± standard errors of the mean. To detect differences between pre-bypass and post-bypass HSP70 levels, Wilcoxon non-parametric signed-rank test was used. Student’s t-test and Mann–Whitney U-test were performed to evaluate differences between the groups of patients. Spearman rank correlation was used to estimate correlations between different variables. The $P < 0.05$ value was considered the statistically significant difference.

3. Results

Analysis of specificity and affinity of monoclonal antibodies to human HSC73/HSP72 showed that the monoclonal antibody N69 recognized the upper band corresponding to HSC73 from human heart and coincided with bovine HSC73 (Fig. 1). The monoclonal antibody 2H9 recognized the lower band corresponding to HSP72, the inducible member of the HSP70 family. To reveal the difference in affinity of these antibodies to their antigens, preparations of pure HSC73/HSP72 isolated from human myocardium and bovine muscle were analyzed by immunoblotting. The bands on blot and on gel stained by Coomassie Blue were scanned, and their intensities were compared. Affinity of 2H9 antibody to HSP72 was higher than affinity of N69 antibody to HSC73 (the HSC73/HSP72 ratio $= 1:5$).

Fig. 2 demonstrates Western blot with the protein samples of a group of patients; the blot was stained with 2H9 antibody. The mean initial (pre-bypass) level of HSP72 in the hearts in patients with coronary disease was 465 ± 30 OU, in patients with cardiac valve disease, 420 ± 40 OU. The initial level of constitutive HSC73 in the above groups was lower than that of inducible HSP72, 200 ± 30 and 200 ± 40 OU, respectively. Using pure HSP72/HSC73 as a standard, we estimated protein concentration in human myocardium as 1 mg for HSC73 and 3 mg for HSP72 per 1 g of total tissue protein.

Unlike the right atria, the peripheral blood lymphocytes isolated from patients before operation had almost equal amounts of HSP72 and HSC73, 290 ± 15 and 270 ± 25 OU, respectively (Fig. 3). No correlation has been revealed between the HSP72/HSC73 contents in lymphocytes and in myocardium.

An elevation of HSP72 and HSC73 contents in myocardium of patients with coronary disease after bypass was observed in 40 and in 23% of cases, respectively. The inverse correlation ($P = 0.04$) was revealed between the pre-bypass HSP72/HSC73 content and the elevated protein level after bypass. The determination of the HSP72/HSC73 content in patients with valvular disease provided similar results.

Further analysis of the HSP70 amount was focused on the group of 33 patients with coronary disease, as this group included the greater number of patients, so the dispersion of age and sex parameters as well as of diagnosis was minimal.
In patients with long-lasting coronary disease (>1 year), the HSP72 induction after cardiopulmonary bypass was higher than in patients with stenocardia or myocardium infarct diagnosed during the last year; the difference between the post-bypass and pre-bypass contents were 130 ± 10 and 85 ± 9%, respectively (P ≤ 0.007). The duration of the cardiopulmonary bypass was shown to affect the HSP72 content to a much greater extent than the aortic crossclamp time (Fig. 4). The post-bypass HSP72 levels at the time periods shorter and longer than 2 h were 430 ± 40 and 620 ± 60 OU, respectively (P ≤ 0.02). The HSP72 content and its induction after bypass did not correlate with the age of patients and with the number of previous infarcts, nor was there any correlation between all the above parameters and the HSC73 level.

All patients with coronary disease were subdivided into two groups depending on the initial HSP72 content: the 1st group had the HSP72 content higher, while the 2nd group, lower than the mean value (465 OU). The analysis of cardiospecific enzymes, aspartate aminotransferase, lactate dehydrogenase, creatine phosphokinase and its MB fraction, showed their activities to be substantially lower in the 1st than in the 2nd group (Fig. 5). The most pronounced differences between the two groups were in the CK-MB activity, 55 ± 16 and 140 ± 21 U/l, respectively, at the first post-operation day (P = 0.009) and 56 ± 14 and 135 ± 24 U/l, respectively, at the second post-operation day (P = 0.03). After the second post-operative day, no correlation was observed between the HSC73, post-bypass HSP72 contents, and the character of their induction, on one hand, and the cardiospecific enzyme activities, on the other hand.

