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Simultaneous accurate quantification of HO-1, CD39, and CD73 in human calcified aortic valves using multiple enzyme digestion – filter aided sample pretreatment (MED-FASP) method and targeted proteomics

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ABSTRACT

Several proteins such as membrane-associated ectonucleotidases: ecto-5'-nucleotidase (E5NT/CD73) and ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1/CD39), and intracellular heme oxygenase-1 (HO-1) may contribute to protection from inflammation-related diseases such as calcific aortic valve stenosis (CAS). Accurate quantification of these proteins could contribute to better understanding of the disease mechanisms and identification of biomarkers.

This report presents development and validation of quantification method for E5NT/CD73, ENTPD1/CD39 and HO-1. The multiplexed targeted proteomic assay involved antibody-free, multiple-enzyme digestion, filter-assisted sample preparation (MED-FASP) strategy and a nanoflow liquid chromatography/mass spectrometry under multiple reaction monitoring mode (LC-MRM/MS). The method developed presented high sensitivity (LLOQ of 5 pg/mL for each of the analytes) and accuracy that ranged from 92.0% to 107.0%, and was successfully applied for the absolute quantification of HO-1, CD39 and CD73 proteins in homogenates of human calcified and non-calcified valves. The absolute CD39 and CD73 concentrations were lower in calcified aortic valves (as compared to non-stenotic ones) and were found to be: 1.16 ± 0.39 vs. 3.15 ± 0.37 pmol/mg protein and 1.94 ± 0.21 vs. 2.39 ± 0.39 pmol/mg protein, respectively, while the quantity of HO-1 was elevated in calcified valves (10.72 ± 1.18 vs. 4.28 ± 0.42 amol/mg protein). These results were consistent but more reproducible as compared to immunoassays.

In conclusion, multiplexed quantification of HO-1, CD39 and CD73 proteins by LC-MRM/MS works well in challenging human tissues such as aortic valves. This analysis confirmed the relevance of these proteins in pathogenesis of CAS and could be extended to other biomedical investigations.

1. Introduction

Calcific aortic stenosis (CAS) is the most common heart valve disorder in the aging population of developed countries [1–4]. It is characterized by progressive fibro-calcific remodeling of the valve leaflets and their thickening that, over the years, evolve to cause severe obstruction to cardiac outflow. The pathobiology of CAS is complex/multifactorial and involves genetic factors, lipoprotein retention and oxidation, chronic inflammation, the differentiation of cardiac valve interstitial cells (VICs) into osteoblasts and active leaflet calcification [4,5]. Although no medical intervention has proved to be effective in delaying or halting its progression promising therapeutic targets include the renin–angiotensin system, lipoprotein(a), receptor activator of NF-κB ligand (RANKL) and ectonucleotidases [4,6]. Currently, the mainstay of treatment for CAS is surgical or transcatheter aortic valve replacement (AVR) with a mechanical or bioprosthetic valve [7,8]. However, some limitations still exist that include increased complications attributable to anticoagulation and the future need for reoperation

Abbreviations: AA, amino acid; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; AVR, aortic valve replacement; BCA, bicinchoninic acid; CAS, calcific aortic valve stenosis; CE, collision energy; CID, collision-induced dissociation; 2-DE, conventional two-dimensional electrophoresis; 2D-DIGE, differential gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; E5NT/CD73, ecto-5′-nucleotidase; ENTPD1/CD39, ectonucleoside triphosphate diphosphohydrolase 1; HO-1, heme oxygenase-1; IS, internal standard; LC-MRM/MS, liquid chromatography/mass spectrometry operated in multiple reaction monitoring mode; MED-FASP, multiple enzyme digestion – filter aided sample preparation; QC, quality control; RANKL, receptor activator of NF-κB ligand; RE, relative error; RSD, relative standard deviation; VICs, valvular interstitial cells

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because of the limited lifespan of biological valves [9]. Re-evaluation of the underlying pathophysiological processes involved is therefore required so that novel therapeutic options can be developed.

A growing body of scientific evidence indicates that purinergic signaling which involves ectonucleotidases plays a critical role in controlling the mineralization of the aortic valve [4,6]. Our previous studies showed that aortic valves are rich in membrane-bound enzymes that catalyze conversion of extracellular adenosine triphosphate (ATP) to adenosine, what is essential in maintaining the balance between pro-inflammatory/pro-aggregatory vs. anti-inflammatory/anti-aggregatory effects [10,11]. Ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1/CD39), which hydrolyzes ATP to adenosine monophosphate (AMP) through adenosine diphosphate (ADP) and ecto-5′-nucleotidase (E5NT/CD73), which degrades AMP to adenosine, are the most important in this extracellular nucleotide degradation cascade [12–14]. The altered expression or activity of ectonucleotidases in the valves may affect their calcification.

Other studies proposed that heme oxygenase-1 (HO-1), a rate-limiting enzyme in heme catabolism, with potent antioxidant and anti-inflammatory properties plays a key role in maintaining cellular anti-oxidant/oxidant homeostasis, which could be important in the initiation and progression of lesions in stenotic valves as well as in differentiation of VICs into osteoblast-like phenotype [15,16]. However, analysis of expression of this protein is challenging and its specific role in pathogenesis of CAS remains unclear.

Thus, there exists an urgent need to develop analytical methods capable of reliable estimate of candidate markers related to this pathology at high throughput and reasonable cost. Targeted proteomics based on liquid chromatography-multiple reaction monitoring (LC-MRM) has emerged as a particularly suitable platform, alternative to immunoassays, for analysis of many proteins involved in the pathogenesis of various diseases, such as CAS [17,18]. MS analysis performed in MRM mode offers the unique possibility to simultaneously and specifically monitor the signatures of hundreds of target peptides that are generated by trypsin digestion of proteins and combined with quantification standards (isotope-labeled or structural analogues) can provide accurate quantitative data for each protein targeted [19–24].

