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Promotion of atherosclerosis in high cholesterol diet-fed rabbits by immunization with the P277 peptide

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ABSTRACT

Previous evidence has proved the ability of immunization with heat shock protein (HSP) 60/65 to induce atherosclerosis. P277, a 24-residue peptide of human HSP60, is a promising peptide vaccine against autoimmune diabetes. But as a fragment of HSP60, its potential ability of promoting atherosclerosis has never been investigated yet.

In the present study, the rabbits fed with normal standard diet or high cholesterol diet were immunized with P277 or PBS emulsified in incomplete Freund's adjuvant 4 times at 4-week intervals. Atherosclerotic lesions of the rabbits receiving P277 treatment and fed with high cholesterol diet increased significantly compared with those of the rabbits receiving PBS treatment and the same diet. However, no obvious lesions were found in the two groups of rabbits fed with the normal standard diet. Significant expression of P277 was detected in the high cholesterol diet-induced atherosclerotic lesions and heat-stressed endothelial cells. Surface exposure of P277 was also observed in the stressed cells. In the subsequent assay of endothelial cells *in vitro*, the purified anti-P277 antibodies mediated a noticeable cytotoxicity to the stressed cells with the participation of complement.

In conclusion, subcutaneous immunization with P277 emulsified in IFA can aggravate the atherosclerosis in high cholesterol diet-fed rabbits. Surface expression of P277 was observed on stressed endothelial cells, and were suggested to mediate the autoimmune attack and promote the disease.

1. Introduction

Peptide P277, a 24-residue fragment of human heat shock protein (HSP) 60, is a promising peptide vaccine against autoimmune diabetes [1–4]. Autoimmune diabetes is an organ-specific progressive autoimmune disease resulting in the destruction of the insulin-producing pancreatic beta cells. One of its known target autoantigens is the HSP60/65 [5]. Peptide P277 of human HSP60 corresponding to positions 437–460 has been firstly identified as an immunomodulatory peptide which contains a target epitope for diabetogenic T cells [6,7]. Then it has been used as an ideal autoantigen to develop vaccines against autoimmune diabetes. The treatment with P277 has been proved effective in arresting beta-cell destruction in both NOD mice [8–10] and clinical patients [1,11], probably by shifting the related immune response from Th1 to Th2 pattern.

In fact, HSP60/65 is an autoantigen shared by several autoimmune diseases. Atherosclerosis, which is one major complication of diabetes, has also proved to be closely associated with the autoimmunity specific to HSP60/65 [12,13]. Previous evidence shows that

Abbreviations: HSP, heat shock protein; HCD, high cholesterol diet; IFA, incomplete Freund's adjuvant; HRP, horseradish peroxidase; FCS, fetal calf serum.

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HSP60 can be expressed in a great amount, on the surface of stressed endothelial cells [14–16]. Due to the high phylogenetic conservation of HSP60/65, the protective immune response directed against pathogen-HSP65 can result in a cross-reaction against self-HSP60 and immune destruction of related cells. Many infections, especially chronic infections have been proven to be associated with atherosclerosis [17–19]. This attack may also result from an autoimmune reaction directed against an altered self-HSP60. It is believed that the injury to endothelial cells initiates the autoimmunity to endothelium at the very first stages of atherosclerosis [13,20–22]. Furthermore, numbers of evidence has already proved that immunization with recombinant HSP65 or materials containing HSP65 can lead to the development of atherosclerotic lesions [23–28].

Based on these reports, questions are raised about whether P277 immunization would trigger atherosclerosis when it is used as a vaccine against autoimmune diabetes. The P277 treatment by subcutaneous immunization with adjuvant can effectively stimulate both the cellular and humoral immunity as the whole HSP60/65 molecule does. In previous studies, HSP60 peptides, covering the whole P277 region (437–460), or overlapping with the P277 region, have been reported to contain proatherogenic T-cell and B-cell epitopes [29–31]. It indicates more possibility that P277-associated peptide may be involved in the auto-attack in atherosclerosis. Previous work in our group has indicated that the immunization with P277 could induce vascular leak syndrome in C57BL/6 mice via endothelial damage [32]. Whether it will promote atherosclerosis is unclear yet.

To address this question, experiments were specifically designed to investigate whether P277 treatment could influence the formation of atherosclerotic lesions in aortas of the rabbits fed with normal standard diet or high cholesterol diet.

