JOURNAL OF PROTEOMICS 96 (2014) 92–102

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot

Diabetic nephropathy induces changes in the proteome of human urinary exosomes as revealed by label-free comparative analysis

Irene Zubiri[a], *Maria Posada-Ayala*[a], *Aroa Sanz-Maroto*[a], *Enrique Calvo*[b], *Marta Martin-Lorenzo*[a], *Laura Gonzalez-Calero*[a], *Fernando de la Cuesta*[c], *Juan A. Lopez*[b], *Beatriz Fernandez-Fernandez*[d], *Alberto Ortiz*[d], *Fernando Vivanco*[a,e,1], *Gloria Alvarez-Llamas*[a,*,1]

[a]*Departmento de Inmunologia, IIS-Fundacion Jimenez Diaz, Madrid, Spain*
[b]*Unidad de Proteomica, CNIC, Madrid, Spain*
[c]*Department of Vascular Physiopathology, Hospital Nacional de Paraplejicos SESCAM, Toledo, Spain*
[d]*Departamento de Nefrologia, IIS-Fundacion Jimenez Diaz-IRSIN-UAM, Madrid, Spain*
[e]*Departamento de Bioquimica y Biologia Molecular I, Universidad Complutense, Madrid, Spain*

ARTICLE INFO

Article history:
Received 13 March 2013
Accepted 29 October 2013

Keywords:
Exosomes
Diabetic nephropathy
Proteomics
Selected reaction monitoring
Label-free quantitation
Depletion

ABSTRACT

Diabetic nephropathy (DN) is a major complication of diabetes mellitus (DM), the most frequent cause of end-stage renal disease (ESRD). Exosomes isolated from urine are considered a rich non-invasive source of markers for renal events. Proteinuria associated with DN patients at advanced stages may result in "contamination" of exosomal fraction by co-precipitation of high abundance urine proteins, making it enormously difficult to obtain a reliable comparison of healthy individuals and DN patients and to detect minor proteins. We evaluated different protocols for urinary exosome isolation (ultracentrifugation-based and Exoquick® reagent-based) in combination with an easy and quick depletion procedure of contaminating high abundance proteins (albumin). The optimal methodology was then applied to investigate the proteome of human urinary exosomes in DN and controls using spectral counting LC–MS/MS analysis followed by selected reaction monitoring (SRM) confirmation. A panel of 3 proteins (AMBP, MLL3, and VDAC1) is differentially present in urinary exosomes from DN patients, opening a new field of research focused on improving diagnosis and follow-up of this pathology.

Biological significance

Diabetic nephropathy (DN) is a progressive proteinuric kidney disease, a major complication of diabetes mellitus, and the most frequent cause of end-stage renal disease. Current markers of disease (i.e. creatinine and urinary albumin excretion) have proven limitations (i.e. some patients regress to normoalbuminuria, kidney damage may be already present in recently diagnoses microalbuminuric patients and renal function may decrease in the absence of

* *Corresponding author at:* IIS-Fundacion Jimenez Diaz, Avenida Reyes Catolicos 2, E-28040 Madrid, Spain. Tel.: +34 91 550 48 00x2202; fax: +34 91 544 82 46.
E-mail address: galvarez@fjd.es (G. Alvarez-Llamas).
[1] Both senior authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jprot.2013.10.037

significant albuminuria). We show here the first study on human DN proteome of urinary exosomes. Proteinuria associated to DN patients resulting in contamination of exosomal fraction and the associated difficulty to reliably compare healthy and disease conditions, are here overcome. A combined methodology pointed to increase exosomal proteome recovery and depletion of high-abundance proteome was here set-up. A total of 352 proteins were here identified for the first time associated to human urinary exosomes. Label-free quantitative comparison of DN urinary exosomes vs control group and SRM further validation, resulted in the discovery of a panel of three proteins (AMBP, MLL3 and VDAC1) which changes in DN, opening a new field of research focused to improve diagnosis and follow-up of this pathology.

