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Decreased expression of programmed death 1 on peripheral blood lymphocytes disrupts immune homeostasis in peripartum cardiomyopathy

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ABSTRACT

Peripartum cardiomyopathy (PPCM) is a disease of unknown pathogenesis. Programmed death 1 (PD1) has been postulated to modulate immune response through potential mechanisms that remain elusive. This study aimed to elaborate the expression and function of PD1 on peripheral blood lymphocytes (PBLs) in the development of PPCM. Specimens of PBLs were performed to determine the expression of PD1 mRNA using fluorescence quantitative RT-PCR, and Th cytokines by ELISA. Immune homeostasis was evaluated with T lymphocyte phenotypes and immunoglobulin (Ig) isotypes as well as complement factors (C). Morphology of lymphocytes was observed using transmission electronic microscope. Significantly elevated levels of interferon (IFN)-γ, percentages of $CD3^+$, $CD4^+$, $CD8^+$ T lymphocytes, and pro-brain natriuretic peptide (BNP), but reduced levels of interleukin (IL)-4, IgG, IgM, IgA, C3, C4, and left ventricular ejection fraction (LVEF) were detected, which were associated with significantly lower of PD1 mRNA expression in PPCM relative to control. Furthermore, PD1 mRNA expression showed significant negative correlation with IFN-γ and $CD3^+$, $CD4^+$, $CD8^+$ T lymphocytes, and proBNP as well as positive correlation with IL-4, IgG, IgM, IgA, C3, C4, and LVEF. The morphologic features of cells indicated that the PBLs in PPCM were in the state of activation. Therefore, decreased expression of PD1 mRNA led to LV dysfunction and functional dysregulation of negative costimulation on cellular immunity. This study provided the first findings that PD1 expression was decreased, which might disrupt immune homeostasis that enhanced cellular immunity was predominant over attenuated humoral immunity that may work in the etiopathogenesis of PPCM.

1. Introduction

Peripartum cardiomyopathy (PPCM) is an idiopathic cardiomyopathy that is characterized by symptoms and signs of heart failure associated with decreased left ventricular systolic function and significant morbidity and mortality, occurring between the commencement of the last month of pregnancy and the end of the fifth month in the post-partum period, in women without pre-existing symptoms, sign or history of heart disease [1,2]. The pathogenesis of PPCM remains controversial but immune response has been proposed [3]. During pregnancy it is conceptualized to be a "T helper type 2 (Th2) phenomenon", implying that as a result of the bidirectional interaction between mother and fetus the maternal immune response is shifted toward humoral immunity dominance linked to Th2 cell. Consequently, increased immunoglobulin synthesis and decreased cell-mediated response are associated with the maintenance of pregnancy. The enhancement of cellular immune disturbs immune homeostasis resulted in detrimental effects on cardiac function. This imbalance of cellular immunity and humoral immunity may contribute to initiating of the pregnancy-associated cardiomyopathy [4].

Programmed death 1 (PD1) and its ligand B7 homolog I (B7H1), have been well-known as a crucial negative costimulatory molecules and play a critical role in down-modulating immune responses and maintaining peripheral tolerance for the favorable immunological environment of a successful pregnancy [5]. Several studies have shown that the engagement of the PD1 with B7H1 depressed Th cell-mediated immune reactivity through reducing expression and secretion of multiple cytokines [6]. Encouraged by these studies, we analyzed impacts of expression of PD1 on immune homeostasis in a cohort of

Abbreviations: PPCM, peripartum cardiomyopathy; PD1, programmed death 1; B7H1, B7 homolog I; PBL, peripheral blood lymphocyte; LVEF, left ventricular ejection fraction; proBNP, pro-brain natriuretic peptide; IFN-γ, interferon-gamma; IL-4, interleukin-4; Th, T helper; Ig, immunoglobulin; C, complement; RT-PCR, reverse transcription polymerase chain reactions; PI3K, phosphatidylinositol 3-kinase; Akt, serine/threonine protein kinase; PIP3, phosphatidylinositol 3-triphosphate.

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PPCM patients in order to provide new insights into the pathogenesis of PPCM.

