Biochimica et Biophysica Acta xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

An in-depth comparison of the male pediatric and adult urinary proteomes ☆

John W. Froehlich [a,d], Ali R. Vaezzadeh [a,d], Marc Kirchner [b,d], Andrew C. Briscoe [a,d], Oliver Hofmann [c], Winston Hide [c], Hanno Steen [b,d,*,1], Richard S. Lee [a,d,**,1]

[a] Boston Children's Hospital, Urological Diseases Research Center, Department of Urology, Harvard Medical School, Boston, MA, USA
[b] Department of Pathology, Boston Children's Hospital and Harvard Medical School, Boston, MA, USA
[c] Department of Biostatistics, Harvard School of Public Health, Boston, MA, USA
[d] Proteomics Center at Boston Children's Hospital, Boston, MA, USA

ARTICLE INFO

Article history:
Received 11 February 2013
Received in revised form 29 April 2013
Accepted 10 May 2013
Available online xxxx

Keywords:
Pediatric urinary proteome
Urinary proteomics
Mass spectrometry
Spectral counting

ABSTRACT

In this study, we performed an in-depth characterization of the male pediatric infant urinary proteome by parallel proteomic analysis of normal healthy adult (n = 6) and infant (n = 6) males and comparison to available published data. A total of 1584 protein groups were identified. Of these, 708 proteins were identified in samples from both cohorts. Although present in both cohorts, 136 of these common proteins were significantly enriched in urine from adults and 94 proteins were significantly enriched in urine from infants. Using Gene Ontology, we found that the infant-enriched or specific subproteome (743 proteins) had an overrepresentation of proteins that are involved in translation and transcription, cellular growth and metabolic processes. In contrast, the adult enriched or specific subproteome (364 proteins) showed an overexpression of proteins involved in immune response and cell adhesion. This study demonstrates that the non-diseased male urinary proteome is quantitatively affected by age, has age-specific subproteomes, and identifies a common subproteome with no age-dependent abundance variations. These findings highlight the importance of age-matching in urinary proteomics. This article is part of a Special Issue entitled: Biomarkers: A Proteomic Challenge.

1. Introduction

Urine is a valuable body fluid for clinical proteomics [1,2]. It has the following advantages: i) readily available in large amounts; ii) can be obtained noninvasively; iii) can be repeatedly prospectively sampled from the same individual for longitudinal studies; iv) it is relatively stable [3] and v) it is less complex in comparison to other systemic body fluids, such as blood-derived body fluids[3]. The urinary proteome is comprised of proteins from ultrafiltration of plasma by the kidneys, in addition to the local secretion and/or shedding of proteins from the genitourinary tract [4], suggesting that it may reflect local and/or distal proteomic changes. Furthermore, the content of the urinary proteome can be significantly affected by normal physiologic and environmental variations [1,2]. Therefore, a detailed examination of the non-diseased, i.e. "normal" population will advance the understanding of the normal variations of the urinary proteome.

Research to define the non-diseased adult urinary proteome has been ongoing for the past decade and the number of the identified proteins has constantly increased. The vast majority of previous efforts have focused on the adult urinary proteome. Adachi et al. defined one of the most comprehensive catalogs of the adult urinary proteome with more than 1500 proteins identified in the urine from 10 healthy adult donors [5]. Marimuthu and colleagues recently found over 600 new proteins in urine by using substantial sample fractionation [6].

