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Short communication

SELDI-TOF MS detection of urinary hepcidin

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ABSTRACT

Hepcidin is a 25-residue hepatic peptide that regulates iron absorption from the diet and tissue iron distribution. Inappropriately low Hepcidin expression is implicated in the pathogenesis of hereditary hemochromatosis and iron-loading anemias, like the thalassemias. Increased hepcidin expression mediates iron retention in the anemias of inflammation and plays a pathogenic role in iron-refractory iron-deficiency anemia (IRIDA). Because of its clinical importance, Hepcidin is expected to be a useful biomarker for diagnosis and management of iron-related disorders. So far an ELISA for human hepcidin and SELDI-TOF-MS based approaches have been applied to monitor urinary and/or serum hepcidin levels. Here we report a modified protocol for SELDI-TOF based detection of human, urinary hepcidin. We show that CM10 Proteinchips are superior to NP20 Proteinchips commonly used in previously reported protocols to sensitively and accurately detect urinary hepcidin. Application of this modified hepcidin assay accurately detects increased hepcidin levels in the urine of sepsis patients.

1. Introduction

Hepcidin (Hamp) is a 25 aminoacid liver-derived peptide essential to maintain iron homeostasis [1]. Additionally, hepcidin is active as an antimicrobial peptide against fungi, gram positive and gram negative bacteria [2]. Hepcidin regulates iron export from enterocytes and macrophages by binding the only known iron export protein, ferroportin [3]. It is synthesized in the liver as an 84-amino acid pre-pro-hormone and matured by proteolysis through a furin cleavage to generate the biologically active 25-aa peptide (Hamp-25) that is secreted into circulation [4]. Hamp-25 leaves the body via secretion through the kidneys [5].

In contrast to the serum, where almost exclusively Hamp-25 (2789.4 Da) is detectable, urine also contains N-terminally truncated peptides of 22 aminoacids (Hamp22; 2436 Da) and 20 aminoacids (Hamp20; 2191.7 Da) [2,5]. Structurally, Hepcidin shows a secondary amphipathic structure and is particularly rich in basic amino-acids that confer a positive charge with an isoelectric point of 8.2 [2]. The eight cysteine residues contained within Hepcidin form four disulfide bridges [5].

Dependent on body iron requirements hepcidin expression must be balanced to prevent iron overload or deficiency. Inappropriately low hepcidin expression causes iron overload in hereditary hemochromatosis due to mutations in HFE, TfR2, HJV or hepcidin as well as in iron-loading anemias such as the thalassemias [6]. By contrast, inappropriately high hepcidin levels as a consequence of mutations in a hepcidin suppressor (TMPRSS6) cause iron-refractory iron-deficiency anemia (IRIDA) [1]. Hepcidin further is a type-II acute-phase protein, whose expression is induced by inflammatory cytokines. It thus contributes to the frequently observed 'anemia of inflammation' which is associated with chronic disease [7].

Because hepcidin levels reveal important clinical information about pathological states measurement of hepcidin in urine or plasma as a biomarker may be interesting to apply in clinical practice. Despite significant efforts, the development of a robust assay to detect hepcidin proved to be problematic. Initially, hepatic hepcidin mRNA expression in the liver was successfully used as a marker, but liver biopsies are rarely clinically indicated. Immunoassays are commercially available for prohepcidin (DRG Diagnostics, Marburg, Germany), however, the assay does not recognize the biologically active Hamp-25 [8]. By contrast, the only available immunoassay against Hamp-25 is not widely available yet as a commercial product [9]. Therefore, several groups have developed assays to detect Hamp-25 based on mass spectroscopy [10–14]. Based on these protocols we recently established the surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry for Hamp-25 in our lab. To further optimize the detection of Hamp-25 for clinical application we compared two ProteinChip surfaces, a weak cation exchange surface (CM10) and a normal phase surface (NP20) for sensitivity

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and linearity of Hamp-25 detection. We then applied our improved protocol on urine collected from sepsis patients to successfully detect elevated urinary Hamp-25 expression.

2. Materials and methods

2.1. Patients

Urine from 8 patients suffering from severe sepsis caused by systemic bacterial infection who were followed at the University Hospital Heidelberg was included in the analysis. Urine was collected after initial antibiotic treatment by using a conventional catheterization-system which was applied during the stay on the ICU. At the time when the samples were taken all patients had increased or decreased white blood cell-counts, highly elevated CRP (measured in the Analysezentrum of the University of Heidelberg), impaired circulation-parameters and increased or decreased body-temperature.

As controls midstream-urine was taken from healthy volunteers (7 males and 5 females). The urine was collected in the morning. Assays for urea and creatinine were performed as part of the standard routine hospital analysis (Analysezentrum Heidelberg).

2.2. Sample preparation and SELDI measurement

Urine samples from sepsis patients and healthy volunteers were aliquoted and stored at −80 °C. Samples were thawed, centrifuged at 3000g for 5 min and supernatants were directly used for SELDI analysis using the on-spot strategy. Normal phase (NP20) and Weak Cation Exchange (CM10) 8-spot ProteinChip Arrays were tested (Bio-Rad, Hercules, CA).