2. Methods

2.1. Patients and healthy control

From October 2013 to November 2015, the patients ($n = 38$) admitted to our hospital were enrolled in the study and newly diagnosed with PPCM according to the definition proposed in a recent position paper from the Heart Failure Association of the European Society of Cardiology Working Group [7]: (1) age between 18 and 37 years, (2) development of congestive heart failure in the last month of pregnancy or within the first five months of delivery, (3) absence of recognizable heart disease before pregnancy, (4) no other identifiable cause for cardiac failure, and (5) left ventricular (LV) systolic dysfunction demonstrated by echocardiographic analysis such as depressed LV ejection fraction (LVEF < 0.55). Exclusion criteria: (1) significant organic valvular heart disease; (2) hypertension and preeclampsia, (3) metabolic disorders such as diabetes mellitus, thyroid disease, and (4) clinical data are not complete. Pregnancy-matched healthy women with confirmed normal cardiac function (echocardiography, LVEF ≥ 0.55) were invited to participate in the study as control ($n = 38$).

All patients gave informed consent according to a protocol approved by the ethics committee of the First Affiliated Hospital of Xi'an Jiaotong University.

2.2. Clinical assessments and blood tests

Clinical assessments such as demographics, onset of symptom and signs during first presentation, heart rate, blood pressure, echocardiography, diseases in pregnancy were obtained from the patients at baseline.

Peripheral blood was extracted from the subjects in a fasting state in the following morning of the admission day. The specimens were collected in sterile tubes containing ethylenediaminetetraacetic acid as an anticoagulant. Plasma and serum were separated by centrifugation at 1500 rpm for 10 min. Aliquots were stored at −80 °C for future analysis. Laboratory workup was performed as routine investigation by hospital laboratories for hemoglobin, alanine transaminase, aspartate transaminase, creatinine, creatine kinase isoenzyme, troponin I and pro-brain natriuretic peptide (BNP). The concentrations of serum interferon (IFN)-γ, interleukin (IL)-4 were measured with commercially available enzyme linked immunosorbent assay (ELISA) kits according to their manufacturer's protocol (Elabscience Biotechnology, Wuhan, China).

2.3. Isolation of the peripheral blood lymphocytes

The venous blood specimen was mixed with equal volume of phosphate buffered saline (PBS: 8.0 g NaCl, 0.2 g KCl, 0.24 g KH_2PO_4, 3.628 g $Na_2HPO_4 \times 12H_2O$, pH 7.4), centrifugated for 10 min at 2000 rpm (Eppendorf, USA). Peripheral blood lymphocytes (PBLs) were freshly isolated from the interface layered on a density gradient on lymphocyte separation medium (Li Rui Biotechnology, Shanghai, China), washed thrice in PBS and cultured for 8 h in 5% CO_2.

2.4. Evaluation of immune homeostasis

Immune homeostasis was evaluated with regard to the balance of cellular immunity and humoral immunity [5]. For cellular immunity, analysis of PBL phenotypes was performed by flow cytometry (FACSCalibur™, Becton-Dickinson, USA) using the Multiset software package according to the manufacturer's protocol. Cells were incubated with fluorescein isothiocyanate- and phycoerythrin-conjugated monoclonal antibodies. Flow cytometric assay was carried out using a Procount Progenitor Cell kit (Becton-Dickinson) for enumeration of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes. Data were analyzed using FlowJo software (Tree Star, Inc., San Carlos, USA).

For humoral immunity, serum samples of immunoglobulins G (IgG), IgA, IgM as well as complement factors 3 (C3) and 4 were measured by nephelometry using Binding site kits (Birmingham, UK). Briefly, the antibody dilution was prepared from filtrated saline solution from each protein for determination. The dilution of the standard serum protein was done for the reference curve. The reference curves and the patient curves were placed together with IgG controls in their respective cell. Antibodies were added and afterward were left to settle for 60 min. The reading was done on a Hoechst nephelometer.

2.5. PD1 and B7H1 mRNA expression analysis

Total RNA was extracted from PBLs with Trizol reagents (Invitrogen), through centrifugation for 1 min at 12,000 rpm. After confirming RNA concentration and assessment of purity with UV absorbance at 260 nm on Nanodrop 1000 spectrophotometer (ThermoScientific, Wilmington, USA), equal amounts of total RNA from each sample were reversely transcribed into cDNAs using reverse transcription kit (Takara, Japan) in accordance with the manufacturer's instructions. Equal amounts of cDNA for each sample were used as template for fluorescence quantitative reverse transcription polymerase chain reactions (RT-PCR). The following sequence-specific primers were designed by using Primer Premier 5.0 software (Applied Biosystem, USA) for PCR amplification: (1) PD1 primers, 5′-GCA CGA GGG ACA ATA GGA-3′ (forward) and 5′-GAC AAT GGT GGC ATA CTC-3′ (reverse); (2) B7/H1 primers, 5′-CAG GGC ATT CCA GAA AGA-3′ (forward) and 5′-CCT CCA TTT CCC AAT AGA C-3′ (reverse); and (3) the housekeeping gene β-actin primers, 5′-CCT GGG CAT GGA GTC CTG TG-3′ (forward) and 5′-TCT TCA TTG TGC TGG GTG CC-3′ (reverse). Thermal cycling was performed with a S1000 Thermal Cycler PCR detection system (Bio-Rad, Hercules, USA). Each reaction contained 3 μl of cDNA, 0.96 μl primer, 3 μl DEPC and 6 μl Taq polymerase. The protocol included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 10s at 95 °C, 30s at 57 °C and 30s at 72 °C, followed by 5 min at 72 °C. Fluorescence signals were collected at 60 °C. Each sample was assayed in triplicate and compared to arbitrary values assigned to a standard melt curves generated for the target gene to obtain relative abundance of amplified produces. These values were then normalized to those of β-actin produces.

