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Mass spectrometric characterization of human serum albumin dimer: A new potential biomarker in chronic liver diseases☆

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ABSTRACT

Human serum albumin (HSA) undergoes several structural alterations affecting its properties in pro-oxidant and pro-inflammatory environments, as it occurs during liver cirrhosis. These modifications include the formation of albumin dimers. Although HSA dimers were reported to be an oxidative stress biomarker, to date nothing is known about their role in liver cirrhosis and related complications. Additionally, no high sensitive analytical method was available for HSA dimers assessment in clinical settings.

Thus the HSA dimeric form in human plasma was characterized by mass spectrometry using liquid chromatography tandem mass spectrometry (LC-ESI-Q-TOF) and matrix assisted laser desorption time of flight (MALDI-TOF) techniques. N-terminal and C-terminal truncated HSA, as well as the native HSA, undergo dimerization by binding another HSA molecule. This study demonstrated the presence of both homo- and hetero-dimeric forms of HSA. The dimerization site was proved to be at Cys-34, forming a disulphide bridge between two albumin molecules, as determined by LC–MS analysis after tryptic digestion. Interestingly, when plasma samples from cirrhotic subjects were analysed, the dimer/monomer ratio resulted significantly increased when compared to that of healthy subjects. These isoforms could represent promising biomarkers for liver disease. Additionally, this analytical approach leads to the relative quantification of the residual native HSA, with fully preserved structural integrity.

1. Introduction

Human serum albumin (HSA) is one of the most studied human proteins, nevertheless its different and multiple functions have attracted and continue to attract the interest of researchers.

In healthy individuals HSA is the most abundant plasma protein with normal values of approximately 40–55 g/L, thus accounting for 50–60% of the measured serum protein [1].

Because of its high intravascular concentration and its strong negative net charge, HSA plays a pivotal role in modulating the distribution of fluids between body compartments [2]. Besides its oncotic capacity, HSA presents other biological properties, such as antioxidant and scavenging activities, binding and transport of many endogenous and exogenous substances, and regulation of endothelial function [1–5]. Clinically, albumin has been employed as a plasma expander in different clinical fields, including acute liver failure and chronic liver disease [6].

HSA is a monomeric multi-domain macromolecule consisting of 585 amino acids, with a molecular mass based on an amino acid sequence of 66,438 Da [7]. The cysteine residue at position 34 (Cys-34) is the only free cysteine of the HSA molecule. It is characterized by a strong nucleophilicity [8] and, because of the high HSA plasma concentration and relatively long half-life, represents the most important scavenger of reactive oxygen species (ROS) in the extracellular compartment [4,8].

HSA is already considered a valuable biomarker in many chronic diseases, such as cancer, liver disease, rheumatoid arthritis, ischemia and post-menopausal obesity [1]. Several post-translational modifications (PTM), possibly affecting the HSA non-oncotic function [9], contribute to the micro-heterogeneity of the molecule in the vascular compartment both in healthy and diseased state [10–14].

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For this reason, in recent years, the interest of researcher is focused on the characterization of HSA structural and functional alterations, particularly those affecting the Cys 34, considered as one of the most promising and innovative biomarkers in different clinical settings [9].

Oxidative stress was far considered as leading event for the HSA structural impairment. Indeed we recently reported that, during cirrhosis, a condition in which oxidative stress play a pivotal role, the proportion of the native unchanged HSA isoform was far lower than the absolute albumin concentration; this is due to the extensive structural changes occurring to the molecule which lead to several additional HSA isoforms [9]. Recently it was reported that, in patients undergoing haemodialysis, the HSA molecule undergoes dimerization process. The resulting complex was hypothesized to be a valuable biomarker of oxidative stress [15]. However, the semi-quantitative determination of HSA dimers used in this work makes difficult its application in the routinely clinical practice. Additionally, to date nothing is known about the chemistry of the dimerization process and therefore its possible impact on the HSA functioning.

The aims of the present work were: first, to tuning-up a valuable analytical approach based on liquid chromatography tandem mass spectrometry (LC–MS) and matrix assisted laser desorption-time of flight (MALDI-ToF) for the detection and relative quantification of albumin dimers in plasma samples; second, to assess which molecular sites were involved in this process and eventually the resulting functional alteration; finally, to establish the role of oxidative stress in promoting the HSA dimerization through *in vitro* experiments and to perform a preliminary assessment of albumin dimers in plasma samples from cirrhotic patients by native protein gel electrophoresis followed by an HPLC-ESI-MS determination of the same complexes.

2. Experimental

2.1. Patients

Peripheral blood samples were obtained from ten cirrhotic patients (mean age 64 ± 11 years; 7 male) enrolled among those admitted to our Hepatological Unit for the onset of a clinical complication of the disease (mean Model for End stage Liver Disease: 17.5 ± 5.4). Exclusion criteria were onco-haematological comorbidities and albumin infusion within the last month. Additionally ten age and sex matched healthy volunteers were enrolled.