1. Introduction

Diabetic nephropathy (DN) is a progressive proteinuric kidney disease characterized by microangiopathy and podocyte and tubular cell injury caused by high glucose concentrations or by secondary mediators of injury. DN is a major complication of diabetes mellitus (DM) and is the most frequent cause of end-stage renal disease (ESRD), accounting for 40% of patients requiring renal replacement therapy [1]. The early stages of DN are asymptomatic. A better understanding of its pathogenesis is required for earlier diagnosis, and for non-invasive monitoring that allows for treatment individualization that prevents progression of the disease. The discovery of novel markers of disease will help clinical diagnosis at earlier stages. Increased serum creatinine as diagnostic value is a late event and renal disease can occur with minimal or no change in creatinine [2]. Assessment of urinary albumin excretion allows earlier diagnosis of diabetic kidney disease, however, some patients regress to normoalbuminuria, histopathological changes in kidney structure may be already present in recently diagnosed microalbuminuric patients, and renal function may decrease in the absence of significant albuminuria [3,4]. Therefore, a deeper knowledge of the pathophysiological mechanisms in DN and the discovery of candidate markers that allow for better disease staging, outcome prediction, and monitoring of the response to clinical intervention are clearly needed.

Biological fluids in general and urine, in particular, have been widely proposed as sources for biomarker discovery. Urine is an ideal non-invasive biofluid as it is quite stable and has been described as undergoing minimal degradation in the bladder and urinary tract [5]. In particular, exosomes are one type of urinary secreted vesicles that have created increasing interest in the scientific community. Exosomes have proven roles in regulating immune response, antigen presentation, RNA and protein transfer, and cell–cell (organ–organ) interaction/signaling [6–8]. As opposed to apoptotic blebs (ABs) and shedding microvesicles (SMVs), they are 50- to 100-nm-sized particles, with density values in the range 1.13–1.19 g/mL. They are formed by the fusion of multivesicular bodies (MVBs, late endosomes) with the plasma membrane and release of their intraluminal vesicles, which are then termed exosomes once in the extracellular space. Exosomes can be considered a mechanism of non-classical secretion of proteins, and in particular they represent a sub-proteome of the whole urine. In this sense, sampling of urinary exosomes results in a significant reduction in the linear dynamic range of protein concentrations and therefore represent a better alternative for detection of minor low abundance proteins (potential markers) that otherwise could be masked by major proteins. However, some of these abundant proteins (i.e., Tamm–Horsfall protein (THP) or albumin in particular diseases) could be co-detected together with the exosomal fraction, depending on the isolation procedure [9].

We hypothesized that different proteins may be found in urinary exosomes of DN patients when compared with controls. Here we evaluated a combination of an ultracentrifugation-based protocol for exosome isolation, previously described as the best option for protein recovery for mass spectrometry (MS) analysis [10], with DTT treatment of the low-speed pellet to improve exosome recovery [11] and the depletion procedure of high abundance proteins co-precipitating with exosome fraction, as an alternative to size exclusion chromatography [9]. The set-up methodology was then applied to examine the proteome of human urinary exosomes as a non-biased approach to investigate protein-level changes in DN in response to physio/pathological conditions. Urinary exosomes from DN patients and healthy subjects were isolated, and their proteomes were analyzed and quantitatively compared by spectral counting LC–MS/MS analysis in an Orbitrap mass spectrometer, which provided sufficient sensitivity and specificity for this approach [12].

2. Materials and methods

2.1. *Patient recruitment and urine collection*

Urine samples from DN patients in advanced disease stages (CKD stages III–V) who did not present other severe diseases (i.e., lung or liver diseases, infectious diseases, or cancer) were collected at the nephrology department of IIS-Fundación Jiménez Díaz, Madrid (Spain) (see Table 1). Sample collection procedures were in accordance with the Helsinki declaration and were approved by the institution's ethics committee. All subjects received all appropriate information and signed a confidentiality agreement. A total of 5 patients were recruited for the discovery phase (LC–MS/MS analysis) together with a group of 5 sex- and age-matched healthy donors with estimated glomerular filtration rate >60 mL/min/1.73 m^2 and urinary albumin/creatinine ratio <30 mg/g who had no history of diabetes, hypertension, or renal or inflammatory disease and at the time of the study were not receiving any medication known to interfere with the studied variables (see Table 2). An additional cohort was recruited, consisting of 3 different patients and 3 controls other than those used in the discovery stage.