4. Discussion

The endogenous mechanisms of cardioprotection from ischemia and reperfusion injury during open heart surgery have been investigated intensively. One of such elements of cardioprotection is inducible HSP72. A high level of HSP72 in myocardium is attributed to a smaller infarct size and to the threshold of heart tolerance to the irreversible ischemic injury in experiments on laboratory animals [18].

Perrault et al. [19] have established that the HSP70 mRNA levels rise 1.5–3 times after bypass. Our study found induction of the HSP72 protein expression only in 40% of patients with a similar difference between the post-bypass and pre-bypass levels. The lack of any observed increase in the HSP72 content in some cases may be due to different duration of surgical procedures and accordingly, to different time period between the pre-bypass and post-bypass biopsies. Thus, the post-bypass HSP72 levels were higher in patients with the cardiopulmonary bypass lasting longer than 2 h. A short period between the pre-bypass and post-bypass biopsies might be insufficient to detect the HSP72 accumulation [20].

The second cause of the lack of the HSP72 induction may be high levels of this protein and a high alignment of inducible HSP72/constitutive HSC73 in human tissues unlike those in laboratory animals [21]. Such a high level may be sufficient to protect cardiomyocytes from ischemic stress. The inverse correlation between the pre-bypass
HSP72/HSP73 content and its increased expression after bypass may indicate that the increase in HSP70 is detected only in cases when the pre-bypass HSP70 level is relatively low. Thirdly, Locke and Tanguay [22] analyzed age differences of the HSP70 expression in myocardium. The ability of cells to induce expression of HSP70 in response to stress decreased with age. Meanwhile, in our study, the mean age of the observed patients was 52 ± 8 years, and the HSP70 protein synthesis might have been modified.

To confirm the cardioprotective role of HSP70 in human, we examined correlation between the HSP70 level in the patients’ heart and the blood markers of cardiomyocyte alteration. Differences in the HSP72 level could be due to various causes such as genetic differences in HSP expression between patients, different duration and severity of disease, and other factors which also can have a role in variations of the HSP72 pre-operational level in the examined population. Inducible HSP72 has been shown to correlate inversely with blood level activities of some cardiospecific enzymes used in clinical practice as markers of myocardium injury. The lower activities of the cardiospecific enzymes in blood and, accordingly, the lower myocardium damage were found in patients with the higher rather than with the mean HSP72 level. We explain the statistically significant correlation only in the case of CK-MB by a higher cardiac specificity of this enzyme. This correlation was observed when the enzyme levels were measured in the blood samples taken at the 1st and 2nd days after operation. Activities of cardiospecific enzymes in the blood samples taken at the 3rd, 5th, and 7th days after operation had no differences, so their activities returned to normal levels. Constitutive HSC73 was not been shown to correlate with any parameters that reflect myocardium injury and, possibly, do not directly participate in the cardioprotection.

Two regularities observed in the present study are the most significant. First, the high initial myocardium level of inducible HSP72 content determines cardioprotection of the heart by HSP70 during cardiac operation. Second, the induction level of HSP72 expression basically is not a marker of cardioprotection but rather reflects severity of the cell stress caused by factors of open heart surgery.

Since no correlation between the lymphocyte and heart HSP72 levels has been found, the problem of choosing a laboratory method to detect HSP72 content outside myocardium remains so far unsolved.

Thus, together with other cell systems of protection, such as antioxidants, inducible HSP72 protein play an important role in cardioprotection from ischemia and reperfusion injury during open heart surgery. Induction of the HSP72 content in the myocardium before cardiac operation may be the point of action and a marker of efficacy of the heart pre-conditioning. The induction of constitutive HSC73 in myocardium possibly indicates its own specific role in myocardium recovery after surgery stress.

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