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Heat shock protein 65 promotes atherosclerosis through impairing the properties of high density lipoprotein

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ABSTRACT

Aim: To explicit whether the functions of high density lipoprotein (HDL) are impaired in murine atherosclerosis by subcutaneous immunization with recombinant mycobacterial heat shock protein 65 (HSP65). **Methods**: C57BL/6 mice were fed a normal chow-diet with non immunization as normal group. ApoE knockout ($ApoE^{-/-}$) mice on high-fat diet were randomly divided into three groups ($n = 8$) and immunized subcutaneously with different concentrations of HSP65 or phosphate-buffered saline (PBS). All animals were treated for 16 weeks. Reverse cholesterol efflux, the anti-oxidant and anti-inflammatory functions of HDL were assayed. Hepatocytes and peritoneal macrophages were isolated to examine the expression of cholesterol transport regulating proteins, including SR-B1, ABCA1, ABCG1, PPAR-γ and LXR-α. **Results**: In HSP65-immunized mice, paraoxonase1 (PON1) activity and the expression of IL-10 were reduced, while High-density lipoprotein inflammatory index (HII), myeloperoxidase (MPO) activity, and the expression of IFN-γ were elevated gradually. The MPO/PON1 ratio amount was significantly higher in HSP65-immunized group than in normal or PBS-immunized group. In addition, compared with normal or PBS-immunized group, cholesterol efflux rate and the expression of regulating proteins were markedly decreased in HSP65-immunized group. The mice immunized with HSP65 developed significantly larger aorta atherosclerotic plaques when compared with PBS-treated littermates. The high MPO/PON1 ratio was correlated with HII, cholesterol efflux rate and atherosclerotic plaques. **Conclusions**: This study demonstrates that subcutaneous immunization with HSP65 impairs the properties of HDL, which may contribute to its important pathogenic role of HSP65 in atherogenesis. Also, MPO/PON1 ratio may be a predictor of AS.

1. Introduction

Atherosclerosis is a chronic inflammatory disease initiated by deposition of subendothelial lipoprotein in the arterial wall [1,2]. This concept has been further extended to incriminate autoimmune factors as enhanced the progression of atherosclerotic plaque [3]. There is considerable evidence that heat shock protein 65 (HSP65), a major autoantigen, contributes to the initiation and development of autoimmunity and atherosclerosis [4,5]. It has been reported that immunization with mycobacterial HSP65 administered subcutaneously accelerated atherosclerosis in mice fed with normal chow diet or high-cholesterol diet [6,7].

Accumulating evidence has demonstrated that microbial infection and inflammation are important proatherogenic factors that influence the lipid and lipoprotein metabolism [8]. Nasal administration of HSP65 induced noticeable HSP65-specific immune tolerance and reduced serum lipids which were resulted from a different immune mechanism other than tolerance [9]. According to these reports, the inflammatory immune reaction induced by HSP65 may also mediate several proatherogenic changes of lipids. However, it remains unclear whether HSP65 could influence the properties of high density lipoprotein (HDL).

HDL is an independent protective factor for coronary artery disease (CAD) [10]. The major cardiovascular protective effects of HDL are attributed to its role in reverse cholesterol transport (RCT) and cholesterol efflux [11]. Furthermore, HDL also has anti-oxidant and anti-inflammatory functions [12]. However, quantifying HDL-C concentration alone only provides limited information about HDL's cardioprotective effects [13]. Reports of marked heterogeneity in

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the particle composition and biologic properties of HDL have reinforced a need for assessment of HDL function [14]. Recent studies have suggested that the ability of HDL to promote RCT from macrophages, correlated with atherosclerosis, independent of HDL-C [11]. Consequently, the assessment of HDL functions has become a novel target to investigate the association between HDL and CAD risk.

Notably, it was found that structural modification and compositional alteration of HDL particles as a result of chronic inflammation and acute phase responses may adversely affect or reverse their normal biological function [15]. There are many possible alterations between this dysfunctional HDL and normal functional HDL. However, whether the immuno-inflammatory reactions induced by HSP65 would shift HDL to dysfunctional HDL has not been investigated.

In our study, we investigated whether subcutaneous immunization with different dose of HSP65 would influence the properties of HDL in Apolipoprotein E knockout (Apoe$^{-/-}$) mice. Furthermore, we explored the underlying relationship among HSP65-specific immune response, the function of HDL and atherosclerosis.

2. Materials and methods

2.1. Animals

Eight-week-old male wild-type C57 BL/6 and Apoe$^{-/-}$ mice, obtained from Peking University (Beijing, China), were maintained at the Animal Holding Unit in Nanfang Hospital. C57BL/6J mice were fed with normal chow-diet as normal group and Apoe$^{-/-}$ mice were fed with high-fat diet (21% fat and 0.15% cholesterol). Three groups of 8 Apoe$^{-/-}$ mice each, were immunized subcutaneously with either phosphate-buffered saline (PBS) or HSP65 (5 μg and 25 μg; StressMarq, Canada). The mice were boosted twice under the same protocol following three and six weeks. All mice were sacrificed 16 weeks later with diethyl ether.

