Peripheral blood mononuclear cell responses to heat shock proteins and their derived synthetic peptides in juvenile idiopathic arthritis patients

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Abstract. Background: Sequence homology and cross reactivity between microbial and human heat shock proteins (hsps) led to the concept that hsps might be involved in the etiopathogenesis of autoimmune diseases. Objective: In our study we stimulated peripheral blood mononuclear cells (PBMC) of patients with juvenile idiopathic arthritis (JIA) and healthy controls with various hsp-derived peptides together with the whole molecules of corresponding hsp. Methods: PBMC were cultured with recombinant human hsp60 (rh-hsp60), rh-hsp70, Mycobacterium bovis hsp65 (M. bovis hsp65), P562–571 human hsp60, P180–188 M. bovis hsp65, P450–463 human hsp70 and P545–554 cytokeratin derived synthetic peptides. Cell responses were measured after incorporation of 3H-thymidine and expressed as stimulation indices. Results and conclusion: We found elevated proliferative response to rh-hsp60, M. bovis hsp65, Mycobacterium bovis hsp65, P562–571 human hsp60 derived peptide in patients with JIA polyarthritis. Significantly elevated proliferation to P180–188 M. bovis hsp65 was found in JIA lasting more than 2 years. None of the particular clinical characteristics (RF, ANA, HLA B27 and disease activity) seemed to be associated with hsp or hsp-derived synthetic peptide proliferative response in the JIA cohort.

Key words: Heat shock proteins – Proliferative response – Juvenile idiopathic arthritis – Hsp-derived synthetic peptides

Introduction

Juvenile idiopathic arthritis (JIA) is characterized by chronic inflammation with three distinct subtypes distinguished on the basis of clinical parameters at the onset of disease [1]: oligoarticular, polyarticular and systemic JIA. In spite of similar histopathological abnormalities in these subtypes there is a striking difference in the clinical course. Oligoarticular JIA has a relative benign clinical course, whereas polyarticular and systemic JIA are usually non-remitting [2]. The aetiology of JIA is still unknown and the pathogenetic mechanisms remain unclear.

Heat shock proteins (hsps) are essential to prokaryotic and eukaryotic cellular organisms through their chaperone function during intracellular (un) folding, assembly and translocation of proteins [3]. Their synthesis is greatly increased in response to a variety of stressful stimuli (temperature, hypoxia, irradiation, infection, inflammation) [4]. Hsps are highly conserved during evolution, which has resulted in extensive amino acid sequence identities between mammalian and microbial hsps. Such sequence homology makes microbial hsps a potential inducer of cross-reactive immune responses to host self molecules that may lead to autoimmunity [5]. In spite of the capacity of microbial hsps to induce autoimmunity through this molecular mimicry [6], immunization with microbial hsps has been shown to lead to protection in virtually all forms of experimental autoimmune arthritis. In the adjuvant-induced arthritis model, it was shown that the protection resulted from the induction of self hsp60-cross-reactive T cells capable of down-regulating inflammation [7]. In several experimental models, T cells responding to hsps play an important role in the regulation of peripheral tolerance and the suppressing pathogenic immune response [8]. New ways for immunotherapy in chronic arthritis may be found in strategies aimed at the restoring the natural regula-
tory responses in patients with JIA, through vaccination with hsp60 or peptides containing defined hsp epitopes.

The aim of our study was to investigate peripheral blood mononuclear cell proliferative responses to hsp and hsp-derived synthetic peptides in a cohort of patients with JIA and compare with healthy controls. Jones et al. [9] suggested that hsp60 might function as a recruiting antigen since the similarity between hsp60 and 19 peptides identified to be known autoantigens in various autoimmune disorders involving rheumatoid arthritis (RA) was described. Human hsp60 (562–571) shares sequence homology with cytokeratin (545–554), one of the known RA autoantigens [9]. Cross reactivity between the nonapeptide (180–188) derived from M. bovis hsp65 and cartilage proteoglycan may cause inflammation to the joints and may be involved in the pathogenic effector mechanism [10]. Finally, hsp70 derived N-terminal-extended 14-mer peptide (TKD, 450–463) was described to be able to stimulate the cytolytic and proliferative activity of NK cells equipotently with full-length hsp70 protein [11].

We investigated also an association between the proliferative response to hsp and hsp-derived synthetic peptides and clinical characteristics like rheumatoid factor (RF), antinuclear antibodies (ANA), HLA B27, the duration and the activity of the disease.

Materials and methods

Ethical and informed consent

Local ethics committee approval and informed consent from all participants in the study were obtained prior to testing.