2.6. Transmission electron microscopic observation

Isolated PBLs were fixed with 2.5% glutaraldehyde (1 M phosphate buffer, PH 7.2) for 1 h, washed well, postfixed with 1% osmium tetroxide for 1 h, dehydrated, and embedded in epoxy resin. Semithin sections were stained with uranyl acetate and lead citrate, stabilized by carbon coating, and examined with a Hitachi electron microscope operated at 75 or 100 kV.

2.7. Statistical analysis

Database management and statistical analyses were performed with SPSS software (Version 13.0, SPSS Inc., Chicago, IL, USA). Statistical graphs were applied to GraphPad Prism 5 (GraphPad software, La Jolla, CA, USA). Continuous variables were expressed as mean ± standard deviation (SD). Comparisons of means between PPCM and control were assessed using independent samples *t*-test. Spearman's correlation coefficient was calculated to disclose relationships between PD1 mRNA expression and immune molecules and Th cytokines. *P* value < 0.05 was considered significant difference.

3. Results

3.1. Baseline clinical profiles of study subjects

The levels of LVEF and proBNP, indicators to the severity of cardiac dysfunction, were diminished and elevated in PPCM patients, respectively. Statistical analysis showed both PPCM and control did differ significantly in LVEF, proBNP and creatinine. However, all other clinical data between the two groups that displayed no significant difference were presented in Table 1.

In addition, the concentration of serum IFN-γ in PPCM tended to be significantly higher than that in control (189.3 ± 42.8 μg/ml vs 161.7 ± 37.3 μg/ml, $P = 0.004$; Fig. 1A). Furthermore, the serum level of IL-4 was displayed significantly lower in PPCM compared with control (79.8 ± 17.4 μg/ml vs 88.7 ± 18.7 μg/ml, $P = 0.036$; Fig. 1B).

3.2. Enhanced cellular immunity and attenuated humoral immunity

For cellular immunity, the percentages of $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes in PPCM remained significantly elevated compared with control ($CD3^+$: 73.62 ± 6.03% versus 66.95 ± 4.55%, $CD4^+$: 52.72 ± 5.62% versus 36.92 ± 3.19%, $CD8^+$: 34.05 ± 4.18% versus 27.72 ± 3.33%, all $P < 0.001$, Fig. 2A). However, for humoral immunity, the levels of IgG, IgM, and IgA in PPCM exhibited significantly lower than those in control (IgG: 10.57 ± 1.56 g/l versus 11.78 ± 3.15 g/l, $P = 0.037$, IgM: 1.34 ± 0.33 g/l versus 1.61 ± 0.32 g/l, $P = 0.001$, IgA: 2.05 ± 0.63 g/l versus 2.46 ± 0.60 g/l, $P = 0.005$, Fig. 2B). Similarly, the difference was compatible with C3 and C4 between PPCM and control (C3: 0.64 ± 0.20 g/l versus 1.60 ± 0.23 g/l, C4: 0.10 ± 0.03 g/l versus 0.31 ± 0.04 g/l, both $P < 0.001$, Fig. 2C). Therefore, elevated $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes and reduced IgG, IgM, IgA, C3, and C4 along with Th cytokines including IFN-γ and IL-4 could provide a valuable tool for evaluating of enhanced cellular immunity and attenuated humoral immunity.

3.3. Decreased expression of PD1 and B7H1 mRNA on PBLs in PPCM

PD1 and B7H1 mRNA expression levels were detected on PBLs in both PPCM and control subjects using fluorescence quantitative RT-PCR (Fig. 2), PBLs in PPCM showed decreased expression of PD1 and B7H1 mRNA compared with control (PD1: 0.65 ± 0.15 versus 0.74 ±