2.2. Blood analyses

At the end of the experiment, the concentrations of serum total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured with an automated biochemical analyzer (Type AU5421, Olympus, Japan). Paraoxonase1 (PON1) activity was measured as previously described [16]. Myeloperoxidase (MPO) activity was measured by a MPO determination kit (Jiancheng Biotech Ltd, China) using commercially available reagents, according to the manufacturer's instructions. Serum interleukin-10 (IL-10) and interferon-gamma (IFN-γ) were quantified by ELISA kits (Assay, USA).

2.3. Detection of anti-HSP-65 antibodies

Recombinant HSP65 (1 μg/ml) in PBS was coated onto flat bottom 96-well ELISA plates by overnight incubation at 4 °C as previously described [17]. After washing with 0.02% PBS Tween and blocking with 1% bovine serum albumin (BSA) in PBS, sera were added in a single dilution (1:50 in PBS) and incubated for 1 h at room temperature. HRP conjugated rabbit anti-mouse IgG (Bioss, China) was added and incubated for 1 h at room temperature followed by four washings with PBS/Tween. Finally, 100 μL of citrate phosphate buffer (0.1 M, pH4.2) containing 0.53 mg/ml of 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma St. Louis, MO, USA) was added, and absorbance was measured after 30 min at 405 nm in a Titertek ELISA reader.

2.4. Assay of cholesterol efflux from macrophages

Cholesterol efflux rate was measured as previously described [11]. RAW264.7 macrophages (American Type Culture Collection) were incubated with DMEM containing 0.2%BSA, 30 μg/ml Ox-LDL, and 1 μCi/ml ^{3}H-cholesterol (Perkin–Elmer) for 24 h. The cells were then washed and incubated in DMEM with 0.2%BSA. After another wash with serum-free medium, efflux mediums containing 2.8% serum from mice were added for 4 h. Subsequently, the incubation medium was collected and filtered through a 0.45 mm glass fiber filter to remove cellular debris before counting radioactivity. The cell monolayers were washed with PBS and lysed with 1 ml of 0.1 M NaOH.

2.5. Serum HDL inflammatory index (HII)

The CFA assay was a modification of a previously published method using PEIPC as the fluorescence-inducing agent [18]. Briefly, HDL was isolated by the dextran sulfate method. To determine the inflammatory/anti-inflammatory properties of HDL, the change in fluorescence intensity as a result of the oxidation of DCFH by PEIPC in the absence or presence of the test HDL was used. DCFH-DA was dissolved in fresh methanol at 2.0 mg/mL and was incubated at room temperature in dark for 30 min, resulting in the release of DCFH. PEIPC solution (10 μL) and 90 μL of HDL-containing dextran sulfate supernatant were divided into black 96-well polystyrene microtiter plates (Corning, USA) and incubated at 37 °C on a rotator for 1 h. 10 μL DCFH solution was added to each well, mixed, and incubated for an additional 2 h at 37 °C with rotation. Fluorescence intensity was determined using a Fluorescent Plate Reader (SpectraMax M5 plate reader, Molecular Devices) set at an excitation wavelength of 485 nm, emission wavelength of 530 nm, and cutoff of 515 nm. Values in the absence of HDL were normalized to 1.0. In this assay, values >1.0 after the addition of the test HDL indicated pro-inflammatory HDL; values <1.0 indicated anti-inflammatory HDL.

2.6. Real-time polymerase chain reaction

Total RNA was extracted from the liver tissues by using RNA Trizol reagent (Gibco RRL, Gaithersburg, USA). Then first-strand cDNA was synthesized by using PrimeScript RT Reagent Kit (Takara, China) according to the instruction from manufacture. The quantitative RT-PCR was performed on iQ5 Real Time PCR Detection System (Bio-Rad, USA) using AllinOne™ Q-PCR Mix (GeneCopoeia Inc, USA). Expression levels of the target genes generated standard curves were normalized against an endogenous reference gene β-actin. The specific primer sequences were listed in Supplementary data (Table 2).

2.7. Protein isolation and western blot analysis

The cellular or liver of Apoe$^{-/-}$ mice was isolated and protein was extracted. Expression of these proteins were measured as previously described. The appropriate primary antibodies include: anti-β-actin (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-scavenger receptor class B type I (SR-B1, 1:500; Abcam, USA), anti-ATP binding cassette transporter A1 (ABCA1, 1:500; Novus), anti-ATP binding cassette transporter G1 (ABCG1, 1:1000; Abcam, USA), anti-liver X receptor α (LXR-α, 1:500; Abcam, USA) and anti-Peroxisome proliferator-activated receptor γ (PPAR-γ, 1:400; Abcam, USA).

2.8. Assessment of atherosclerosis

Quantification of atherosclerosis plaques was performed by calculation of lesion size in the aortic sinus as previously described