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PDIA3, HSPA5 and vimentin, proteins identified by 2-DE in the valvular tissue, are the target antigens of peripheral and heart infiltrating T cells from chronic rheumatic heart disease patients

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ABSTRACT

Rheumatic fever (RF) is a post-infectious autoimmune disease due to sequel of group A streptococcus (GAS) pharyngitis. Rheumatic heart disease (RHD), the major manifestation of RF, is characterized by inflammation of heart valves and myocardium. Molecular mimicry between GAS antigens and host proteins has been shown at B and T cell level. However the identification of the autoantigens recognized by B and T cells within the inflammatory microenvironment of heart tissue in patients with RHD is still incompletely elucidated. In the present study, we used two-dimensional gel electrophoresis (2-DE) and mass spectrometry to identify valvular tissue proteins target of T cells from chronic RHD patients. We could identify three proteins recognized by heart infiltrating and peripheral T cells as protein disulfide isomerase ER-60 precursor (PDIA3), 78 kD glucose-regulated protein precursor (HSPA5) and vimentin, with coverage of 45%, 43 and 34%, respectively. These proteins were recognized in a proliferation assay by peripheral and heart infiltrating T cells from RHD patients suggesting that they may be involved in the autoimmune reactions that leads to valve damage. We also observed that several other proteins isolated by 2-DE but not identified by mass spectrometry were also recognized by T cells. The identified cardiac proteins are likely relevant antigens involved in T cell-mediated autoimmune responses in RF/RHD that may contribute to the development of RHD.

1. Introduction

Group A streptococcus (GAS)-infected individuals display different clinical manifestations, ranging from non-invasive pharyngitis to invasive diseases such as necrotizing fasciitis. Acute rheumatic fever (ARF), sequelae of GAS pharyngitis, remains the most common cause of acquired heart disease in children and young adults [1]. ARF is characterized by inflammation of the myocardium and heart valves, mainly the aortic and mitral valves, which leads to the life-threatening rheumatic heart disease (RHD) in 30%–40% of patients. The patients usually develop mitral and/or aortic regurgitation and mitral stenosis and subsequently myocardial failure.

RF is considered a post-infectious autoimmune disease. Molecular mimicry between GAS antigens and host proteins has been shown [2,3]. Cross recognition of streptococcal antigens and heart tissue proteins has been evidenced at the B and T cell level [2,3].

Serum antibodies from RF patients recognize simultaneously streptococcal carbohydrate antigen *N*-acetyl-glucosamine and specific regions of cardiac myosin in the myocardium and laminin in the valves [4]. These cross-reactive antibodies may bind to the valvular endothelium and basement membrane, generating inflammatory signals that may up-regulate the expression of adhesion molecules, facilitating lymphocyte extravasation into the valve [5]. Immunohistochemical analysis of rheumatic valves showed a predominant infiltration of CD4 + T cells in the valve surface endothelium and a high expression of HLA-DR molecules in

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valvular fibroblasts [6,7]. Our group previously shown that heart-infiltrating T cells from RHD patients recognized simultaneously streptococcal M5 protein, mainly the N-terminal region located at the 86–103 amino acid residues, and heart tissue proteins isolated by molecular weight (MW) [3]. Peripheral and heart-infiltrating T cell clones also cross-recognize streptococcal M protein peptides and specific regions of the human cardiac myosin β-chain located at the light meromyosin and S2 fragment [8,9]. In addition, the light meromyosin and S2 myosin regions induced the production of inflammatory cytokines such as TNF-α and IFN-γ by T cells from RHD patients [8,9], reinforcing previous results that showed an inflammatory pattern of cytokine production in valve tissue [10].

Despite all the progress in understanding the immune response in RF/RHD, the molecular characterization of the target autoantigens recognized by T and B cells within the inflammatory microenvironment of heart tissue in patients with RHD is still not completely elucidated. Several pieces of evidence emphasize the role of cardiac myosin, laminin and vimentin as the target antigens of the humoral and cellular immune response observed in RF/RHD patients [4,11–13].

In the present work, we used a proteomic approach to molecularly identify new myocardium and valvular autoantigens recognized by heart infiltrating and peripheral T cells from RF patients. We identified novel heart tissue proteins such as disulfide isomerase ER-60 precursor (PDIA3) protein and a 78 kD glucose-regulated protein precursor (HSPA5) that were recognized by peripheral blood mononuclear cells and heart infiltrating T cell clones from chronic RHD patients. In addition, we also identified vimentin and showed the recognition of this protein by peripheral and heart infiltrating T cells from chronic RHD patients and confirm previous work that emphasized the role of vimentin as the autoantigen target of antibodies from RF patients.

2. Materials and methods

2.1. Patients

Patients were followed for at least 5 years at the Heart Institute (InCor) and at the Children's Hospital, University of São Paulo. Patients were diagnosed according to Jones' Criteria [14]. All protocols were approved by the Institutional Review Board of the University of São Paulo, and written informed consent was obtained from parents of patients under 18 years old participating in the study.

