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Histological and proteomic profile of diabetic versus non-diabetic dilated cardiomyopathy

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ABSTRACT

Background: Diabetic cardiomyopathy (DbCM) is indistinguishable from idiopathic dilated cardiomyopathy (IDCM) as specific histological and/or biochemical markers are unavailable.
Methods and results: Comparative histology, electron microscopy, morphometry for cell volume composition and myocardial fibrosis, reactive oxygen species (ROS), polymerase chain reaction for cardiotropic viruses, immunohistochemistry for nitrotyrosine, inducible nitric oxide synthase (iNOS), 8-hydroxydeoxyguanosine (8-OH-dG) and proteomics have been evaluated in endomyocardial biopsies from 9 patients (pts) (5 male and 4 female, mean age 61 ± 13 years) with DbCM (left ventricular end-diastolic diameter 65 ± 2.3 mm; ejection fraction 27 ± 6) and type 2 diabetes mellitus and 9 pts with IDCM (mean age 60 ± 9 years) matched for sex, age and severity of left ventricular (LV) dysfunction. Controls were surgical biopsies from 9 pts with mitral stenosis and normal LV dimensions and function.
No qualitative morphological changes were observed between DbCM and IDCM although mitochondrial damage and myofibrillolysis appeared more pronounced in DbCM. ROS were 5 times higher in DbCM than in IDCM and controls and were associated with higher expression of cytoplasm iNOS and nitrotyrosine and nuclear 8-OH-dG. Apoptosis was 14 times higher in DbCM than in IDCM and 41 times higher than in controls. Proteomic profile showed in DbCM a reduced expression of proteins related to beta-oxidation and detoxification pathway.
Conclusions: DbCM is a distinctive ROS-mediated disorder with oxidative damage of myocyte's structural proteins and DNA causing cell dysfunction and death. Reduced expression of beta-oxidation proteins suggests a decline of energy production and of mitochondrial function.

1. Introduction

Patients with diabetes mellitus (DM) may develop myocardial dysfunction independently of the occurrence of known causes such as coronary artery disease or hypertension. This distinct entity suggesting a specific involvement of myocardiocytes is currently categorized as diabetic cardiomyopathy (DbCM). DbCM was originally described in 1972 by Rubler et al. [1], who reported data from 4 DM patients with heart failure without evidence of hypertension, coronary artery disease, valvular, or congenital heart disease. Since then several pathological studies have followed, reporting intracellular hyperglycemia to be associated with various metabolic derangements including lipid and protein glycosylation, reactive oxygen species (ROS) production and increased expression of receptors for advanced glycation end-products (RAGE) and RAGE ligands such as S100A8, S100A12 and HMGB1 [2–3]. These biochemical events have been associated with pro-inflammatory effects, via NFkB activation, and oxidation of DNA and structural proteins causing cell dysfunction and death [4]. However, to what extent these mechanisms influence the development of cardiac dysfunction in human DM is still unknown. In addition, the biological functions involved are still poorly defined while their recognition may provide distinctive markers toward IDCM and pathogenetic clues to potentially improve disease treatment and prognosis. The aim of the study is to compare histological, ultrastructural, immunohistochemical and proteomic analysis between DbCM and IDCM in order to assess distinctive markers and pathways among these poorly defined entities.

Abbreviations: PTS, Patients; DM, Diabetes mellitus; DbCM, Diabetic Cardiomyopathy; ROS, Reactive oxygen species; RAGE, Advanced glycation end-products; IDCM, Idiopathic dilated cardiomyopathy; LV, Left ventricular; 2D-DIGE, Two-dimensional difference gel electrophoresis; rMnSOD, Human manganese superoxide dismutase.

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2. Materials and methods

2.1. Patients' characteristics

Out of 36 patients with type 2 DM and heart failure registered in our Institution in the last five years, nine (5 male and 4 female, mean age 63.5 ± 10.5 years, in treatment for diabetes for 11.4 ± 2.6 years, glycosylated hemoglobin at admission 6.3 ± 0.3) were enrolled in the study as being devoid of hypertension, coronary artery disease at CT scan, valve disease or systemic disorders. Results were compared with nine patients with idiopathic dilated cardiomyopathy (IDCM) matched for age, sex and degree of left ventricular (LV) dilation and dysfunction. Patients with IDCM were considered those presenting progressive LV or biventricular dilatation and dysfunction with normal coronary angiography, normal valvular pattern, no hypertension and no systemic disease potentially responsible of cardiac damage. Controls were 9 patients with mitral stenosis and normal LV dimension and function, undergoing surgical valve replacement. These endomyocardial fragments, although not obtained from healthy individuals, derived from a not overloaded chamber and were considered the nearest samples to a normal endomyocardial tissue [5].

2.2. Cardiac study

Study population and controls underwent non-invasive cardiac examinations including electrocardiogram (ECG), Holter monitoring, 2D-echocardiogram and cardiac magnetic resonance imaging (MRI) and invasive studies, including coronary and left ventricular angiography and left or bi-ventricular endomyocardial biopsy, after approval by the local Ethics Committee and the patients' informed consent. Six to eight endomyocardial samples per patient were taken cut and processed for histology, electron microscopy and polymerase chain reaction (PCR) for the most common cardiotropic viruses [6].

