Cellular autoimmunity to cardiac myosin in patients with a recent myocardial infarction

Miriam Moraru, Arie Roth, Gad Keren, Jacob George*

Department of Cardiology, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel
Affiliated to the Tel Aviv University, Sackler School of Medicine, Tel Aviv, Israel

Received 21 November 2004; received in revised form 7 February 2005; accepted 19 February 2005
Available online 27 April 2005

Abstract

After a large myocardial infarction (MI) a progressive remodeling process can occur that results in a severe mechanical dysfunction of the heart. Among the determinants of remodeling, immune mediated inflammation has been suggested as an important contributor. However, the role of autoimmunity to cardiac antigens that are released to the circulation has not been sufficiently addressed. Cardiac myosin is a contractile protein that is unique to the myocardium and has been shown to induce a humoral immune response in patients after MI, and to trigger an autoimmune myocarditis in experimental rats and mice. In the current study, we evaluated humoral and cellular immune responses to myosin in patients with and without a history of recent MI.

Eighteen patients with MI, 2 weeks to 4 months prior to initiation of the study, and eighteen control subjects were enrolled. Peripheral blood mononuclear cells (PMBC) were obtained and subjected to priming with different concentrations of cardiac myosin. Interferon-gamma and TNF-alpha were measured in conditioned medium obtained from the cultured PMBC upon priming with cardiac myosin. Humoral immune responses were also assessed by evaluating IgG anti-myosin antibodies.

We have found that a third of the patients with the recent history of MI and none of the control subjects had a proliferative response to cardiac myosin evident by stimulation indices greater than 1.5. No differences were detected between the patients and controls with regard to IFN-gamma and TNF-alpha secreted by their PMBC, nor were there differences in the serum levels of IgG anti-myosin antibodies.

Thus, in patients with a recent history of MI, cellular autoimmunity to cardiac myosin is present as compared with controls. It remains to be determined whether this autoimmune response is associated with an adverse outcome.

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Keywords: Myocardial infarction; Cellular immunity; Myosin; Lymphocyte

1. Introduction

Functional deterioration after myocardial infarction progresses beyond the acute ischemic event. This process, which is mediated in part by activation of immune and neurohumoral pathways, culminates in adaptive changes in ventricular size, shape and function, and is known as left ventricular remodeling [1,2].

Myocardial infarction triggers cellular migration into the infarct zone, which includes macrophages, monocytes and neutrophils, that assemble to produce and secrete cytokines and growth factors, such as: TNF-α, INF-γ, IL-2, IL-6 [2–6].

Following myocardial necrosis, intracellular sequestered cardiac antigens are released to the blood stream. The exposure to the immune system of these previously ‘concealed’ molecules, that may have been modified in the infarct milieu, could potentially trigger autoimmune responses evident by the production of ‘anti-heart antibodies’, and also by the activation of antigen-sensitized cytotoxic T lymphocytes [7,8].
Myosin, one of the contractile proteins of the sarco-
meres, is released from necrotic myocytes to the blood following acute myocardial infarction (AMI), and is thus may be considered as a candidate autoantigen against which an immune reaction is expected to develop. Indeed, there is evidence supporting the role of cardiac myosin as an important antigen in the initiation of inflammatory processes of a number of cardiac disorders, such as myocarditis, rheumatic heart disease and post-pericardiody-
tomy syndrome [2,9–11]. These observations are further supported by experimental animal models that confirm the occurrence of myocardial inflammation following immu-
nization against cardiac myosin [12–14].

The role of T cells in the pathogenesis of experimental autoimmune myocarditis has been demonstrated by employing adoptive transfer studies in which the disorder was transferred by antigen-sensitized lymphocytes [15,16]. The possible association between autoimmune to myocardial antigens and myocardial inflammation following MI was suggested by Maisel et al. [17]. The authors demonstrated that transfer of splenic lymphocytes from rats after myocardial infarction led to a T cell-mediated autoimmune myocarditis in the healthy syngeneic rats.

In the present study, we tested the hypothesis that cellular autoimmune response to cardiac myosin is present in a subset of patients after myocardial infarction. For this purpose, patients with recent myocardial infarction were compared to subjects with ischemic heart disease, assessing the proliferative reactivity of their lymphocytes to the cardiac myosin.