The NP20 array was pre-rinsed with 10 μL of ultrapure H_2O (Braun – Melsungen, Germany) and 7 μl urine was subsequently applied on each spot. CM10 arrays were activated with 10 μL of 10 mM HCl for 10 min. 7 μL urine samples were preincubated for 5 min with 3 μl ammonium acetate buffer (0.1M; pH6) and then applied to the CM10 ProteinChip surface.

In both cases, the urine-loaded arrays were incubated for 30 min in a humidity chamber with constant agitation. NP20 ProteinChips were then washed three times with ultrapure water (Braun – Melsungen, Germany) while CM10 ProteinChips were washed with 30 mM ammonium acetate (pH = 6) and air-dried. Then, 1 μl of energy absorbing matrix [EAM; Sinapic acid (SPA) dissolved in 50% ACN/0.5% TFA (Acetonitrile/Trifluoroacetic acid)] was added to each spot, air dried and reapplied. ProteinChips were read using a PBS IIc SELDI mass spectrometer (Bio-Rad – Hercules, CA). The instrument was externally calibrated using a standard reference for low molecular weight molecules [ACTH, Bovine insulin (Sigma) and synthetic human hepcidin (Peptides international-Louisville, KY)].

Data acquisition was performed using the following parameters: High mass was set to 50 000 Daltons, Mass optimization from 1500 to 10 000 Daltons, Laser intensity 180, Detector sensitivity 9, Mass Deflector 1500 Daltons, 2 warming shots at intensity 185 (without warming shot collection), acquisition of 50 shots every 5 positions from 27 to 87.

2.3. Peak identification and statistical analysis

Peak analysis and identification, baseline subtraction and normalization against total ion current (TIC) were performed using the Ciphergen ProteinChip® Software Version 3.2. Statistical analysis (Mann–Whitney and Spearman non-parametric test) was performed using the GraphPad Prism 4.0 software package and Microsoft Excel.

3. Results and discussion

3.1. CM10 protein arrays more accurately and sensitively detect HAMP-25

Hamp-25 contains several cationic residues and forms an amphipathic structure. We therefore compared two types of ProteinChips, a weak cation exchange (CM10) and a normal phase (NP20) surface to detect Hamp-25 in a sensitive and linear manner. Representative spectra obtained with both arrays are shown in Fig. 1A and B. Both surfaces successfully detect Hamp-25 at the expected molecular weight of 2789 Da in addition to the shorter peptides Hamp-22 (2436 Da) and Hamp-20 (2192 Da). Importantly, the CM10 ProteinChips detect the strongest signal for all three hepcidin peptides, suggesting a higher binding capacity for the hepcidin peptides.

We next investigated which ProteinChip detects Hamp-25 with the least experimental error in independent measurements. Therefore, the same urine sample was spotted three times on either CM10 or NP20 ProteinChips. As shown in Fig. 1C and D the standard deviation of independent measurements was smaller using the CM10 ProteinChip as represented by the error bars and by comparison of the standard deviation values of 12.9% for the CM10 ProteinChip versus 70% for the NP20 ProteinChip. We next investigated the linearity of the Hamp-25 signal of the same undiluted sample, together with serial dilutions of the urine using PBS in triplicate with both CM10 and NP20 arrays. While we did not detect an absolute linearity between dilution factor and signal intensity, the signal intensities obtained on the CM10 protein surface best reflected upon the fold dilution of the urine. Taken together our data show that hepcidin is sensitively and accurately detected by the SELDI-TOF-MS assay in the urine, that measurements show good reproducibility between independent experiments and that linearity between sample dilution and signal intensity is sufficiently high to semi-quantitatively detect Hamp-25 levels.

3.2. Normalization of hepcidin signals to creatinine and urea

To correlate arbitrary signal intensity units measured by SELDI-TOF mass spectrometry with specific amounts of hepcidin, we established a calibration curve by using serial dilutions of synthetic Hamp-25 (44 pg–350 pg; Peptides International-Louisville, KY) on CM10 ProteinChip Arrays. As shown in Fig. 2A, there is a strong linear correlation between hepcidin concentration and signal intensity detected by SELDI-TOF measurements ($R^2 = 0{,}93$) in line with previously reported results using IMAC-30 ProteinChips [14].

The amount of urine produced by an individual depends on several factors including the state of hydration, activity, size and health. We therefore assessed two urinary markers, creatinine and urea, for normalization of the hepcidin signal. We screened the morning urine of 12 healthy volunteers (7 males and 5 females, medium age 28 years) for hepcidin levels using CM10 ProteinChip Arrays. Based on our previous optimizations (Fig. 1C) we opted for a 33% dilution of the urine samples in PBS to reach the optimal detection range for hepcidin. Hepcidin levels were measured in arbitrary signal units and then converted to hepcidin values (in pg) using the external reference as depicted in Fig. 2A. We then normalized the data to the respective creatinine (mg/dl) or urea (mg/l) values. As shown in the Fig. 2B and C, both creatinine as well as urea values directly correlate with the hepcidin signals. Statistical analysis using the non-parametric Spearman test indicates correlation with a P-value of 0.0026 for creatinine ($R = 0.7832$) and 0.0051 for urea ($R = 0.7483$). This experiment suggests that in principle both creatinine and urea values are suitable to normalize for hepcidin levels in the urine.