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Fusion protein His-Hsp65-6IA2P2 prevents type 1 diabetes through nasal immunization in NOD Mice☆

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ABSTRACT

Human heat shock protein 60 (Hsp60), is an endogenous β-cells autoantigen, it could postpone the onset of insulitis and sooner type 1 diabetes mellitus. P277 is one of Hsp65 determinants at position 437–469 of amino acids cascaded. Meanwhile, it's already well-known that there were several better anti-diabetic B epitopes, such as insulinoma antigen-2 (IA-2). Currently, fusion protein IA2P2 has constructed in order to enhance its pharmacological efficacy. In addition, added homologous bacterial-derived Hsp65 and His tag were beneficial to protein immunogenicity and purification separately. So, finally we examined a fusion protein His-Hsp65-6IA2P2 could regulate Th2 immune response and reduce natural diabetic incidence in NOD mice. We constructed two express vector pET28a–His-Hsp65-6P277 and pET28a–His-Hsp65-6IA2P2. After purification, we observed that triple intranasal administration of these two fusion protein in 4-week-old NOD mice maintained normal blood glucose and weight, with a lower diabetic or insulitis incidence. Consistent with induced splenic T cells proliferation and tolerance, His-Hsp65-6IA2P2-treated mice performed reduced IFN-γ and increased IL-10 level. In conclusion, we suggested that fusion protein His-Hsp65-6IA2P2 could be reconstructed and purified successively. Furthermore, nasal administration of this fusion protein could rebalance T cells population and prevent T1DM.

1. Introduction

As a serious genetic autoimmune disease, T1DM process is influenced by disease-associated autoantigen and determined by dendritic cells or T cells recognition of islet autoantigenic epitopes [1].There is no panacea for diagnosed T1DM including insulin injection, oral drug, panacea transplantation etc., but postponing the onset of insulitis can be induced by endogenous β-cells autoantigens [2], for example, Insulin, glutamic acid decarboxylase65 (GAD65), insulinoma antigen-2 (IA-2) and heat shock protein 60(Hsp60) [3]. Positively correlated with the severity of disease, high titer of anti-Hsp60 antibody and T-cells reaction to its epitopes had detected in diabetic patients. Also, natural antibodies to Hsp60 had detected in T1DM-resistant NOD mice [4].

One of Hsp60 determinants is VLGGGCALLRCIPALDSLTPANED (P277) at position 437–469 [5].It is P277 merely has anti-inflammatory influence by upregulating Treg cells population and function [6].Recently, Th1 epitope (P1: VLGGGCALLRC) was shown located on N-terminal of P277 peptide, related to anti-P277 antibodies and contributed to atherosclerosis, while the C-terminal is Th2 epitope (P2: IPALDSLTPANED) [7]. However, the single P2 peptide fragment was weaker than the whole P277 against diabetes, suggesting that cross-talk between T and B cells epitopes was also beneficial to T1DM prevention. In addition, our team had demonstrated that 6-repeated P277 could enhance its effect on anti-T1D characteristics [8]. So we selected the 6-repeated IA2P2 peptide which is a combinatorial peptide between B-cell insulinoma antigen (IA-2) epitope and P277 peptide Th2 epitope [9,10]. We have noted that this new peptide IA2P2 performed better than P277 in STZ-induced diabetic mice (unpublished data). Furthermore, human Hsp60 is almost 50% homologous to mycobacterial heat shock protein65 (Hsp65), so it's likely to produce cross-immune response to self-Hsp60 when simulated by bacterial Hsp65. Upon this finding, researches have shown that the onset of β-cells destruction is associated with the spontaneous development of anti-Hsp65 T-lymphocytes [11]. In other words, reaction to Hsp65 may confer greater susceptibility to T1DM [12] by enhanced immunogenicity. Also fusion protein Hsp65-6P277 was performed with a better effect than P277 itself in our lab [8]. On these basis, we constructed a new protein vaccine His-Hsp65-6IA2P2 (His as the affinity tag for purification) to clarify its anti-T1DM effect and relative mechanisms.

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Nasal vaccination will activate the immune system effectively [13].Vaccination produces a strong systemic immune response without impact on mucosal infection. In contrast, as an important part of mucosal immune system, nasal immunization will generate both mucosal and systemic immune response because of richness of blood vessels in the nasal cavity. Further, the presence of common mucosal immune system ensures immune response in the remote mucosal sites. Immunology studies in both animals and human showed that intranasal immunization might produce a sufficient immune protection against a variety of pathogens through mucosa [14].Without complex manipulations, special equipment and cross-infections caused by injections, nasal vaccination is easily accepted by large numbers of people. Similar to human T1DM progress, the body immunity declined and disease developed progressively from birth to death in NOD mice as a typical animal model [15].Thus, nasal immunization in NOD mice which provides an optimal animal fundament for human use.

Our group had constructed expression vectors pET28a-His-Hsp65-6P277 and pET28a-His-Hsp65-6IA2P2, transformed into *Escherichia coli* BL21 (DE3). Female NOD mice were treated by these two purified protein via nasal cavity, monitoring physiological status and immune responses. In our research, experimental group treated by Hsp65-6IA2P2 fusion protein maintained normal blood glucose and lower morbidity, compared with control groups. Our study supported that Hsp65-6IA2P2 fusion protein through nasal immunization could delay the onset or the pathogenicity of T1DM in NOD mice, possibly since Th1/Th2 and Th17/Tregs cells imbalance had been reset to prevent β cells-specific inflammation.