2. Materials and methods

2.1. Study population

Two groups of patients were selected:

Group A: 18 patients that have suffered myocardial infarction in the recent past from 2 weeks up to 4 months prior to the blood sampling. In all patients peak CPK were at least 1000 IU and all experienced MI associated chest pain at least for 4 h. All patients underwent primary PTCA and none received prior thrombolytic therapy. In 12 patients, the culprit artery was patent and subsequently dilated and stented whereas the six additional patients, total occlusion was evident and the artery was success-
fully opened. All patients received either epifibatide or tirofiban initiated during the PTCA and continued for 24 h.

Group B: 18 controls without a history of ACS in the recent past up to one year prior to the study.

The severity of myocardial injury was assessed by myocardial enzyme release (peak CPK) and by left ventricular function (echocardiography and catheter ventri-
culography). Patients with disorders associated with immune suppression or those taking immunosuppressive drugs were excluded from the study.

2.2. Separation of peripheral blood mononuclear cells (PBMCs) from the whole blood

Peripheral venous heparinized blood samples were diluted 1:2 with PBS (Phosphate Buffer Saline), gently layered over a ficoll-gradient and centrifuged at 2000 rpm, PBMCs were collected, washed twice with PBS, and resuspended at a density of 1 × 10^6 cells/ml in RPMI. Serum from patients and controls was also collected and frozen until further use.

2.3. Lymphocyte proliferation assay

PBMCs (100 μl of suspension 1 × 10^6 cells/ml) from patients and controls were incubated in triplicate wells with purified porcine cardiac myosin (sigma) at different final concentrations (1, 2.5, 5 μg/ml), and also with RPMI and PHA (100 μl/well of each substrate), at 37 °C. On the 6th day of incubation the culture PMBC were pulsed with radioactive thymidine and incubated for an additional 24 h. Thymidine incorporation was measured using a β-counter.

2.4. Cytokine levels in the conditioned media

PBMCs (100 μl of suspension 0.5 × 10^6 cells/ml) from patients and controls were incubated in triplicate wells with purified cardiac myosin (2.5 μg/ml), and also with RPMI and PHA, at 37 °C, for 3 days. At the end of the 3rd day of incubation, supernatants from stimulated cultures of PBMCs were collected for the measurement of TNF-α and INF-γ using Duoset ELISA kits (R and D Systems) according to the manufacturer’s instruction.

2.5. Assessment of serum anti-myosin antibody levels by ELISA

Plates were coated overnight at 4 °C with human cardiac myosin (10 μg/ml). Next, plates were washed 3 times with a solution containing 0.05% TWEEN 20 in PBS at pH=7.4, and then blocked with 1.5% BSA in PBS for 2 h at room temperature. After another series of washings, samples of diluted serum 1:30 (in diluent solution 0.15% BSA in TBS) from patients and controls were added to the plates and incubated on a shaker for 2 h at room temperature. Following washings, alkaline phos-
phate conjugated detection antibody in a diluent solution was added to the plates for 1 h at room temperature. Next, the plates were washed and the substrate pNPP in NaHCO₃+MgCl₂ at pH=9.6 was added for 30 min. Absorbance was determined using an ELISA-plate reader at 450 nm.
2.6. Statistical analysis

We used double-tailed Student’s $t$ test in order to determine differences between continuous variables and Chi square test to sample differences between patient and control groups. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Study population

Table 1 presents a comparison of the demographic data and risk factors of our patient population versus the control group. Regarding the risk factors, no significant differences in the occurrence of hypertension, hyperlipidemia and smoking were noted, while the frequency of diabetes mellitus was twice as higher in the control group as in the patient group (33.3% versus 16.7%). Also, the incidence of positive family history of ischemic heart disease (IHD) was higher among our controls than among our patients (27.8% versus 16.7%).

3.2. Cellular immune responses to cardiac myosin

In order to compare the extent of lymphocyte proliferation, stimulation indices (SI) were employed, calculated as the average total counts per minute of antigen-stimulated cells divided by the average total counts per minute of unstimulated cells.

