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Shotgun analysis of plasma fibrin clot-bound proteins in patients with acute myocardial infarction

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ABSTRACT

Introduction: The presence and amount of the proteins within a plasma clot may influence clot properties, like susceptibility to fibrinolysis, however, the clot proteome has not yet been extensively described. The aim of the study was to investigate the protein composition of clots of four patients with acute myocardial infarction (AMI) in two time points: in the acute ischemic phase and two months later during the standard therapy.
Materials and Methods: Shotgun proteomic method (2DLC-MS/MS) was used to investigate time-dependent protein composition changes of clots prepared *ex vivo* from citrated plasma of the peripheral blood of patients with AMI.
Results: Proteomic analysis revealed a total number of 62 proteins identified in all 8 samples grouping into several distinct functional clusters (e.g. cholesterol transporter activity, immunoglobulin binding and peptidase regulatory activity). The protein signatures of clots differed significantly depending on time after ACS, showing 30% greater variability in protein composition of the clots prepared in the plasma two months after the onset of AMI. Several proteins potentially involved in clot formation and resolution showed an interesting pattern of changes over time.
Conclusion: We provided the first qualitative analysis of proteomes of fibrin clots generated *ex vivo* in plasma taken from patients with AMI showing differences between clots generated in the acute ischemic phase and those prepared two months later. It might be hypothesized that differences involving proteins of potential influence on within-clot fibrinolysis and clot stability may partially explain time-dependent changes in the clots structure and firmness in patients with AMI.

Introduction

Acute myocardial infarction (AMI) results from rupture or erosion of an atherosclerotic plaque with the subsequent formation of thrombus leading to partial or complete occlusion of the coronary artery [1]. Occluding thrombi contain large amounts of fibrin and bloods cells, in particular platelets and erythrocytes. The composition of intracoronary thrombus evolves over time with the increase in fibrin content and decrease in platelet content, as evidenced by scanning electron microscopy [2]. Importantly, a highly variable structure of a fibrin clot has a major impact on fibrinolysis that determines the patency of vessels [3,4]. More dense, less porous plasma fibrin clot structure associated with attenuated fibrinolysis has been shown in patients with AMI [5]. The prothrombotic plasma fibrin clot phenotype within the first hours of myocardial ischemia is at least in partly related to enhanced inflammatory state, oxidative stress and platelet activation [5].

It has been demonstrated that fibrin clot network binds in a covalent and non-covalent manner a large variety of proteins, including α_2-antiplasmin, serotransferrin, vitronectin and fibronectin [6–8]. From a functional point of view, binding of fibrinolysis activators and inhibitors is of vital importance for clot stability and persistence in the vessels. It is likely that such specific composition of bound proteins within a clot varies among individuals and undergoes transient changes in disease states. The presence and amount of the proteins within a plasma clot may influence clot properties, like susceptibility to fibrinolysis, however, the plasma clot proteome has not yet been extensively described. The application of proteomic technologies represents novel approach to multifaceted plasma clot analysis [9]. Recently, Talens et al. using two-dimensional electrophoresis (2DE) with the subsequent liquid chromatography – mass spectrometry (LC-MS) analysis, classified the

Abbreviations: AMI, acute myocardial infarction; ACS, acute coronary syndrome; 2DE, two-dimensional electrophoresis; LC-MS, liquid chromatography – mass spectrometry; HDL, high-density lipoprotein; STEMI, ST segment elevation myocardial infarction; NSTEMI, non-ST segment elevation myocardial infarction; CK-MB, serum creatine kinase MB; CRP, C-reactive protein; 2DLC, two-dimensional liquid chromatography; FASP, Filter Aided Sample Preparation; ACN, acetonitryle; TFA, trifluoroacetic acid; DTT, dithiothreitol; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1- propanesulfonate; SCX, strong cation exchange; MS/MS, tandem mass spectrometry; HCD, Higher energy Collisional Dissociation; TPP, Trans-Proteomic Pipeline.

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non-covalently bound proteins of plasma clots formed *in vitro* from plasma of healthy volunteers to three functional groups, e.g. blood coagulation, protease inhibition and High-Density Lipoprotein (HDL) metabolism [10]. Interestingly, two-thirds of the newly identified proteins were associated with HDL particles, which led to the hypothesis that the HDL presence in the clot may be of some importance for clot structure and function, as suggested by data on increased clot permeability and susceptibility to lysis in subjects with elevated HDL cholesterol concentrations [11]. To our knowledge, there have been no data on protein composition of plasma fibrin clots in patients with cardiovascular diseases, in particular in those with AMI.

This work was aimed to investigate the protein composition of clots prepared ex vivo from citrated plasma of the peripheral blood of patients with AMI using the shotgun proteomic method. We compared protein compositions of fibrin clots in the acute ischemic phase and 2 months later during the standard therapy, when potent activation of blood coagulation is suppressed by natural inhibitory mechanisms and/or therapy introduced.

Methods

Patients

Citrated plasma was obtained from four patients admitted to the hospital for symptoms typical of AMI and at least one of the following: persistent ST-segment or T-wave abnormalities, elevated cardiac troponin, or creatine kinase-MB; or documented coronary disease [12]. The exclusion criteria were: acute cardiovascular event within the last 3 months, heart failure, serum creatinine >2 mg/dL, known cancer, severe liver injury, diabetes, autoimmune disease, known alcohol abuse, treatment with oral anticoagulants, time delay > 24 h from symptom onset, cardiogenic shock previous coronary artery bypass surgery. All patients took aspirin at least 1 hour before the study. None of the subjects received thienopyridines prior to blood collection.