While there have been significant efforts to characterize the adult urinary proteome, there have been comparatively few efforts to characterize the pediatric urinary proteome outside of biomarker discovery studies [7,8]. To date, there have been no studies that determine if the pediatric urinary proteome is distinct from the adult urinary proteome. In rodents, we previously demonstrated that the urinary proteome changes drastically with normal postnatal maturation [9]. In this study we perform an in-depth analysis of the normal male infant urinary proteome and then compare it to normal healthy adult males and the current reported literature. This analysis identifies proteins that are enriched in or specific to infant urine, and proteins that are present independent of age. We identified three distinct subproteomes: 1) infant subproteome, 2) adult subproteome, and 3) common subproteome. Using spectral counting, we identified a subset of proteins in the common subproteome to be enriched or diminished based on age. To our

Abbreviations: LTQ-FT, Linear Trap Quadrupole Fourier Transform; GeLC–MS, Gel Electrophoresis Liquid Chromatography Mass Spectrometry; FDR, False Discovery Rate; GO, Gene Ontology; BiNGO, A Biological Network Gene Ontology Tool

☆ This article is part of a Special Issue entitled: Biomarkers: A Proteomic Challenge.

* Correspondence to: H. Steen, Boston Children's Hospital, Department of Pathology, 300 Longwood Avenue, Enders 1130, Boston, MA 02115, USA. Tel.: +1 617 919 2629; fax: +1 617 730 0168.
** Correspondence to: R.S. Lee, Boston Children's Hospital, Department of Urology, 300 Longwood Avenue, Hunnewell—390, Boston, MA 02115, USA. Tel.: +1 617 355 7796; fax: +1 617 730 0474.
E-mail addresses: hanno.steen@childrens.harvard.edu (H. Steen), richard.lee@childrens.harvard.edu (R.S. Lee).

[1] Equal contribution.

1570-9639/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbapap.2013.05.008

knowledge, this is the first in-depth interrogation of the normal male infant urinary proteome and demonstrates that the non-diseased urinary proteome changes between infants and adults. This information is critical to future proteomic-based clinical studies on infant urine.

2. Experimental section

2.1. Urine sample acquisition and storage

Urine samples and demographic data were obtained from a urine specimen registry at Boston Children's Hospital using an IRB approved protocol. For the adults, clean catch voided samples were obtained from six healthy adult males. All adult patients completed self-reported health questionnaires to determine eligibility. No patients had reported urologic or other major systemic abnormality. All infant urine samples were obtained via a sterile, 5 French (1.67 mm) pediatric feeding tube utilizing standardized protocols at Boston Children's Hospital. The feeding tube is approximately 1/4 the diameter of a normal pediatric male urethra. This method is the current clinical gold standard for collecting a one-time urine sample in non-toilet trained pediatric patients, because it is atraumatic and sterile. Catheterized urine samples have the lowest risk of introduction of contamination from skin or skin flora bacteria, as opposed to other methods such as a bagged urine sample. Samples were obtained from six healthy infant males undergoing minor penile surgery. Detailed medical histories were obtained from the patient record to determine eligibility. All patients were full term babies, with no prenatal or postnatal abnormalities except for a minor distal penile cosmetic abnormality. Macroscopic urinalysis and culture were negative for all samples. Samples were centrifuged at 4000 ×*g* for 60 min at 4 °C to remove debris. Protein concentration was measured by spectrophotometry (BioRad, CA) and samples were divided into 100 μg aliquots and stored at −80 °C for one time use. Table 1 details the demographic data of each individual per cohort.

2.2. Ethanol precipitation

9 volumes of cold ethanol were added to each urine aliquot, centrifuged at 2500 ×*g* for 25 min at 4 °C. All of the supernatant except for ~200 μl was removed. The pellet was resuspended in the remaining supernatant, transferred to a 1.5 ml Eppendorf tube, and then dried in the vacuum centrifuge (Speedvac, Thermo Savant) at room temperature.

2.3. One dimensional gel electrophoresis (1-DE)

100 μg of protein was resuspended in 21 μl of 100 mM ammonium bicarbonate (NH_4HCO_3) and 8 μl 4X LDS sample buffer (Invitrogen). 1 μl of 1 M DTT was added prior to incubation for 50 min at 50 °C to reduce the sample. Samples were alkylated with 2 μl of 40% acrylamide at room temperature for 10 min. Samples were heated at 70 °C for 10 min and then all samples from one age group were loaded onto a single 4–12% bis-Tris precast gel (Invitrogen). Gels were run at 100 V and stained overnight in colloidal blue as per manufacturer protocol (Invitrogen).