A cut-off point was chosen at SI = 1.5 in order to define responder patients. These patients were regarded so if their

Table 1
Clinical data, risk factors and medication of the study population

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Patients ($n = 18$)</th>
<th>Controls ($n = 18$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.61±2.179</td>
<td>60.16±2.607</td>
<td></td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>22%/78%</td>
<td>28%/72%</td>
</tr>
<tr>
<td>Risk factors:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTN</td>
<td>61.11%</td>
<td>72.22%</td>
</tr>
<tr>
<td>HL</td>
<td>72.22%</td>
<td>66.66%</td>
</tr>
<tr>
<td>DM</td>
<td>16.66%</td>
<td>33.33%</td>
</tr>
<tr>
<td>Smoking</td>
<td>61.11%</td>
<td>55.55%</td>
</tr>
<tr>
<td>Family history of IHD</td>
<td>16.66%</td>
<td>27.77%</td>
</tr>
<tr>
<td>Beta-blockers</td>
<td>27.77%</td>
<td>50%</td>
</tr>
<tr>
<td>Ca antagonist</td>
<td>11.11%</td>
<td>16.66%</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>33.33%</td>
<td>38.88%</td>
</tr>
<tr>
<td>Hypoglycemias</td>
<td>16.66%</td>
<td>33.33%</td>
</tr>
<tr>
<td>Statins</td>
<td>33.33%</td>
<td>38.88%</td>
</tr>
<tr>
<td>Other</td>
<td>44.44%</td>
<td>88.88%</td>
</tr>
</tbody>
</table>

Table 2
Stimulation indices of lymphocyte proliferation to myosin (concentration: 2.5 μg/ml) in patients versus controls

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>SI</th>
<th>Control no.</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.9</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>0.7</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
<td>4</td>
<td>2.2</td>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>5</td>
<td>1.2</td>
</tr>
<tr>
<td>6</td>
<td>0.6</td>
<td>6</td>
<td>1.4</td>
</tr>
<tr>
<td>7</td>
<td>0.6</td>
<td>7</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>2.8</td>
<td>8</td>
<td>0.9</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>9</td>
<td>0.3</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>10</td>
<td>1.4</td>
</tr>
<tr>
<td>11</td>
<td>1.3</td>
<td>11</td>
<td>0.9</td>
</tr>
<tr>
<td>12</td>
<td>0.8</td>
<td>12</td>
<td>0.5</td>
</tr>
<tr>
<td>13</td>
<td>0.5</td>
<td>13</td>
<td>0.5</td>
</tr>
<tr>
<td>14</td>
<td>3.7</td>
<td>14</td>
<td>1.4</td>
</tr>
<tr>
<td>15</td>
<td>0.5</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>0.2</td>
<td>16</td>
<td>0.7</td>
</tr>
<tr>
<td>17</td>
<td>0.2</td>
<td>17</td>
<td>0.1</td>
</tr>
<tr>
<td>18</td>
<td>0.5</td>
<td>18</td>
<td>0.1</td>
</tr>
<tr>
<td>Average</td>
<td>1.194±0.2299</td>
<td>0.7722±0.09928</td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>6 (33.33%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Proliferative response of PMBC to cardiac myosin. Lymphocytes were cultured with cardiac myosin at different concentrations (1, 2.5 and 5 μg/ml, respectively), and the stimulation indices were calculated.
PMBC responded to cardiac myosin by an SI ≥ 1.5, as opposed to non-responder patients (those with SI < 1.5) (Table 2).

Six of our 18 patients (33%) following MI exhibited a significant cellular autoimmune response to cardiac myosin. PBMCs were incubated with cardiac myosin at different concentrations (1, 2.5 and 5 μg/ml), yet this did not influence lymphocyte proliferation with a mean number of responders of ~ 30% among the patients as compared to none of the control subjects (p < 0.05) (Fig. 1).

We investigated a possible correlation between the cellular reactivity of the responders and the severity of myocardial injury, as reflected by their peak CPK level, but no such relationship was found (data not shown). Likely, no significant association was evident between the SI values and the time interval from the occurrence of MI to the time of blood sampling where cellular reactivity was assessed.

3.3. Levels of pro-inflammatory cytokines

Levels of pro-inflammatory cytokines produced and secreted by specific antigen-sensitized lymphocytes were measured in order to evaluate the possible activation of cellular immunity. For this purpose, supernatants of cardiac myosin-stimulated cultures of PBMCs were collected and levels of interferon-gamma (INF-γ) and tumor necrosis factor alpha (TNF-α) were measured. Our results reflected no significant differences between the patient and the control groups in this regard (Fig. 2).

