Life Sciences 158 (2016) 111–120

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Plasma proteomics for the assessment of acute renal transplant rejection

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ARTICLE INFO

Article history:
Received 31 March 2016
Received in revised form 8 June 2016
Accepted 28 June 2016
Available online 5 July 2016

Keywords:
Mass spectrometry
Biomarker
Plasma proteomics
Serum amyloid A

ABSTRACT

Renal transplant is the best treatment for patients with chronical kidney disease however acute graft rejection is the major impediment to success in renal transplantation leading to loss of the organ the first year after transplantation. The aim of this study was to identify plasma proteins that may be early biomarkers of acute rejection of renal allograft, developing a diagnostic model that avoids the loss of the transplanted organ. Shotgun proteomics (LC-MS/MS) method was used to analyze a set of thirty-one plasma samples, including 06 from patients with acute graft rejection after transplantation (rejection group/Rej-group) and twenty-five from renal transplant patients with stable renal graft function (control group/Ct-group). As results nineteen proteins were upregulated in the rejection group compared to the control group, and two proteins were downregulated; and three were present exclusively in the rejection group. After analysis, we selected four proteins that were related to the acute phase response and that were strongly associated with each other: they are alpha-1 antitrypsin (A1AT), alpha-2 antiplasmin (A2AP), serum amyloid A (SAA) and apolipoprotein CIII (APOC3). We think that simultaneous monitoring of SAA and APOC3 can provide insights into a broad profile of signaling proteins and is highly valuable for the early detection of a possible acute renal graft rejection.

Statement of significance of the study: In this study we did plasma shotgun patients with and without acute rejection of renal allograft. In a clinical setting an acute rejection is typically suspected upon an increase in plasma creatinine and renal biopsy. But these methods are late and unspecific; sometimes the rejection process is already advanced when there is an increase in serum creatinine.

Therefore, it is necessary to find proteins that can predict the allograft rejection process. In our study were able to identify changes in the concentration of plasma protein belonging to a network of protein interaction processes the acute phase response.

We believe, therefore, that development of a routine diagnosis of these proteins can detect early acute rejection of renal allograft process, thus preventing its loss.

1. Introduction

The renal transplant (renal TX) is considered the best treatment option for patients with chronical kidney disease (CKD), especially by improving the quality of life of these patients by eliminating the need for hemodialysis sessions per week. Survival and quality of life are significantly higher among transplant patients when compared to those who are on dialysis awaiting transplantation [1].

However, even with the progress in development of immunosuppressive therapies many patients lose the allograft after the first year of transplantation. It has numerous causes for the loss of renal graft, such as acute tubular injury, toxic effects of drug treatments, viral graft infections and chronic allograft nephropathy [2–4]. Patients whom experience an acute rejection (AR) after renal transplantation have an increased risk of developed chronic allograft and reduce long-term graft survival [5].

Before going into the tools for rejection diagnosis is must be realized that acute rejection is a heterogeneous condition concerning the involved renal structures, the mechanisms of immunological injury, and the clinical presentation [6]. Major targets of immunological injure include tubular epithelial cells, glomerular capillaries and larger renal vessels. Categorization of this multiplicity has been found in BANNFF classification [7]. The increase in serum creatinine in periods after transplantation is suspicious of rejection that will be confirmed by renal biopsy [8].

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http://dx.doi.org/10.1016/j.lfs.2016.06.029
0024-3205/

However, the biopsy protocol is not intended to be used routinely, must assess individual variability in evaluation biopsies, the costs and procedural risk need to be considered [9].

Therefore, the reliable identification of potential for early biomarkers acute rejection allograft is extremely necessary.

Last generation techniques involving genomics and proteomics have been used to identify the proteins with altered expression even before acute rejection is detected [10]. Although a genome remains static and relatively unchanged, a proteome demonstrates significant variations between individuals and conditions [11].

Proteomics have been extensively applied to several fields of medical research and technical progress in proteomics is growing [12–14]. Proteomics is an emerging technique for discovery of biomarkers that can be used for non-invasive diagnoses and prognoses to potentially increase the efficacy of treatment. Significant amounts of data that have been obtained from proteomics have already brought a better understanding of the pathophysiology of renal diseases and the identification of biomarkers and therapeutic targets [15–19].

Advances in proteomic technologies rely to a great extent on mass spectrometry (MS) instrumentation, protein and peptide separation techniques, computational data analysis tools, and the availability of complete sequence databases for many species. Ever since the analysis of complex protein mixtures using "shotgun" proteomics was developed, a strategy based on the combination of protein digestion and MS/MS-based peptide sequencing has become widely adopted [20–23]. A high demand for accuracy is placed both on the power of separation techniques, considering the extremely high complexity of the proteome samples, and on the sensitivity of detection methods, enabling the search for low abundant proteins or peptides [24–26].

In our study we used proteomic analysis to identify proteins whose expression has been changed during the process of graft rejection.

2. Materials and methods

2.1. Transplanted patients

Thirty-one adult subjects (67% male and 33% female), aged between eighteen and fifty-nine years and had first living donor kidney transplants were included in this study. Written informed consent was obtained from each participant. The study design was performed in accordance with the Declaration of Helsinki (1967) 2013 revision and approved by the Ethics Board of Federal University of Sao Paulo, Sao Paulo, Brazil, under the number 1081/09.

