Myocardial Proteomic Profiling Helps Identify Different Forms of Heart Disease

Gökhan Baykut[1], Matthias Witt[1], Maria Bergquist[2], Franz Mayer-Posner[1], Per Håkansson[3], Hans-Reinhard Zerkowski[4], Doan Baykut[4] and Jonas Bergquist[2]
[1]Bruker Daltonik GmbH, Bremen, Germany, [2]Analytical Chemistry, Uppsala University, Uppsala, Sweden, [3]Department of Ion Physics, Uppsala University, Uppsala, Sweden, [4]University Hospital Basel, Basel, Switzerland

Objective

Understanding the differences of proteomic profiles of myocardial tissue has crucial importance for gaining insight into molecular mechanisms of cardiac disease Myocardial insufficiency is increasingly expected to be a result from alterations in gene and protein expression. Protein patterns may change faster in close relationship with the appearance of disease, making up an adequate identification of proteome characteristics important. If different etiologies and pathways of cardiac disease progression can be represented in form of unequivocal proteomic patterns, then advanced but simple molecular diagnostics tools can be developed based i.e. on blood tests.

Introduction

Coronary (= ischemic) heart disease (CD) and valve related diseases (VD) have different origins. In coronary disease, myocardial changes are based on a reduced oxygenation in myocardial tissue by low perfusion, while valve diseases, particularly aortic valve disease lead to a hypertrophic conformation of the tissue by altered mechanical workload and/or dynamic properties of blood flow (Fig.1).

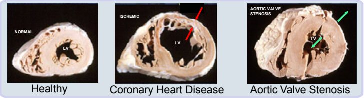

Fig. 1: Changes of heart morphology in different forms of cardiac disease

Methods

Proteomic profiles of myocardial tissue in two different forms of heart disease, CD and VD, were investigated by LC-FT-ICR mass spectrometry. Myocardial samples from 10 patients with isolated CD and from 10 patients with isolated VD were collected during elective cardiac surgery. Patient selection was made in such a way that all CD patients included in this study were healthy in terms of VD and all selected VD patients were healthy in terms of CD. Sample collections were in accordance with the regulations of the Ethical Committee of the University of Basel, that after a prior written agreement of each patient, any tissue specimen which is subject to be discarded during the surgical intervention is allowed to be examined for scientific purposes. Before starting the extracorporeal circulation, right atrial appendages were collected, which are actually subject to removal and discarding for venous cannulation.

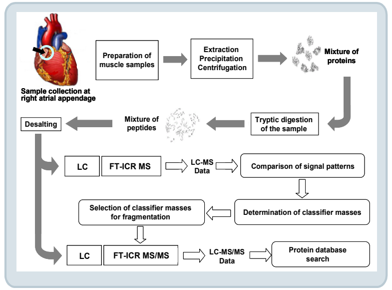

Fig. 2: Sample preparation and analytical processes

All specimens were in the range of 1 cm3. After removal, the samples were immediately washed in Krebs-Henseleit solution (NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.25 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, glucose 11 mM, pH 7.4) and fixed on wax plates at room temperature. After separation from the epicardium, the trabecular tissue was shock-frozen in liquid nitrogen and stored at -75 ºC. The tissues were homogenized in 8M urea, 0.4M NH_4HCO_3 followed by carbamido-methylation and tryptic digestion. The digests were SPE extracted (Spec-Plus C_{18} AR, Ansys Diagnostics) vacuum-centrifuged to dryness and then redissolved before injection into the LC. Acetonitrile and acetic acid were purchased from Merck. Urea, ammonium carbonate, and iodoacetamide were obtained from Sigma and were used without further purification. Dithiothreitol was purchased from Amersham Biosciences. All used chemicals were of analytical grade. Trypsin, sequence grade from bovine pancreas was obtained from Roche Diagnostics. Water was purified with a Milli-Q system of Millipore. Fused silica capillaries were obtained from Polymicro Technologies [1].