Mean level of INF-γ of MI patients was 100.5 ± 56.41 pg/ml compared to controls 41.07 ± 27.40 pg/ml (p = 0.3571). No correlation between lymphocyte proliferation and the levels of cytokine production was established.

3.4. Anti-myosin IgG antibodies

In order to investigate generation of humoral immunity to myosin, levels of anti-myosin IgG antibodies were evaluated. No significant differences were evident between patients (OD: 14.22 ± 1.594) and controls (OD: 14.33 ± 1.535, p = 0.9603) (Fig. 3).

4. Discussion

Activation of the immune system constitutes one of the pathways to post-infarct myocardial injury, triggering ventricular remodeling that culminates in myocardial dysfunction [2]. When myocardial necrosis evolves, previously sequestrated cardiac antigens are exposed and presented to the immune system, triggering both cellular and humoral immune responses, consisting of activation of cytotoxic T cells and production of anti-heart antibodies [7,8,19–21].

Many studies investigated the antigenicity of myosin, supporting its role as a candidate offending antigen in the development of an inflammatory process in various cardiac disorders the best characterized of which is experimental myocarditis [9–11,18]. Rat models of experimental autoimmune myocarditis (EAM), an inflammatory disease of the ventricular walls, were developed by immunization with human cardiac myosin [12–14]. Adoptive transfer of autoimmune myocarditis was achieved by transferring splenic and lymph-node cells primed with cardiac myosin,
from diseased rats into healthy syngeneic rats. Studies attempting transfer of myocarditis by anti-myosin antibodies have not been consistently reproduced, thus supporting a more pronounced importance of the cellular autoimmune response in EAM [15,16]. In a similar murine model study, investigators have provided direct evidence of autoimmune myocardial injury induced by adoptive transfer of activated splenocytes after myocardial infarction [17].

Our study was designed to test the hypothesis that cellular autoimmune reactions to cardiac myosin can be detected in patients after acute myocardial infarction. This is, to our knowledge, the first study in humans addressing this question. We selected patients that had evidence of myocardial necrotic damage in the context of their ischemic event, in order to assure that sufficient systemic exposure to cardiac myosin had occurred. We limited the timing of assessment of cellular immunity to myosin to a period between 2 weeks up to 4 months after the acute myocardial event, as this matches the time period during which an inflammatory response is expected to develop. We demonstrated for the first time, evidence of cellular autoimmunity to cardiac myosin in a significant subset of patients after MI.

Unexpectedly, a more extensive myocardial injury was not associated with a stronger cellular autoimmunity to myosin, yet this may have been obscured to the relatively small number of patients and the great variability between individuals.

As one of the presumed mechanisms of post-infarction myocardial injury involves local activation of T-helper 1 lymphocytes, we assessed levels of pro-inflammatory cytokines produced and secreted by myosin-sensitized lymphocytes. No significant differences were noticed with regard to secretion of either INF-γ or TNF-α between patients and controls. However, Th1 and Th2 responses are not confined to INF but rather include a wide array of additional cytokines and therefore it is possible that a more detailed analysis of the cytokine profile would have resulted in significant changes reflecting a Th switch between the subjects and the controls.

Several studies have shown high serum titers of anti-heart antibodies in a variety of cardiac disorders [9–11,19]. We evaluated levels of serum anti-myosin IgG antibodies and found no significant difference between patient and control groups. We suggest that this finding derives from the fact that these are low affinity antibodies and a larger sample size is needed to obtain statistically significant differences.

We are aware that our study presents several limitations. The study population, consisting of 18 patients versus 18 controls, though common for this type of study design (assessing lymphocyte reactivity), represents a relative small sample. It is possible that with a larger study group, more conclusive data could be obtained. Another shortcoming of our study is the lack of clinical correlation. A long-term follow-up with clinical evaluation of cardiac function by echocardiography or ventriculography would have added valuable information concerning the association between post-infarct cellular immune responses and future outcome. Further studies are needed to clarify this issue and the significance of antigen specific cellular autoimmunity in the development of post-infarct ventricular remodeling and progression to heart failure. At this point however, there is no experimental and clinical data to support the contribution of myosin specific cellular immune responses to syndromes associated with myocardial damage.

In conclusion, this study provides for the first time, direct evidence for myosin cellular autoimmunity in humans after acute myocardial infarction, and further research will be required to elucidate the significance of these observations.

References