The inclusion criteria for this study were patients who had first living donor kidney transplants that were non-human leukocyte antigen (HLA) identical living-related, who were on immunosuppressive regimens that consisted of tacrolimus, azathioprine and prednisone. All drug doses were adjusted based on clinical findings.

The decision to perform a renal biopsy was based on serum creatinine increases of 25% over baseline with clinical rejection signs. A pathologist analyzed all biopsies, and rejection was classified according to the revised Banff grading system. Only patients who presented acute cellular rejection (BANFF IA, IB, IIA, IIB and III) were considered for this study (the group with acute rejection) [7].

2.2. Plasma collection

Plasma was collected into Vacutainer K_2 tubes with EDTA (BD, USA) and *Complete Mini* protease inhibitor tablets (Roche, Germany). Samples were centrifuged at 1700 *g* at 4 °C for 15 min, and the plasma was stored at −80 °C. All samples were collected seven days after the transplant day (D7) and on the day of kidney biopsy suspected of graft rejection (RD). Collection was always performed in the morning before the administration of immunosuppressive.

2.3. Albumin depletion protocol

Albumin was removed by affinity chromatography, using a HiTrap Blue HP column (GE Healthcare, Sweden) according to the manufacturer's protocol and as described in the literature [27].

Samples were diluted in binding buffer (20 mM sodium phosphate, pH 7.0) at a 1:1 ratio, filtered through a 0.45 μM filter and centrifuged at 2000 *g* at 4 °C for 20 min. The column was activated using ten volumes of binding buffer, 1 mL at a time. Following this 300 μL of plasma was introduced onto the column, and it was washed ten times with binding buffer. The eluted fraction was concentrated in a *SpeedVac concentrator* (Thermo/Savant, USA).

The protein concentration of the plasma was measured by a Coomassie method described by Bradford [28](Bio-Rad, USA). Bovine serum albumin (BSA) was used as a standard protein. The protein concentration was determined by reading absorbance at 595 nm in an Infinite F200 ELISA plate reader (Tecan, USA). The values were calculated as mg/mL.

2.4. Tryptic digestion and sample preparation for mass spectrometry (MS) analysis

Plasma (40 μL) from each of the twenty-five patients (control group) was used to generate a pool of 1 mL. The rejection group was not pooled, and samples from this group were prepared separately. The proteins were denatured with 0.2% *RapiGest™* Protein Digestion surfactant (Waters, USA). Immediately thereafter, they were heated at 80 °C for 15 min, centrifuged and reduced using 100 mM dithiothreitol (DTT). Next, they were heated at 60 °C for 30 min, followed by cooling to room temperature and centrifugation. The samples were alkylated with 300 mM iodoacetamide (Sigma-Aldrich, EUA) for 30 min at room temperature in the dark. The proteins were digested using porcine trypsin (Promega, EUA) at a ratio of 1:50 (w/w trypsin/total protein) at 37 °C overnight. The digestion was stopped by the addition of 5% TFA (trifluoroacetic acid -Thermo Scientific, USA). After the incubation, the samples were centrifuged at 15,300 *g* (Centrifuge 5403, Eppendorf, EUA) at 4 °C for 30 min. Supernatants were transferred into glass vials (Waters, USA) and combined with an internal standard (50 fmol yeast alcohol dehydrogenase, MassPREP, USA). They were then loaded into the LC-MS/MS.

2.5. Nano ultra performance-liquid chromatography (UPLC) tandem electrospray ionization (ESI) conditions

Peptides were concentrated and desalted on a C18 Symmetry column (5 μm, 20 mm × 180 μm) (Waters, USA) at a flow rate of 10 μL/min and then subsequently fractionated on-line in a strong cation exchange (SCX) 300 Å column (5 μm, 23 mm × 500 μm, Waters, USA) via 8 subsequent injections with ammonium formate (Merck, Germany) (50–200 mM) and acetonitrile (Merck, Germany) (5–30%). Each SCX fraction was separated on a High Strength Silica column (HSS - T3) (1.8 μm, 75 μm × 10 cm) (Waters, USA) using a binary gradient from two to 40% acetonitrile and 0.1% formic acid at a 400 nL/min flow. Masses were recorded in a Synapt HDMS (Waters, UK) with a resolving power of 10,000 [29]. All analyses were performed in positive-mode nanoelectrospray. The MS was calibrated with a solution of GFP (Human [Glu1] -fibrinopeptide B 100 fmol/μL). A doubly-charged ion ($[M + 2H]^{2+}$) was used for initial single point calibration (L_{teff}), and MS/MS fragment ions of GFP were used to obtain the final instrument calibration. The same solution was used to "lock mass" with 30 s of acquisition rates.

Proteomic experiments were performed in MS^E data independent analysis where spectra were acquired during alternating low (3 eV) and elevated (12-40 eV) collision energies applied to a module trap 'T-Wave' collision cell. Argon was used to promote collision-induced dissociation. A scan time of 0.6 s in each mode in a range of *m/z* 50 to 2000 was employed. An "RF offset" was set in the analyzer to allow