Digested samples were dissolved in 250 µL 0.1% trifluoroacetic acid (TFA), split into 5 vials each of 50 µL sample solution and SpeedVacced to dryness at medium temperature. Each dried vial of sample was dissolved in 50 µL 0.1% TFA and diluted 1:10 with 0.1% formic acid. An aliquot of 5 µL of the sample solution was injected on an RP column for FT-ICR-NanoLC/MS/MS analysis. Mass spectra were acquired with an APEX Qe FT-ICR of Bruker Daltonics (Fig. 3) equipped with a 9.4 Tesla superconducting magnet (Bruker). Samples were ionized using an "Apollo II" ESI source. NanoLC-MS/MS measurements were performed with an

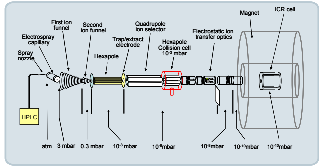

Fig. 3: Schematic view of the used FT-ICR MS system

Ultimate 3000 NanoLC system (Dionex). The on-line nanospray source was equipped with a 70°-angled off-axis sprayer and a 10µm i.d. Picotip adapter (New Objective). The needle was on ground potential and at a few mm distance to the ESI orifice. The ESI capillary entrance voltage was -1500V. A PepMap C_{18} nano LC column (75µm x 15cm, 5µm, 120Å, Dionex) was used for compound separation at a flow rate of 200nL/min. Peptides were first captured on a C_{18} pre-column for desalting of the sample and eluted column to the Pepmap column with a gradient for peptide separation (solvent A: water with 0.1% formic acid, solvent B: acetonitrile with 0.1% formic acid, 0 min 2% solvent B, 5min 2% solvent B, 175min 40% solvent B, 190min 50% solvent B, 191min 90% solvent B, 205 min 90% solvent B, 206min 2% solvent B, 250min 2% solvent B). After loading on the pre-column the sample was washed for 5min with 0.1% TFA for desalting using a 30µL/min flow and then switched to gradient flow. The default parameter setting was used for collision energy calculation for CID fragmentation. FT-ICR mass spectra were acquired using ApexControl 1.0 software with data-dependent MS/MS acquisition. Ion accumulation time was set to 0.5 s for the MS scan and 2s for the MS/MS. The mass range was m/z 246 to 2000 for MS and MS/MS spectra using 512k data points. The mass spectra were Fourier transformed after sine apodization. For further processing of the data including mass deconvolution and mgf-file generation for database search DataAnalysis 3.3 was used. Mass spectra were processed with Biotools 3.0 for database searches and result interpretations. MASCOT search engine (Matrix Science) was used for protein identification with the Swissprot database. Search criteria included one missed cleavage, taxonomy human, fixed modification carbamidomethyl and variable modifications oxidation of methionine. Tolerance for the MS/MS search was set to 4 ppm for the parent mass and 0.01 Da for the fragment masses. Mass spectra were externally calibrated with a siloxane mixture of the background using a two parameter calibration.

Results

The LC-MS data obtained from CD and VD samples were compared to each other using a special pattern recognition method [1,2]. The differences represent the classifier peptide masses of digested proteins in each case. No interference between samples representing the two diseases was seen. Highly and less characteristic peaks could be distinguished in classification for all representative samples. In VD, a closer accumulation pattern of spots was observed, indicating more specific protein classification than in CD. The classification using 240 characteristic peaks in the CD group and 90 characteristic peaks in the VD group analyzed by LC-FT-ICR MS resulted in clearly different distribution patterns (Fig. 4). Dots and squares represent the two cardiac disorders accumulated on either side of the diagonal with increased distance from it related to the specification for the particular type of disease. Samples from myocardial tissue with CD are located above the diagonal, and samples from myocardial tissue with VD are located below the diagonal. In order to identify the proteins from classifier peptides, collision-induced dissociation MS/MS experiments prior to FT-ICR mass analysis of these selected ions have been performed.

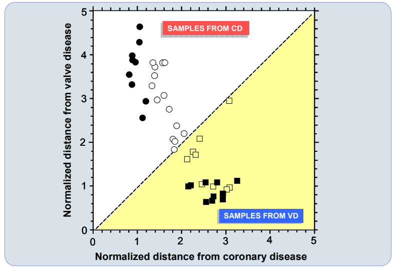

Fig. 4: Coronary (circles) and valve (squares) diseases show clear differences in proteomic pattern characteristics as they are located at different sides of the diagonal. Each point represents an individual sample where filled symbols represent supervised classified training samples while open symbols represent unsupervised classified test samples. All samples with exception of two were unambiguously correctly classified [1].