Peripheral blood mononuclear cells (PBMC) were collected from patients with chronic RHD ($n = 10$) to evaluate cellular immune response. Myocardium and valvular tissue biopsies also obtained from patients with chronic RHD ($n = 5$) undergoing valve correction surgery were used for the establishment of heart-infiltrating T cell lines and T cell clones.

2.2. Mononuclear cells obtained from peripheral blood

Peripheral blood mononuclear cells (PBMC) were purified by Ficoll–Hypaque (d = 1.077 g/l, Amersham Biosciences, Piscataway, USA) gradient and centrifugation at 300 × *g* for 25 min. Cells were collected, resuspended and washed three times in PBS. Cells were then resuspended in RPMI (RPMI 1640, Gibco Invitrogen Cell Culture, Carlsbad, CA, USA). The total number of cells and the percentage of viability were determined by Trypan blue exclusion (Sigma-Aldrich, St Louis, MO, USA).

2.3. Establishment of heart infiltrating T cell lines and T cell clones

Heart infiltrating T cell lines were established from six surgical fragments of five patients with RHD undergoing valve correction surgery. Heart tissue was slightly minced, placed in flat bottom 96-well plates (Becton & Dickson, Lincoln Park, NJ, USA) filled with Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Life Technologies, Carlsbard, CA, USA) supplemented with 2 mM L-glutamine (Invitrogen), 10% pooled normal human serum, antibiotics (Gentamycin 40 μg/ml and Peflacyn 20 μg/ml) and 40 U/ml of human recombinant IL-2 (PrepoTech, Rocky Hill, NJ, USA) on a HLA-DR-matched feeder layer of peripheral blood mononuclear cells (PBMC, 10^5 cells/well), irradiated at 5000 rad. All T cell lines were further expanded with irradiated HLA-DR matched feeder cells and phytohemagglutinin (PHA-P, 2.5 μg/ml; Sigma-Aldrich Corporation, St Louis, MO, USA).

T cell clones were obtained by limiting dilution (0.5 cell/well) from established T cell lines in the presence of 10^5 HLA-DR matched irradiated PBMC, PHA-P (2.5 μg/ml) and IL-2 (20 U/ml). Plates that had more than 15% of the wells with lymphoblasts growing were discarded.

2.4. Heart tissue protein preparation

Cardiac protein homogenates were prepared from samples of human cardiac tissue fragment (left atrium and mitral valve) collected up to 8 h *post-mortem*. Heart tissue samples were homogenized in 1% SDS (w/v) in the presence of protease inhibitors. The supernatant was collected after centrifugation at 2000 × *g* for 10 min at 4 °C. The total amount of protein was determined using a Bicinchoninic Acid (BCA) protein assay as described by the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL, USA).

2.5. Two-dimensional electrophoresis (2-DE)

2.5.1. Isoelectric focusing/SDS-PAGE

Isoelectric focusing (IEF) was carried out on a pH gradient using a carrier ampholyte mixture (pH 3.6–9.2) (BioRad, Hercules, CA, USA). Protein homogenates (50 μg of left atrium and 220 μg of mitral valve) were diluted in isoelectric focusing buffer (9.5 M urea, 2% Nonidet-P40, 2% ampholyte mixture and 5% beta 2-mercaptoethanol) and run for 16 h at 420 V on a 13 × 0.5 cm chamber.

The second dimension was carried out on a 5–15% polyacrylamide gel (SDS-PAGE) for 90 min at 250 W. Gels were stained with Coomassie blue and scanned in the transmission mode on a GS-710 Imaging Densitometer (Bio-Rad) and analyzed using the software Melanie 2D gel analysis (BioRad, Hercules, CA, USA). After background subtraction, the protein spots were automatically defined and quantified with the feature detection algorithm as described elsewhere [15]. Spot intensities were expressed as relative volumes in percentages by integrating the OD of each pixel in the spot area (vol) and dividing by the sum of volumes of all spots detected in the gel. One of the gels was selected as a reference gel to which each other gel used in the analysis was aligned and matched, according to the landmarks.

2.5.2. Rotofor cell system/SDS-PAGE

Isoelectric focusing was carried out using a 2% carrier ampholyte mixture (pH 4.9–8.0) (BioRad, Hercules, CA, USA) in a Rotofor device (Bio-Rad, Hercules, CA, USA), equipped with a mini focusing chamber with 20 fractionation compartments. Mitral valve homogenate (4.5 mg) was diluted in isoelectric focusing buffer (5 M urea, 1% Nonidet-P40, 2% ampholyte mixture and 2.5% 2-mercaptoethanol) and applied and run for 5 h at 12 W with an external refrigerated water bath set at 4 °C. Anolyte and catholyte were, respectively, 0.1 M phosphoric acid and 0.1 M sodium hydroxide. All fractions were collected and concentrated using 10 kDa UltraFree® Centrifugal Filters (Millipore, Millipore Corporate Headquarters, MA, USA) to 250 μl under vacuum. The protein