Each gel lane was cut into twenty-four similar sized pieces. Gel fractions were washed for 30 min in 200 mM NH_4HCO_3, pH 7.8 at 37 °C. These fractions were then dehydrated by incubation for 30 min in 200 mM NH_4HCO_3, pH 7.8/ACN (4:6 v/v) at 37 °C, followed by rehydration for 30 min in 50 mM NH4HCO3, pH 7.8 at 37 °C. Following incubation in 100% acetonitrile for 2 min, 0.1 μg of trypsin in 50 mM NH_4HCO_3, pH 7.8 was added to each sample. The proteolysis was performed overnight at 37 °C. The supernatant was subsequently recovered into microcentrifuge tubes and a second peptide extraction from these gel pieces was carried out with 0.1% trifluoroacetic acid (TFA) in 60% ACN for 5 min. Peptide-containing liquid fractions were pooled, dried under vacuum and resuspended in 25 μl 0.5% formic acid/5% Acetonitrile.

2.4. LC–MS/MS

All LC–MS/MS analyses were performed on an LTQ-FT Ultra mass spectrometer (Thermo). The LC columns (15 cm × 100 μm ID) were packed in-house (Magic C18, 5 μm, 100 Å, Michrom BioResources, into PicoTips, New Objective, Woburn, MA). Samples were analyzed with a 60 min linear gradient (0–35% acetonitrile with 0.2% formic acid) and data were acquired in a data dependent manner, in which MS/MS fragmentation is performed on the 6 most intense peaks of every full MS scan.

2.5. Database searching and validation

The 200 most intense fragment ions of each raw product ion spectrum were used for searches against the *Homo sapiens* database (2012_05) using Protein Pilot. Default search settings were utilized. A fragment ion search tolerance of 0.8 Dalton (Da) was permitted. The peptide level score cutoff for each of the runs was adjusted to ensure a 1% false discovery rate throughout the experiments [10], and proteins were regrouped to ensure parsimony. All identified proteins required at least 2 or more unique peptides within in at least one of the samples studied.

2.6. Spectral count quantification

Spectral counts were used as a surrogate measure of protein abundances [11]. We implemented a data analysis method that accounts for variability between samples to normalize relative protein abundance. Briefly, the total number of tandem MS spectrum matching peptides from a protein was divided by the total sum of the spectral count values in that sample. The normalized spectral counts for each protein were then compared across the infant and adult cohorts. Normalization is particularly important in urine due to a potentially high level of variation between individuals [12]. Statistically significant proteins were

Table 1
The demographic information and number of proteins and peptides identified in each of the (A) adult and (B) infant samples. Cohort-wide numbers of unique proteins and peptides are also reported along with their % coefficient of variations (*cv*) and the mean ages of each cohort.

	Adult 1	Adult 2	Adult 3	Adult 4	Adult 5	Adult 6	Adult total*	*cv*
Age (years)	23	31	33	36	32	35	31.67 (mean)	–
Proteins	547	435	609	521	599	629	936	12.9
Peptides	3912	2934	4582	3786	4775	5628	10,170	21.8
Spectra	13,840	9889	16,333	15,888	21,725	25,159	102,834	
	Infant 1	Infant 2	Infant 3	Infant 4	Infant 5	Infant 6	Infant total*	*cv*
Age (years)	0.8	0.6	1.5	2.1	0.5	0.7	1.03 (mean)	–
Proteins	640	572	878	483	644	693	1356	20.3
Peptides	5625	3956	7745	4164	5340	6315	17,569	25.5
Spectra	19,063	12,760	33,066	19,079	14,298	15,355	113,621	

Please cite this article as: J.W. Froehlich, et al., An in-depth comparison of the male pediatric and adult urinary proteomes, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbapap.2013.05.008