2.3. Endomyocardial biopsy

All patients underwent right and left cardiac catheterization, coronary with ventricular angiography and bi-ventricular endomyocardial biopsy. Endomyocardial biopsies were performed in the septal–apical region of both ventricles, which were approached by a 7-F (501-613A Cordis) long sheet and identified on an X-ray view using flashing of contrast medium. Three to five samples per ventricular chamber were taken, cut and processed for routine histological and histochemical analysis and for transmission electron microscopy. Two to four endomyocardial samples were snap-frozen in liquid nitrogen, stored at −80° and used for PCR, ROS assessment, protein analysis and assessment of myocardial anti-oxidant reserve.

2.4. Histology and electron microscopy

For histological analysis the endomyocardial samples were fixed in 10% buffered formalin and paraffin embedded. Five micron thick sections were stained with hematoxylin & eosin, Masson thrichrome. For electron microscopic examination, biopsies from DbCM and IDCM patients were fixed with 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide and embedded in Epon resin as previously described [7].

2.5. Immunohistochemistry

The presence of oxidative stress was evaluated by anti-inducible nitric oxide synthase (iNOS) (mouse monoclonal, Santa Cruz Biotechnology) and anti-nitrotyrosine (mouse monoclonal, Santa Cruz Biotechnology) antibody as previously described [6]. Intensity of immunostaining was semi-quantitatively evaluated as absent (grade 0), mild (grade 1), moderate (grade 2) and strong (grade 3).

Oxidative stress at the nuclear level was evaluated by immunofluorescence for 8-hydroxydeoxyguanosine (8OH-DG).

For indirect immunofluorescence tissue section was deparaffined, thoroughly dehydrated and incubated with Goat Serum (dilution 1:10, Sigma). After treatment with Proteinase K, 8-hydroxydeoxyguanosine (dilution 1:20 over-night at 4 °C, mouse monoclonal, Santa Cruz Biotechnology) was applied as primary antibody, and Goat Anti-Mouse FITC (dilution 1:30, Sigma) was used as secondary antibody. In situ ligation of hairpin probes with single-base 3′ overhangs were used to measure cardiomyocyte apoptosis (hairpin 1) and necrosis (hairpin 2) [8]. Cardiomyocytes were labeled by alpha-sarcomeric actin antibody staining (clone C5C, Sigma, St. Louis, MO, USA). Nuclei were visualized with 4′, 6-diamidino-2-phenylindole (DAPI). Sections were analyzed at a confocal microscope.

2.6. Molecular biology study

PCR and reverse transcription RT–PCR analysis was performed on two frozen endomyocardial biopsy samples to search for the most common DNA (adenovirus, cytomegalovirus, parvovirus B19, Epstein Barr virus, Human herpes virus 6, herpes simplex virus 1 and 2) and RNA (enterovirus, influenza virus A and B, hepatitis C virus) cardiotropic viruses as described [9].

2.7. ROS measurement assay

To measure the level of ROS in the heart, we performed a 2′7′ dichlorofluorescin diacetate assay. Frozen heart tissue was homogenized in 5 mL of Tris–Hcl 40 mM (pH 7.4) with Ultraturrax. Protein concentration was assessed by the Bio-Rad protein assay. 2′7 ′ dichloroflurescin diacetate 5 μM (Molecular Probes) was utilized for the assay from a 500 μM stock. The tissue samples were diluted in a range from 10,000 to 2500 times. The spectrum was analyzed from 480 to 525 nm at different times (T0, T10, T30) using a spectrophotometer Jasco FP-777. The levels of ROS were expressed as intensity fluorescence (IF) normalized for grams of tissue and micrograms of proteins.

2.8. Proteomic analysis

Biopsies were homogenized in 2-D sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% sulfobetaine 3–10, 1% amidosulfobetaine 14 and 10 mM Tris–HCL pH 8.5). Proteins were precipitated using the 2-D clean-up kit (GE-Healthcare) and re-suspended in 2-D sample buffer.

Thirty micrograms of proteins were labeled with Cy3 or Cy5 accordingly to manufacturer's instructions (GE-Healthcare). The same amount of internal standard was labeled with Cy2. A dye-swapping scheme was used to ensure that samples from different groups were labeled either with Cy3 or Cy5. Proteins were subjected to IEF using non-linear 3–10 pH range dry strips on a IPGphor II (GE-Healthcare), as described [10]. Second dimension electrophoresis was performed on 12% polyacrylamide gels in Tris-glycine SDS-PAGE running buffer (Bio-Rad).

Cy2, Cy3 and Cy5-labeled images were acquired on a Typhoon 9400 scanner (GE-Healthcare). Images were imported in DeCyder v6.5, 2-D Differential Analysis Software (GE-Healthcare) for spot co-detection, normalized volume ratio calculation, simultaneously match all spot maps and to identify spots with statistical variations.

Gels were then stained with SyproRuby (Bio-Rad); spots were excised from the gels using a ProPic Spot picker (Genomic Solutions) and subjected to in-gel tryptic digestion [11]. MALDI-MS and MALDI-MS/MS were performed on an Applied Biosystems 4800 Proteomics Analyzer, as described [10]. MS and MS/MS data interpretation was carried out with a GPS Explorer software (Version 3.6, Applied Biosystems), searching the NCBI human database using the MASCOT search algorithm. Monoisotopic precursor ion tolerance was set at 30–50 ppm and MS/MS ion tolerance at 0.3 Da. MASCOT scores greater than 63,