In this study, we have developed a MRM-MS-based quantification method for detecting selected proteins (HO-1, CD39 and CD73) associated with CAS. After a thorough validation, the method has been successfully used to determine the protein levels in human valve samples obtained from control subjects and patients suffering from CAS. The present study is the first one reporting MRM-MS assay allowing the sensitive and reproducible determination of the HO-1, CD39 and CD73 proteins in human valve homogenates. The obtained results demonstrated excellent analytical performance of the assay developed and highlighted the importance of the proteins determined in the pathogenesis of CAS.

2. Material and methods

2.1. Chemicals and reagents

Tris(hydroxymethyl)aminomethane (Tris), NaCl, Triton x-100, β-mercaptoethanol, urea, thiourea, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), ammonium bicarbonate, tris (2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), iodoacetamide, and a broad-spectrum protease inhibitor cocktail were purchased from Sigma-Aldrich (all more than 97% pure; Poznan, Poland). Trypsin/Lys-C protease mix, used for protein digestion, was from Promega (Madison, WI, USA), while C_{18} silica bonded SPE cartridges (100 mg/1 mL, Sep-Pak type) were supplied by Waters (Milford, USA). The BCA protein assay kit was purchased from Merck (Darmstadt, Germany). All solutions were prepared using high-purity water and LC/MS-grade solvents (acetonitrile (ACN), methanol, Sigma-Aldrich). All other chromatography-grade chemicals were obtained from Sigma-Aldrich.

Table 1
Baseline clinical characteristics of the control subjects and CAS patients.

Clinical variables	Control subjects (n = 5)	CAS patients (n = 12)	P-value
Age (years)	48 ± 5	72 ± 2	< 0.001
Gender (male/female, n)	4/1	7/5	NS
BMI (kg/m^2)	25.3 ± 1.4	27.5 ± 1.1	NS
Smoking (%)	40.0	33.3	NS
Fasting blood sugar (mg/dL)	97.0 ± 8.7	104.7 ± 6.8	NS
Triglycerides (mg/dL)	81.3 ± 16.8	142.3 ± 15.5	0.037
Cholesterol (mg/dL)			
Total	161.0 ± 10.6	212.0 ± 12.6	0.028
LDL	102.0 ± 6.4	133.3 ± 8.6	0.043
HDL	43.7 ± 5.6	50.3 ± 4.1	NS
eGFR (mL/min/1.73 m^2)	83.0 ± 5.8	61.8 ± 3.9	0.009
Aortic valve area (cm^2)	–	0.78 ± 0.08	N/A
Aortic peak gradient (mmHg)	–	72.8 ± 8.2	N/A
LVEF (%)	53.0 ± 4.6	49.6 ± 4.0	NS
Hypertension (%)	40.0	50.0	NS
Diabetes (%)	0.0	16.7	NS
Coronary artery disease (%)	0.0	41.7	< 0.001
Hypercholesterolemia (%)	20.0	41.7	0.02
Medication (%)			
ACEIs/ARBs/MRAs	40.0	33.3	NS
Statins	20.0	66.7	0.001
β-blockers	60.0	75.0	NS
Diuretics	40.0	50.0	NS
Anticoagulants	20.0	83.3	< 0.001

Values are denoted as mean ± SEM or %.
BMI: body mass index; LDL: low-density lipoprotein; HDL: high-density lipoprotein; GFR: glomerular filtration rate; LVEF: left ventricular ejection fraction; ACEIs: angiotensin-converting enzyme inhibitors; ARBs: angiotensin II type I receptor blockers; MRAs: mineralocorticoid receptor antagonists; N/A: not applicable; NS: not significant.

Synthetic peptide standards were obtained from Lipopharm (Poland). Their purity and masses were verified with the use of a high-resolution, accurate-mass LC/MS system (Ulitmate 3000, Dionex, Thermo Scientific, San Jose, CA, USA; MicrOTOF-QII, Bruker Daltonik GmbH, Bremen, Germany).

2.2. Samples and study population

Samples were obtained from patients operated at the Department of Cardiac and Vascular Surgery of the Medical University of Gdansk. Heart valves with degenerative calcific aortic stenosis (n = 12) were obtained from patients of both sexes (7 male, 5 female), with an average age of 72 (± 2) years, who underwent aortic valve replacement due to severe degenerative stenosis (Table 1). Patients with mitral valve disease, aortic regurgitation, or any suspicion of rheumatic disease were not included in this study. Control macroscopically normal valves (n = 5, Table 1) were obtained from patients undergoing cardiac transplantation or Bentall procedure. This study was conducted in accordance with the recommendations of the Helsinki Declaration and it was approved by the Bioethics Committee at the Medical University of Gdansk. Written informed consent was obtained from all the patients prior to their inclusion in the study.

2.3. Processing of aortic valves and sample preparation prior to LC/MS analysis

Aortic valves were processed according to Martin-Rojas et al. protocol with minor modifications [25,26]. Briefly, the valves were processed within a maximum of 2–3 h after surgery having maintained the tissues at 4 °C in RPMI medium. They were washed three times in phosphate-buffered saline to eliminate blood contaminants, and the aortic valve leaflet was then ground into a fine powder in liquid nitrogen with a mortar. A total of 0.2 g of the powder was resuspended in 600 μL of protein extraction buffer (Tris 10 mM [pH 7.5], 500 mM NaCl,