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Mass spectrometric characterization of limited proteolysis activity in human plasma samples under mild acidic conditions

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ABSTRACT

We developed a limited proteolysis assay for estimating dynamics in plasma-borne protease activities using MALDI ToF MS analysis as readout. A highly specific limited proteolysis activity was elicited in human plasma by shifting the pH to 6. Mass spectrometry showed that two singly charged ion signals at *m*/*z* 2753.44 and *m*/*z* 2937.56 significantly increased in abundance under mild acidic conditions as a function of incubation time. For proving that a provoked proteolytic activity in mild acidic solution caused the appearance of the observed peptides, control measurements were performed (i) with pepstatin as protease inhibitor, (ii) with heat-denatured samples, (iii) at pH 1.7, and (iv) at pH 7.5. Mass spectrometric fragmentation analysis showed that the observed peptides encompass the amino acid sequences 1–24 and 1–26 from the N-terminus of human serum albumin. Investigations on peptidase specificities suggest that the two best candidates for the observed serum albumin cleavages are cathepsin D and E. Reproducibility, robustness, and sensitivity prove the potential of the developed limited proteolysis assay to become of clinical importance for estimating dynamics of plasma-borne proteases with respect to associated pathophysiological tissue conditions.

1. Introduction

Free micro-vascular tissue transfer has become the gold standard to cover large or complex tissue defects of the human body that occur after trauma, upon infection, by tumor removal, or due to congenital deformities. In spite of substantial progress in the field, the rate of flap failure still lies between 2% and 10% of all operations [1]. Up to 25% of the transferred tissue has to be revised in a second operation because of complications [2–4]. Complications are mostly caused by thrombotic closure of the afferent arterial or the efferent venous vessels of the transplant. Common reasons of thrombotic closure are technical problems like kinking of blood vessels and pairing disproportionate vessel diameters of donor and recipient sites [5]. Once the perfusion of the transplant is impaired the success of the whole procedure is at stake. A partial or complete loss of transplant (flap failure) is a demanding situation for both, the patient and the surgeon. Free tissue transfer pathophysiologically involves prolonged duration of ischemia during transplantation that can potentially harm the tissue and threaten the success of the operation [6]. Apart from ischemia, the so-called "ischemia or reperfusion (I/*R*) injuries" are unavoidable. Here, a local inflammatory reaction starts after an episode of ischemia as soon as the blood flow is restored [7,8].

Experience shows that the information obtained via clinical inspection of a transplanted flap is often not sufficient and valuable time might be lost for rescuing the transplant, e.g. once the perfusion of the transplant is impaired. A short time interval between

Abbreviations: DHB, 2,5-dihydroxybenzoic acid; FA, formic acid; TFA, trifluoroacetic acid; ACN, acetonitrile; MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; ECD, electron capture dissociation; CID, collisional induced dissociation; ToF, time of flight; QIT, quadrupole ion trap; Q, quadrupole; ISD, in-source decay; FT-ICR, Fourier transform-ion cyclotron resonance; RF, radio frequency; FWHM, full width at half maximum; MRM, multiple reaction monitoring; HSA, human serum albumin.

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impairment of perfusion and revision surgery reduces flap failure. Hence, the focus of clinical interest is on the development of monitoring procedures that can detect problems of transplant perfusion as early as possible. Monitoring tissue-related processes on the molecular level should enable to assess individual transplant quality at a much earlier time, i.e. when clinical signs are not yet visible. We hypothesized that lysosomal and endosomal proteases such as cathepsin D and cathepsin E could be released from tissue during ischemia into the blood stream, similar to events that have been reported in connection with severe tissue damage [9]. As lysosomal or endosomal proteases have pH optima of approx. 5.5–6.5 [10], we intended to initiate the assumed proteolytic activity in plasma by dropping the pH to 6, despite the fact that the samples of interest should normally not have suffered from such severe injuries. An ideal measure for monitoring flap quality is studying plasma proteins from blood that passed through the transplanted tissue because plasma protein compositions reacts dynamically with respect to tissue condition changes.

With the ability to investigate multiple plasma protein abundances in parallel, mass spectrometry has become an attractive approach for studying such precious clinical samples [11,12]. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI ToF MS) is an advanced technique that can detect hundreds of peptide and/or protein ion signals from body fluids in one measurement with good reproducibility and robustness. Hence, MALDI ToF MS has enabled the multi-parametric analysis of the reservoir of proteins derived from high-abundant endogenous circulating molecules. Plasma proteome profile analyses by mass spectrometry encompass determination of abundance differences of intact plasma proteins using affinity-enrichment methods in combination with MALDI mass spectrometry [12–15]. Since mass spectrometric protein analyses are capable to monitor dynamic changes of protein/peptide abundances in plasma they are ideal for discovery of novel biomarkers. Another advantage of mass spectrometric proteome analysis is that they do not require prior knowledge of the pathophysiological mechanisms underlying the condition of interest as long as the signature of recorded signals remains differential [16]. An alternative approach is peptide-based quantitative analysis of plasma proteins by MRM mass spectrometry upon enzymatic digestion of plasma proteins [17–20] when target proteins are known. As a consequence, mass spectrometric measurements of the entire set of protein abundances or abundance changes in biological fluids allow the representation of proteome signatures for a particular pathology.