This study was approved by the Bioethics Committee of the Jagiellonian University. All patients gave written informed consent.

Laboratory Investigations

Blood samples were drawn from an antecubital vein with minimal stasis within 15 minutes upon admission, prior to primary coronary angioplasty. The patients were drawn within the first 12 hours since the chest pain onset.

Another sample of venous blood was drawn in the same patients 8–12 weeks later while on standard therapy post-MI; over that period of time no recurrent ischemia or bleeding were observed in the 4 patients. All the patients declared regular drug administration. The patients received the standard therapy as recommended by the European guidelines for STEMI and NSTEMI, including aspirin 75 mg/d, clopidogrel 75 mg/d, and simvastatin 40 mg/d [13].

Lipid profiles, blood cell counts, glucose, creatinine, CK-MB and cardiac troponin T were assayed by routine laboratory techniques. Fibrinogen was determined using the Clauss method. High-sensitivity CRP was measured by immunoturbidimetry (Siemens, Marburg, Germany).

Blood samples (9:1 of 3.2% trisodium citrate) for clot permeability and proteomic analysis were centrifuged at 2560 *g* for 20 min within 20 minutes of collection, immediately frozen, and stored in aliquots at −80 °C until further use.

Plasma Clot Preparation

A fibrin clot was prepared using an assay by Pieters et al. [14]. Briefly, to 100 μL of citrate plasma was added 20 mmol/L calcium chloride and 1 U/mL thrombin (Merck, USA). This mixture was placed into plastic tubes, which were put into a wet chamber. After 120 minutes of incubation, tubes were connected to a reservoir of a buffer (0.05 mol/L Tris HCl, 0.1 mol/L NaCl, pH 7.5), which rinsed a fibrin gel for one hour.

Clots were transferred immediately to protein lysis buffer (7 M urea, 3 M tiourea, 4% CHAPS, 2% DTT with protease inhibitors) and stored at −80 °C until assayed

2DLC-MS/MS

Samples were thawed, vortexed and left at 25 °C for 30 min to ensure maximal protein solubilization, and finally centrifuged at 12,000 g for 15 min. Supernatant was harvested and the protein concentration was determined by the Bradford method [15]. Next, samples were washed by acetone precipitation, reduced, alkylated and digested with sequencing grade trypsin (Thermo Scientific, Rockford, IL USA) according to the FASP protocol [16]. Peptide maps were lyophilized, resuspended in 2% acetonitryle (ACN) and 0.1% trifluoroacetic acid (TFA) and purified with MacroSpin C18 columns. Finally, peptides were lyophilized and stored at – 80 °C. Prior to 2DLC-MS/MS analysis each sample was resuspended in 2% ACN and 0.1% FA and injected on a Poros 10S SCX column (300 μm i.d. x 25 cm, Thermo Scientific Dionex). Peptide fractions were then eluted by consecutive 11 salt plugs injections (1 mM, 2 mM, 5 mM, 10 mM, 20 mM, 50 mM, 100 mM, 200 mM, 500 mM, 1000 mM and 2000 mM NaCl solutions, respectively) and concentrated on a trap column (Acclaim PepMap100 RP C18 75 μm i.d. x 2 cm column, Thermo Scientific Dionex). Each fraction was then injected on-line on PepMap100 RP C18 75 μm i.d. x 15 cm column (Thermo Scientific Dionex) and the peptides were separated in 55 min 7-55% B phase linear gradient (A phase – 2% ACN and 0.1% formic acid; B phase – 80% ACN and 0.1% formic acid) with a flow rate of 300 nl/min by UltiMate 3000 HPLC system (Thermo Scientific Dionex) and applied on-line to a Velos Pro (Thermo Scientific, USA) dual-pressure ion-trap mass spectrometer. The main working nanoelectrospray ion source (Nanospray Flex, Thermo Scientific) parameters were as follows: ion spray voltage 1.7 kV and capillary temperature 250 °C. Spectra were collected in full scan mode (400 – 1500 Da), followed by five Higher energy Collisional Dissociation (HCD) MS/MS scans of five most intense ions form the preceding survey full scan under dynamic exclusion criteria. Collected data were analyzed by the X!Tandem search algorithm (The GPM Organization) and statistically validated with PeptideProphet under the Trans-Proteomic Pipeline (TPP) suite of software (Institute for Systems Biology). Search parameters were set as follows: taxonomy: human (SwissProt release 2014_03), enzyme: trypsin, missed cleavage sites allowed: 1, fixed modification: carbamidomethyl, variable modifications: oxidation of methionine; parent mass error – 1.5 to + 3.0 Da and peptide fragment mass tolerance: 0.7 Da. Protein identifications with FDR below 2% were considered as correct matches. ClueGO [17] was used for gene ontology annotation of the identified protein network under the Cytoscape software environment [18]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [19] via the PRIDE partner repository with the dataset identifier PXD001109

Results

Characteristics of the AMI patients during the acute phase of ischemia following admission to hospital and 8–12 weeks after the event are presented in Table 1.

All identified proteins are listed in Table 2. Dots refers to the presence of particular proteins in plasma clots prepared from blood taken on the two time points (quantitative data based on the label–free spectral counting annotated to particular patients are shown in Suppl. Table 1). Proteomic analysis revealed a total number of 62 proteins identified in 8 samples with the average of 42% of total identifications in each sample. During the acute phase of AMI and 8–12 weeks later, we identified 3 fibrinogen chains and albumin in plasma clots as expected. Prothrombin was present in 7 samples except one