2. Materials and methods

2.1. Construction of E. coli expressing His-Hsp65-6IA2P2

Based on our two constructed plasmids: pET28a-His-Hsp65-6P277 and pUC19-6IA2P2, the rest enrichment IA2P2 fragment of the latter, double digested by *Nhe*I and *Hin*dIII, had been inserted into the same double-digested former plasmid to get the recombinant plasmid pET28a-His-Hsp65-6IA2P2 whose 6P277 sequences were displaced by 6IA2P2. This plasmid was then transfected into *E. coli* BL21 (DE3) through electroporation, verified by sequencing.

2.2. Expression and purification of protein

Lactose induction time of *E. coli* BL21 (DE3), containing pET28a-His-Hsp65-6P277 or pET28a-His-Hsp65-6IA2P2, respectively, was determined by measuring the growth curve. And the harvesting time was done by induced curve. Targeted compounds were obtained after bacteria flask, lactose induction, nickel affinity and DEAE-52 chromatography, SDS-PAGE identification, desalination and freeze-drying process, successively. Finally, they were tested by western blotting.

2.3. Animal experiments

Female 4-week-old NOD mice were purchased from Beijing HFK bioscience Co., LTD, China. Experiments with animals were performed according to the guidelines of National Institutes of Health and approved by China pharmaceutical university animal ethic committee. All the mice were kept in specific pathogen-free conditions in a 12-h dark/light cycle.

Three groups of 12 randomly divided mice were immunized by nasal inoculation per 100 μg three times on week 4,7,10. Protein were all dissolved by sterile PBS (pH 7.4, 0.01 mol/L) into 4 μg/μL.

Weight was measured at week 4, 7, 10, and then once in weeks till 25th week. Data from 27, 28, 30 weeks were also needed, so do the whole blood glucose from mice's caudal vein. Blood was taken from the orbital veins twice per month, and then serum was segregated for detection and cryopreservation for storage. Mice that the whole blood glucose was ≥11 mmol/L for consecutive weeks were regarded as onset. Number of disease and death were counted.

Once morbidity of control group had been equal to 60% at week 21, blood glucose of each health mouse was measured in caudal vein (T = 0 min) after an overnight fasting (Oral glucose tolerance test, OGTT). Then health mice were given glucose (500 g/L, dissolves in RO water) by oral gavage and measured as above at 30, 60, 75, 90,120,180 min.

2.4. Histopathology and immunohistochemistryl

Mice were killed through cervical vertebra dislocation. And then, pancreas were harvested, fixed by 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (Google organisms, China). The degree of insulitis was scored as followed: no insulitis (without cell infiltration); peri-insulitis (infiltrated occupying <25% of the islet mostly around them); mild insulitis (infiltrated occupying <50% of the islet area); severe insulitis (infiltrated ≥50% of the islet area). About one hundreds of mice were scored from each groups.

Islet cells were analyzed by immunohistochemistry. Sections were stained with anti-insulin antibodies, CD4 antibodies and CD8 antibodies (Google organisms, China), respectively, then observed under a laser scanning confocal microscope.

2.5. Splenocytes proliferation assay and cytokine production assay

Splenocytes were isolated from 30-week-old NOD mice, ground into scattered cells and incubated with red blood cell lysis buffer. Cells were re-suspended and incubated in 6-well plates at 10^7 cells/well in 2 mL RPMI-1640culture medium (10%FBS, 100 U/mL streptomycin). The stimulated protocols in three groups were performed as followed: the negative blank without the additional besides medium, 5 μg/mL ConA (Sigma, USA) as positive; and PBS,10 μg/mL His-Hsp65-6P277 or 10 μg/mL His-Hsp65-6IA2P2 as stimulated agents in their corresponding immunized group respectively. Cells were incubated for 72 h and proliferation was assessed by addition of WST-8 (Beyotime, China) for 2 h and measured at 570 nm with a 630 reference wave in a Microplate reader (ST-360, KHB, and China).

Cells supernatants were collected by centrifugation for cytokine production assay. The levels of IL-10 and IFN-γ were determined using a R&D ELISA system (R&D, USA).

2.6. Flow cytometric analysis

Single splenocytes suspension was all stained by an anti-CD4 antibody. Then, stained cells were stained again with another specific transcription factors antibodies Foxp3 (also consistent with anti-CD25 antibody), RORγt, Gata-3, or T-bet respectively. All staining processes were performed according to the manufacturer's instructions. Cells were detected using a BD Biosciences FACSVerse™ flow cytometer.

2.7. Statistical analysis

Results were performed as means ± the standard error of mean (SEM) and analyzed using GraphPad Prism 5 software. Comparisons between groups were performed using one-way analysis of variance (ANOVA) after *t*-test. And $p < 0.05$ was considered significant.

3. Results

3.1. Preparation of fusion protein His-Hsp65-6I'A2P2 and His-Hsp65-6P277

The IA2P2 gene (from plasmid pUC19-6IA2P2) was successfully digested by *Nhe*I and *Hin*dIII and the fragment was showed by electrophoresis (Fig. 1B). Then it was inserted into pET28a-His-Hsp65 (from plasmid pET28a-His-Hsp65-6P277 constructed by our lab previously)