In this paper we describe the development of a fast and reliable assay for plasma profiling by which limited proteolysis events are monitored by MALDI mass spectrometry upon mild acidification. Both, substrate and protease that are linked to the observed peptide products in plasma have been identified and characterized in a systematic fashion.

2. Materials and methods

2.1. Clinical specimen collection

The study was approved by the Institutional Review Board of the University Hospital Zurich, Switzerland, and blood samples were taken after written informed consent was given by all donors. Samples were provided by the Division of Cranio-Maxillo-Facial and Oral Surgery and by the Division of Plastic Surgery and Hand Surgery of the University Hospital Zurich. Blood samples were taken intraoperatively. Human blood (1.5 ml each) was taken from the free flap tributary artery before anastomosis as well as from the effluent vein after anastomosis by using S-Monovette® Lithium Heparin syringes (Monovette®, Sarstedt, Germany) during free flap transplantation. Blood samples were immediately subjected to sedimentation of blood cells by centrifugation at 2000 g at room temperature for 15 min. Plasma was aspirated, sterile filtered (0.2 μm pore size) and divided into aliquots (100 μl each) [21]. Aliquots were stored at −80 °C prior to further analysis.

2.2. Analysis of intrinsic proteolytic activity in human plasma

A volume of 10 μl of plasma was diluted with 90 μl 0.1% TFA, reaching a final pH of 6.0. Heat denaturing was done by heating 10 μl of 1:10 with 0.1% TFA diluted plasma for 10 min at 50 °C and 75 °C, respectively, directly after mixing. Inhibition of proteolysis was done by adding 0.8 μl Pepstatin A solution (0.048 mg/ml in EtOH, Sigma Aldrich, Taufkirchen, Germany) to 40 μl of 1:10 with 0.1% TFA diluted plasma, directly after dilution. Acidification to a final pH of 1.7 was achieved by adding 40 μl DHB solution (5 mg/ml in ACN/0.1% TFA in H_2O (33/67 v/v), Sigma Aldrich) to 16 μl of diluted plasma (1:10 with 0.1% TFA). Preparation of a diluted plasma solution with pH 7.5 was accomplished by adding 90 μl of 50 mM NH_4HCO_3 to 10 μl of 1:10 with 0.1% TFA diluted plasma and by adding 40 μl DHB solution (5 mg/ml in ACN/0.1% TFA in H_2O (33/67 v/v), Sigma Aldrich). Samples were incubated for 0, 5, 10, 20, 40, 80, 160 min, and up to 16 h, respectively. Each time-course experiment was performed in duplicate.

2.3. Mass spectrometric profiling of human plasma peptides

For MALDI MS analysis, 0.8 μl of diluted plasma of each time point was mixed with 2 μl matrix solution (5 mg/ml DHB in ACN/0.1% TFA (33/67, v/v)) on an MTP Anchor Chip 600/384 target (Bruker Daltonics, Bremen, Germany). After drying on air, 0.8 μl DHB matrix solution was added to the peptide mixture and dried on air. A further 0.8 μl of DHB solution was added and the preparation was allowed to dry again. Samples from each time point were prepared in duplicate for recording two measurements, each. Peptide mixtures were analyzed with a Reflex III MALDI ToF mass spectrometer (Bruker Daltonics) equipped with a SCOUT source and operated in high vacuum ($2–3 \times 10^{-7}$ hPa). Acceleration voltage was set to 20 kV [22]. In positive ion reflector mode mass range was set to m/z 680–3500. Then, 400 laser shots were added up for recording one spectrum. For measurements in positive linear mode (mass range m/z 800 to 9000) 1000 laser shots were summed up per spectrum. Measurements were externally calibrated using either the Peptide Calibration Standard I (Bruker Daltonics) which contains bradykinin 1–7 ($[M+H]^+$ 757.40), angiotensin II ($[M+H]^+$ 1046.54), angiotensin I ($[M+H]^+$ 1296.68), substance P ($[M+H]^+$ 1347.74), bombesin ($[M+H]^+$ 1619.82), renin substrate ($[M+H]^+$ 1758.93), ACTH clip 1–17 ($[M+H]^+$ 2093.09), ACTH clip 18–39 ($[M+H]^+$ 2465.20), and somatostatin 28 ($[M+H]^+$ 3147.47), or with Peptide Calibration Mix II (LaserBio Labs, Sophia-Antipolis, France) which contains bovine insulin B chain ($[M+H]^+$ 3495.9), bovine insulin ($[M+H]^+$ 5733.6), aprotinin ($[M+H]^+$ 6511.5), and bovine ubiquitin ($[M+H]^+$ 8564.8). Mass spectra were processed and analyzed using the FlexAnalysis 2.4 and BioTools 3.0 programs (Bruker Daltonics).

2.4. Semi-quantitative analysis of MALDI mass spectra

The on-average most robust ion signal in all mass spectra was observed at m/z 1933.99. The ion signals with varying intensities over time were detected at m/z 2753.44 and m/z 2937.56, respectively. Quotients of the intensities of the ion signals at m/z 2753.44/1933.99 were formed and the resulting ratio values were determined for each time point. Double measurements were averaged and standard deviations were calculated as previously reported [12]. The Origin statistics software (version. 6.1G; OriginLab