Patients

Patients from the Outpatient Department of Rheumatology, University Hospital Motol in Prague, were involved in the study. The underlying diseases fulfilling the ILAR criteria for the diagnosis of JIA [1] were recruited consecutively. Peripheral blood heparinized samples were collected from 48 patients (16 males and 32 females) aged 7–37 years (median = 16). 7 children suffered from systemic JIA, 30 had polyarticular JIA and 11 oligoarticular JIA. 6 patients were rheumatoid factor (RF) seropositive, 9 patients had antinuclear antibodies (ANA) and 11 patients were HLA B27 positive. Clinical disease activity was assessed using the physician’s global assessment of overall disease activity and categorised as remission (n = 27) and active disease (n = 21). 10 patients in remission were without therapy. Patients were treated depending on the stage of the disease with non-steroid antirheumatics (NSAIDs), corticosteroids and/or disease modifying antirheumatics (DMARDs). 41 patients had established disease (patients with duration of the disease over 2 years).

Healthy controls

38 healthy controls (20 males and 18 females) aged 3–37 years (median = 17.5) were included in the study.

Antigens

The following antigens were used: recombinant M. bovis hsp65 (Lionex, Braunschweig, Germany); rh-hsp60 (Lionex, Braunschweig, Germany), rh-hsp70 (Stressgen Biotechnologies, Victoria, Canada) used at 1 µg/ml and PHA (Sigma Biosciences, St Louis, MO, USA) used at 5 µg/ml. All the peptides (sequences given in Table 1) were synthesized manually by solid phase method on 4-methylbenzhydrylamine resin using Boc/Bzl strategy, which was described previously [13]. Three-fold molar excess of protected amino acid was used in the coupling step. Dicyclohexylcarbodiimide/1-hydroxybenzotriazole activation was used. After synthesis the peptide-resin was washed with DMF, methyl tert-butylether and dried. The detachment of the peptide from the resin and the side chains deprotection were carried out by liquid hydrogen fluoride-anisole (9:1). The obtained peptides were purified by reverse phase high performance liquid chromatography (RP-HPLC), which indicated the purity of the peptides more than 95 %. The peptide sequences were assigned by amino acid analysis and fast atom bombardment mass spectrometry (FAB-MS) spectra. Lyophilised peptides were entirely soluble in deionised sterile water and used at 5 µg/ml.

Table 1. Synthetic peptides used in the study.

<table>
<thead>
<tr>
<th>Source</th>
<th>Residue No.</th>
<th>Amino acid sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>545–554</td>
<td>GMGGGGLGGGG</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>562–571</td>
<td>GMGGGGMGGG</td>
<td>9</td>
</tr>
<tr>
<td>3&lt;br&gt;M. bovis hsp65</td>
<td>180–188</td>
<td>TFGLQLELT</td>
<td>12</td>
</tr>
<tr>
<td>4&lt;br&gt;human hsp70</td>
<td>450–463</td>
<td>TKDNLLGRFELSG</td>
<td>11</td>
</tr>
</tbody>
</table>

Proliferation assay

PBMC from fresh heparinized blood were obtained following centrifugation on Ficoll-Pague (Amersham Biosciences, Little Chalfont, UK) at 360 g for 40 min. A total of 1 × 10⁶ cells were cultured in 1640 RPMI (Cambrex, Walkersville, USA) with 10% heat-inactivated fetal calf serum (Sigma, St Louis, MO, USA), 10 mmol/l HEPES (Sigma, St Louis, MO, USA), 2 mmol/l glucoseamine (Gibco Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, St Louis, MO, USA) in triplicate in 250 µl volume microtiter tissue culture plates (Corning Inc., Corning, NY, USA) with the appropriate concentration of antigen for 7 days. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂. Proliferation was measured by [³H]-thymidine (each well was pulsed with 1 µCi) incorporation over the next 18 h of culture. PBMC responses were expressed as stimulation index (SI): the mean counts per minute (cpm) in the presence of antigen divided by the mean cpm without antigen.

Statistical analysis

Proliferative responses to antigens were compared between the cohorts of patients and healthy controls by using Mann-Whitney U test. Significance level was established at P < 0.05. Post hoc power calculations were carried out to evaluate whether the study was sufficiently powered, and to inform future research. NCSS-PASS 2005 software was used for the analysis.

Results

To evaluate whether the study was adequately powered to detect differences between the groups, the post hoc power analysis was carried out where a statistically non-significant result was obtained. The result of power analysis of at least 0.80 was assessed as sufficient. Calculations which did not