MS/MS experiments indicated that mainly adenylate kinase isoenzyme was upregulated in CD samples. In VD samples alpha cardiac actin or cytoplasmic actin, desmin, lumican precursor and myoglobin were upregulated. The identification details are shown in Table 1.

Protein ID in Coronary Disease Samples	Peptide Sequence	Sequence Tag	m/z (charge state)	Mascot Scores	Total Mascot Score
(P00568) Adenylate kinase isoenzyme 1 (EC 2.7.4.3) (ATP-AMP transphosphorylase) (AK1) (Myokinase) KAD1_HUMAN	GQLVPLETVLDMLR	64 - 77	792.447 (2+)	105	105
(P62736) Actin, aortic smooth muscle (Alpha-actin-2) ACTA_HUMAN	MQKEITALAPSTMK	315 - 328	774.912 (2+)	18	18

Protein ID in Valve Disease Samples	Peptide Sequence	Sequence Tag	m/z (charge state)	Mascot Scores	Total Mascot Score
(P60709) Actin, cytoplasmic 1 (Beta-actin) ACTB_HUMAN **or** (P68032) Alpha-cardiac actin ACTC_HUMAN	IWHHTFYNELR IWHHTFYNELR	85 - 95 87 - 97	505.922 (3+) 758.379 (2+), 505.922 (3+)	70 53, 61	184
(P17661) Desmin DESM_HUMAN	FLEQQNAALAAEVNR	127 - 141	837.426 (2+)	90	90
(P51884) Lumican precursor (Keratan sulfate proteoglycan lumican) (KSPG lumican) LUM_HUMAN	ILGPLSYSK LKEDAVSAAFK	297 – 305 171 - 181	489.288 (2+) 589.825 (2+)	29 60	89
(P02144) Myoglobin MYG_HUMAN	HPGDFGADAQGAMNK	119 - 133	505.894 (3+)	71	71
(P12111) Collagen alpha-3(VI) chain precursor CO6A3_HUMAN	VAVVQYSDR	1067 - 1075	518.776 (2+)	59	59
(P09493) Tropomyosin 1 alpha chain (Alpha-tropomyosin) TPM1_HUMAN	MEIQEIQLK	141 - 149	566.309 (2+)	36	36
(P02768) Serum albumin precursor ALBU_HUMAN	KYLYEIAR	161 - 168	528.299 (2+)	29	29
(P02511) Alpha crystallin B chain (Alpha(B)-crystallin) (Rosenthal fiber component) (Heat-shock pro CRYAB_HUMAN	HFSPEELK	83 - 90	493.751 (2+)	28	28
(P09669) Cytochrome c oxidase polypeptide VIc precursor (EC 1.9.3.1) COX6C_HUMAN	KAGIFQSVK	67 - 75	489.293 (2+)	28	28
(P12235) ADP/ATP translocase 1 (Adenine nucleotide translocator 1) (ANT 1) (ADP,ATP carrier protein ADT1_HUMAN	TAVAPIER	23 - 30	856.487 (1+)	24	24
(P35555) Fibrillin-1 precursor FBN1_HUMAN	TICIETIK	843 - 850	489.271 (2+)	23	23
(P19429) Troponin I, cardiac muscle (Cardiac troponin I) TNNI3_HUMAN	AKESLDLR	162 - 169	466.264 (2+)	19	19

Table 1: Upregulated proteins identified in CD and VD cases

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Surprisingly, myosin heavy chain alpha and beta isoforms, as well as troponin T could be identified in the upregulated lists of *both* diseases with high total mascot scores. However, the peptides through which these proteins are identified are entirely different in both cases. Factors influencing the digestion process including post translational modifications do suppress the appearance of certain peptides, due to different folding geometries. Glucosylation, e.g. can even completely block the tryptic digestion [3]. Thus, if the same protein is differently modified in the two investigated diseases, their digestion can lead to different peptides. In our ongoing study, we will apply ECD & ETD for fragmentation, which usually help retain most post translational modifications.

References

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Conclusions

- An analytical approach to heart disease at molecular level is possible by using LC-FT-ICR MS
- This method can help identify differences in protein structures between various etiologies of heart disease.
- Changes in proteomic patterns may reflect a generalized process in myocardial tissue which also can be accessed through LC-FT-ICR MS.
- Since protein-based transformations are evident before the clinical symptoms, a breaktrough in molecular diagnostics of the heart disease can be expected.

Clinical Proteomics