2. Materials and methods

2.1. Preparation of peptide P277

Peptides were synthesized by using an automated multiple peptide synthesizer following the company's protocols for *N-a*-fluorenylmethoxycarbonyl (Fmoc) synthesis. The sequence of native P277 is VLGGGCALLRCIPALDSLTPANED. Two cystein residues were substituted with two valine residues for more chemical stability. This substitution has proved to be with no influence to its immunological activity, according to the previous reports of Elias and Cohen [33]. The sequence of P277 used in the current study is VLGGGVALLRVIPALDSLTPANED.

2.2. Animals and immunization

Thirty-two male New Zealand White rabbits weighing between 1800 and 2250 g were obtained from Jiangsu Academy of Agricultural Sciences, Nanjing, P.R. China, individually housed in wire-bottomed cages at room temperature. They were free to water and fed a normal standard diet or high cholesterol diet (HCD) containing 0.5% cholesterol. The rabbits were separated into 4 groups of 8 each. Two groups of rabbits fed with normal standard diet, as well as the other two groups of rabbits fed with HCD, received the immunization with P277 or PBS, respectively. Rabbits were immunized subcutaneously 4 times at 4-week intervals, at 6 deposits in the back. The peptide solution or PBS was emulsified with incomplete Freund's adjuvant (IFA) (Sigma, USA). Each 1 ml of emulsion (1 ml/rabbit) consisted of 0.5 ml peptide solution (1 mg/ml) and 0.5 ml IFA. HCD was started from one week after the first immunization, and to the end of the experiment.

Male C57BL/6 mice, obtained from Shanghai SLAC Laboratory Animal Co., Ltd., Nanjing, P.R. China, were treated following a similar schedule to produce the anti-P277 antibodies used for immunohistochemistry. Briefly, two groups of mice ($n = 10$ for each group) were subcutaneously immunized with 0.1 ml (1 mg/ml) P277 solution or PBS, emulsified with 0.1 ml IFA, 3 times at 4-week intervals.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Purified P277 (10 μg/ml) was coated onto flat bottom 96-well ELISA plates (Costar, USA) (100 μl/well) overnight at 4 °C. After blocking with 5% bovine serum albumin, each well was incubated with 100 μl serum sample diluted 1:100 for 1 h at 37 °C, followed by 100 μl horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (SouthernBiotech, USA) with a dilution of 1:10000. After washing, it was incubated with 50 μl 0.01% 3,3′,5,5′-tetramethylbenzidine (TMB) and 50 μl 0.24% H_2O_2-urea solubilized in 0.2 M Na_2HPO_4-0.1 M citrate buffer (pH 5.5) for 30 min at 37 °C. The reaction was stopped by addition of 50 μl 2 M H_2SO_4. Then the OD_{450} value was measured.

2.4. Serum lipids determination

Blood was taken after a 16-h fast. Values of serum total cholesterol were measured by a Hitachi automatic analyzer (model-7150, Tokyo, Japan).

2.5. Analysis of atherosclerotic lesions

Rabbits were sacrificed at week 16 of the experiment. The aortas were removed intact from the aortic arch to the iliac bifurcation and cut longitudinally. In order to visualize the areas of fat deposition, these aortas were fixed in 10% formalin for 4 days, and then stained with 0.2% (w/v) Sudan III in 75% ethanol (v/v). The percentage of Sudan III staining area in entire aorta area was quantified by the MapInfo Professional software (version 7.0; http://www.mapinfo.com).

2.6. Antibody preparation

Recombinant P277 was coupled to NHS-activated Sepharose 4FF (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. To prepare the anti-P277 antibodies, pooled high-titer rabbit or mouse anti-P277 antisera were inactivated and precipitated twice with 50 and 33% saturated $(NH_4)_2SO_4$ respectively. The precipitated IgGs, dialyzed overnight against PBS, were added to the column. After 30-min incubation, 20 mM Gly-HCl buffer (pH 2.5) was added to elute antibodies. The eluant was immediately neutralized with 0.5 M NaOH, and then dialyzed against PBS. Anti-P277 reactivity was measured by ELISA. Control antibodies were prepared from the serum of PBS-treated rabbits or mice with the same method.

2.7. Immunohistochemical staining of aortas

Serial 4-μm thick frozen sections were fixed in acetone for 10 min. The sections were subsequently placed in a humidified chamber, overlaid with the purified mouse anti-P277 antibodies or control antibodies (diluted 1:10), and incubated overnight at 4 °C. After washing with PBS, the sections were incubated with HRP-conjugated goat anti-mouse IgG (Boster, Wuhan, China) (diluted 1:400) for 40 min at 37 °C. Finally, these sections were washed and developed for 20 min at room temperature by using a substrate solution of 0.05% 3,3′-diaminobenzidine and 0.012% hydrogen peroxide in 10 mM Tris–HCl (pH 7.5), then counterstained with hematoxylin.