Original Research Papers

Th1 and Th2 cell responses of type 1 diabetes patients and healthy controls to human heat-shock protein 60 peptides AA437-460 and AA394-408

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Abstract. Rationale: Type 1 diabetes mellitus (T1) is considered to be an immune mediated disease. Based on previous findings it might be suggested that heat shock protein 60 (Hsp60) could be involved in the mediation of the development of the disease. Furthermore a bias toward Th1 immune response was observed in T1D patients where the level of Th1 cytokines was elevated, while the level of Th2 was decreased.

Aim of the study: To determine Th1 (IFN-γ) and Th2 (IL-13) cytokine levels in T1 diabetic and control subjects as well as to determine whether there is a shift towards Th1 or Th2 immune response.

Materials and methods: ELISPOT (Enzyme-linked ImmunoSPOT) analysis was employed to differentiate antigen specific T-cell responses of a Th1 (IFN-γ) or Th2 (IL-13) type. 11 T1 diabetic patients and 9 healthy controls were investigated. For T-cell stimulation, we used a polyclonal mitogen or Tetanus toxoid (TT) as positive controls and two peptide antigens Hsp60 AA394–408 and Hsp60 AA437–460.

Results: In case of Hsp60 AA437–460 we found significantly decreased Th2 response in patients, although there was no significant difference in Th1 response. In case of Hsp60 AA394–408 and positive controls there was no significant difference.

Conclusion: Comparing the control and diabetic subjects a significant shift towards Th1 response in T1 diabetes mellitus for Hsp60 AA437–460 was observed.

Key words: Interferon-γ – Interleukin-13 – Diabetes – Heat shock protein 60 – p277

Introduction

Numerous studies suggested that T1 diabetes mellitus can be considered an immune system mediated disease [1] leading to destruction of beta cells in the pancreas and resulting in the lack of insulin production. In the past decades several studies proposed new cellular mechanisms of the beta cell destruction process [2].

Since T1 diabetes is the result of an autoimmune process, the disease could potentially be influenced by the modification of the inflammatory autoimmune process. Therefore it is important to identify the target antigens to develop an effective immunotherapy.

As candidate targets in the autoimmune process, the family of 60 kD heat shock proteins (Hsp60, Hsp65) in beta cells could be considered. Antibodies and T-cells reactive to Hsp60 were detected in mice with autoimmune diabetes induced by the beta cell toxin, streptozotocin (STZ) [3]. T-cells reactive to a peptide antigen, corresponding to a conserved Hsp60 domain of amino acids 437–460 have been observed in STZ susceptible male mice of the C57BL/KsJ strain [3]. Moreover, both Hsp65 reactive T-cells and antibodies have been detected in NOD/Lt mice in the course of the autoimmune insulitis [4]. In extension of these results, vaccination with different Hsps (Hsp60, Hsp65) and Hsp peptide fragments, like p277 (our Hsp60 AA437–460 peptide), prevented the development of low-dose STZ induced diabetes in C57BL/KsJ

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mice [5] and spontaneous diabetes in NOD mice [6, 7, 8, 9]. Heat shock proteins are not the only candidate targets in the autoimmune diabetes, though. Significant proliferative responses to the 65 kilo Dalton glutamic acid decarboxylase (GAD65) were observed in T1 diabetic patients [10]. Furthermore, autoantibodies against GAD65, insulin, tyrosine phosphatase IA-2, and other islet cell antigens have been used to diagnose and to predict the onset of T1 diabetes mellitus as well [11, 12, 13]. Some trials also suggested that presence of GAD antibodies could identify a subset of latent autoimmune diabetes in adults (LADA) and predict incipient insulin dependency [14, 15]. Supporting previous findings, elevated levels of antibodies specific for a conserved epitope of Hsp60 and Hsp65 were found in serum of T1 diabetic children. This peptide (Hsp60 394–408) shares homology with GAD [16].

T-cell immunity plays a pivotal role in the pathogenesis of T1 diabetes. Both CD4+ and CD8+ T-cell subsets have been shown to be involved in the diabetogenic process [17] but these data were mainly obtained in NOD mice [18]. Furthermore, newly diagnosed type 1 diabetes patients, similar to prediabetic and newly diabetic NOD mice, show heightened T-cell responses to Hsp60 and its peptides [19]. In newly diagnosed T1 diabetic patients the production of Th1 cytokines was elevated, whereas Th2 cytokines were less inducible upon stimulation by mitogens [20]. Clinical Th1 cytokines was elevated, whereas Th2 cytokines were heightened T-cell responses to Hsp60 and its peptides [19]. Furthermore, newly diagnosed type 1 diabetes patients, but these data were mainly obtained in NOD mice [18].

**Material and Methods**

**Subjects**

A total of 11 T1 diabetic patients (4–11 days after insulin initiation) were included into this study. Patients were eligible if the time period between the first adequate treatment with insulin and blood sampling was less than 2 weeks. The patients were recruited from the German Diabetes Center and IIIrd Department of Internal Medicine, Semmelweis University during 2002–2003 with the recent diagnosis of diabetes mellitus type 1. Diagnostic criteria were immediate insulin requirement upon diagnosis, and only lean subjects with a typical history of ketosis, polyuria, polydypsia weight loss and elevated HbA1c levels were included. C-peptide levels were not used as diagnostic criteria.

Nine healthy volunteers were included in the study as controls. The basic characteristics of the patients and controls are presented in Table 1. As regards gender distribution, the composition of the patient and control groups are not homogenous, further experiments would be needed to equilibrate this difference.

**Peptide synthesis**

Synthetic peptides representing regions of human Hsp60 were synthesized by Boc/Bzl strategy using 4-methylbenzhydrolamine resin (0.96 mmol/g capacity). The following side chain protecting groups were used: benzyl ester (OBzl) (Glu, Asp), benzyl (Bzl) (Thr, Ser), 2-chloro- benzoxyl-carbonyl (Cl-Z) (Lys), tosyl (Arg). The N'-Boc protecting group was removed with 33 % (v/v) trifluoroacetic acid/dichloromethane (2 + 20 min), the resin was neutralized with 10 % (v/v) diisopropyl- ethylamine/dichloromethane. The coupling was performed with disopropyl-carbodiimide/1-hydroxy benzo-triazole (DIPC/HOBt) method, using 3fold molar excess of amino acid derivatives. The success of the coupling was monitored with ninhydrin or isatine test [22, 23]. The peptides were cleaved from the resin with liquid HF containing 10 % (m/v) p-cresol at 0°C for 1.5 h. The crude products were purified by RP-HPLC.

Two peptide amides were synthesized: Hsp60 394LAKLSDGVAVV-408 and Hsp60 437VLGGGVALLRVIPALDSLTPANED460. In the current study, we tested Hsp60 peptides representing the immunodominant epitope Hsp60 AA437–460 [21] and the GAD like domain of Hsp60 AA394–408 [16].

**ELISPOT assay**

Capture anti-cytokine antibodies (for IFN-γ and for IL-13) were diluted 1 : 100 in sterile PBS and coated to plastic ELISA plates, 50 µl/well and left at +2°C to +8°C overnight. After overnight incubation each well was rinsed 5 times with sterile PBS supplemented with 0.05 % Tween-20. The plates were blocked with 1 % BSA in PBS overnight at +2°C to +8°C. After blocking, triplicates of 100 µl cell suspension per well were added and the plates were incubated at 37°C with 5 % CO₂ for 5 hours to capture secreted cytokines. Biotinylated antibodies (for IFN-γ and for IL-13) were diluted 1 : 100 in PBS supplemented with 1 % bovine serum albumine (BSA).