At the time of inclusion in the study, 9 mL of peripheral blood were withdrawn from the brachial vein and collected in a sterile tube containing ethylenediaminetetraacetic acid (EDTA); the samples were then centrifuged at 3000 rpm for 10 min to obtain plasma samples. Informed written consent was obtained from both patients and controls; the study protocol was approved by the ethical committee of our institution and was carried out according to the 1975 Declaration of Helsinki.

2.2. Plasma sample preparation

Plasma samples were diluted 1:100 with water and filtered with a 0.22 μm filter (Merck KGaA, Darmstadt, Germany).

For the oxidation experiments, plasma (99 μL), without any previous dilution, was incubated at 37 °C with and without *tert*-butyl hydroperoxide 20 and 200 mM (1 μL). At the expected time 2 μL of the solution were diluted with water (1:100), filtered and injected in the LC–MS system.

2.3. HSA dimeric form characterization by LC–MS analyses

For the LC–MS analysis of plasma, the method we previously reported [10] was employed and slightly adapted. The chromatographic separation of HSA from other plasma proteins was performed on Agilent 1200 HPLC System (Walbronn, Germany) by using a Phenomenex Jupiter C4 column (5 μm, 300 Å, 150 mm × 2.0 mm i.d.). Mobile phases A [water/acetonitrile/formic acid (99/1/0.1, v/v/v)] and B [acetonitrile/water/formic acid (98/2/0.1, v/v/v)] were used to develop a gradient. The solvent gradient was set as follows: 10–70% B, 15 min; 70% 3 min; 70–90% B, 1 min; 90–90% B, 1 min and 90–10% B, 2 min. The flow rate was set at 0.15 mL/min. The column was equilibrated with the mobile phase composition of the starting conditions for 10 min before the next injection. The injection volume was 5 μL.

Mass spectrometry analysis was carried out on a Quadrupole-Time of Flight hybrid analyser (Q-ToF Micro, Micromass, Manchester, UK) with Z-spray electrospray ion source (ESI). The ESI-Q-ToF source temperature was set at 100 °C, the capillary voltage at 3.5 kV and the cone voltage at 42 V. The scan time was set at 2.4 s and the inter scan time at 0.1 s. The cone gas flow was set at 100 L/h and the desolvation gas at 500 L/h. The mass chromatograms were recorded in total ion current (TIC), within 1000 *m*/*z* and 1700 *m*/*z*. The HSA baseline-subtracted spectrum (*m*/*z* 1084–1534) was deconvoluted onto a true mass scale using the maximum entropy (MaxEnt1)-based software supplied with MassLynx software. Output parameters were as follows: mass range 61,500–138,000 Da and resolution 2 Da/channel. The uniform Gaussian model was used, with 0.5 Da width at half height. The isoforms relative percentage abundance was calculated by dividing the isoform intensity obtained from the deconvoluted and baseline-subtracted spectrum by the sum of all isoform intensities and multiplying them by 100. Data were analysed by Excel.

2.4. MALDI-ToF analysis

MALDI-ToF MS analysis was performed using a Voyager DE Pro (Applied Biosystems, Foster City, CA) equipped with a pulsed N2 laser operating at 337 nm. Positive ion spectra were acquired in linear mode over an *m*/*z* range from 50,000 to 200,000 using a 25,000 V accelerating voltage, a 22,000-V grid voltage, and a delay extraction time of 150 ns. The spectrum for each spot was obtained by averaging the result of 120 laser shots. External mass calibration was performed using the single- and double-charged ions of bovine serum albumin (Sigma–Aldrich). The analysis was performed by spotting on the target plate 1 μL of the sample mixed with an equal volume of the matrix solution, 30 mg/mL sinapinic acid in 1:1 (v/v) ACN/H_2O containing 0.1% (v/v) trifluoroacetic acid (TFA).

Plasma was analyzed by MALDI-ToF analysis both after dilution (1:10) with water and after ultrafiltration procedure. For the ultrafiltration, Amicon Ultra with 3 K cut-off (Millipore) were employed. In detail, 50 μL of plasma were diluted to 500 μL with water and ultra-filtrated at 4 °C, 4000 rpm for 1 h. A second washing step with 400 μL of water was performed. The concentrated and purified sample was finally subject to MALDI-ToF analysis.

2.5. HSA tryptic digestion and LC–MS analysis

To identify the binding site for the HSA dimer formation, plasma sample from a cirrhotic patient containing approximately 95 μg of HSA was diluted 1:10 with 10 mM ammonium bicarbonate, pH 8.0 and digested using trypsin at a mass ratio of 1:50 enzyme/protein overnight at 37 °C. The hydrolysis was stopped by addition of 1% formic acid (FA) and the sample was analysed by two-dimensional chromatographic approach and mass spectrometric detection.

The first dimension RP-LC separation was performed on Agilent 1200 HPLC System (Walbronn, Germany) by using a Zorbax Extend-C18 RP column (3.5 μm, 80 Å, 100 mm × 2.1 mm i.d.). Mobile phases A [water/acetonitrile (98/2, v/v), adjusted pH to 10.0 using ammonium hydroxide] and B [acetonitrile/water (98/2, v/v), adjusted pH