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Molecular anatomy of ascending aorta in atherosclerosis by MS Imaging: Specific lipid and protein patterns reflect pathology

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ABSTRACT

The molecular anatomy of healthy and atherosclerotic tissue is pursued here to identify ongoing molecular changes in atherosclerosis development. Subclinical atherosclerosis cannot be predicted and novel therapeutic targets are needed. Mass spectrometry imaging (MSI) is a novel unexplored ex vivo imaging approach in CVD able to provide in-tissue molecular maps. A rabbit model of early atherosclerosis was developed and high-spatial-resolution MALDI-MSI was applied to comparatively analyze histologically-based arterial regions of interest from control and early atherosclerotic aortas. Specific protocols were applied to identify lipids and proteins significantly altered in response to atherosclerosis. Observed protein alterations were confirmed by immunohistochemistry in rabbit tissue, and additionally in human aortas. Molecular features specifically defining different arterial regions were identified. Localized in the intima, increased expression of SFA and lysolipids and intimal spatial organization showing accumulation of PI, PG and SM point to endothelial dysfunction and triggered inflammatory response. TG, PA, SM and PE-Cer were identified specifically located in calcified regions. Thymosin β4 (TMSB4X) protein was upregulated in intima versus media layer and also in response to atherosclerosis. This overexpression and localization was confirmed in human aortas. In conclusion, molecular histology by MS Imaging identifies spatial organization of arterial tissue in response to atherosclerosis.

1. Introduction

Atherosclerosis is usually the underlying cause of a fatal event (e.g. myocardial infarction, stroke) following the silent and progressive occlusion of the arteries. The lesion starts with the formation of fatty streaks and endothelial activation, followed by lipid accumulation and monocyte and platelet recruitment and inducing proliferation and migration of vascular smooth muscle cells (VSMC) from the media to the intima layer [1–3]. Progressively, atheroma plaque develops silently and this complex scenario cannot be seen as a simple sequence of unrelated facts, but as a complex network of interconnected molecules involved in multiple reactions operating simultaneously [4,5].Thus, mechanisms underlying atherosclerosis development are still not fully understood.

Molecule-specific localization is essential in the study of atherosclerosis disease, considering that specific alterations occur in the different arterial layers (intima and media). Spatial resolved mass spectrometry imaging (MSI) can generate molecular profiles directly from the tissue, providing its molecular histology and allowing visualization of proteins, lipids and metabolites at their tissular location [6–8]. Clinical investigation using spatial resolved mass spectrometry imaging has provided important contributions for a range of diseases [9,10] in biomarker discovery, patient prognosis and survival [11,12], response to treatment [13,14] or evaluation of heterogeneous tumors [15,16]. In cardiovascular diseases, MALDI-MSI represents a novel ex vivo imaging tool to investigate atherosclerosis, offering the unique advantage to investigate the physiopathological changes taking place directly in-tissue and retaining the histopathological context of the observations. Importantly,

Abbreviations: H&E, hematoxylin & eosin staining; IHC, immunohistochemistry; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; MSI, mass spectrometry imaging; PA, diacylglycerophosphates; PE-Cer, phosphoethanolamines; PG, diacylglycerophosphoglycerols; PI, glycerophosphoinositols; SFA, saturated fatty acids; SM, sphingomyelins; TG, triglycerides; TMB4SX, thymosin β4 protein.

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MALDI-MSI allows high spatial integrity as demanded by the small dimensions of these structures.

Using MALDI-MSI, this is the first approach map combining proteins and lipids in the atherosclerotic lesion and healthy arterial tissue, pursuing a look into atherosclerotic mechanisms, and identification of novel therapeutic targets directly at tissue level and which may be potentially released to plasma.

2. Material and methods

2.1. Animal model

A rabbit model of atherosclerosis was developed as previously published [17]. Briefly, twelve male New Zealand white rabbits were divided in two study groups fed with standard or cholesterol enriched diet. All animals were housed in individual cages in an air-conditioned room under a 12:12-h light–dark cycle. Principles of laboratory animal care were followed and all experimental procedures were approved by the Animal Care and Use Committee of the IIS-Fundación Jiménez Díaz, according to the guidelines for ethical care of the European Community. Animals were sacrificed after 13 weeks.

2.2. Tissue collection, matrix deposition and MSI spectral acquisition

We followed standard guidelines for tissue collection and preservation prior to MS imaging analysis [18,19]. In synthesis, the ascending aortic section of each animal was dissected, rinsed in phosphate-buffered saline and immediately snap frozen by immersion in liquid nitrogen without any fixation. Aorta samples were stored at −80 °C. For the MALDI-MSI experiments, a total of 36 tissue sections (3 sections per animal, 12 animals), 15 μm thick, were cut at −22 °C and mounted onto indium–tin–oxide coated glass slides (Bruker Daltonik, Bremen, Germany) using a cryostat (Leica Mycrosystems GmbH, Wetzlar, Germany). The tissue sections (n = 36) were stored at −80 °C before measurements, for which they were slowly brought to temperature in a freeze dryer. The matrix solution was uniformly deposited onto the washed tissue using the ImagePrep automated spraying device (Bruker Daltonik). MSI experiments were performed with an UltrafleXtreme MALDI-ToF (Bruker Daltonik), using 30 μm pixel size and 500 laser shots per pixel (100 laser shots per position of a random walk within each pixel) as previously described [20]. Data acquisition and visualization were performed using FlexControl 3.0 and FlexImaging 2.1, respectively (both from Bruker Daltonik).

Three different MALDI-MSI protocols were applied for the detection of proteins [20], lipids [16] and metabolites [21,22]. Briefly, aortic tissue sections were washed in isopropanol by immersion prior to protein analysis. A solution of 20 mg/mL sinapinic acid (SA) in 10 mL 4:4.2:1.8 acetonitrile:methanol:0.05% TFA was used as matrix. Spectra were acquired in linear positive mode. The m/z values measured were in the range from 2 to 20 kDa. Public libraries of MALDI-MSI assignments, MSiMass list database [23] and MaTisse [24], were used to assign identities to the most significantly altered protein molecular feature using a mass tolerance of ±3 Da, as commonly used cut-off criteria for tentative identification in MALDI-MSI experiments [25]. Confirmation of protein identification was performed by IHC analysis as detailed below. For lipid analysis 20 mg/mL DHB in 10 mL 4:4.2:1.8 acetonitrile:methanol:0.05% TFA was used as matrix. Spectra were acquired in reflector negative and positive modes in the mass range 500–1200 Da. 9-Aminoacridine (9AA) (10 mg/mL) in 70%MeOH was used for metabolites/lipids, and spectra were acquired in reflector negative mode in the mass range 0–1000 Da. For identity assignment, tissue samples were also analyzed on a 9.4T Solarix FT-ICR (BrukerDaltonik, Bremen, Germany) mass spectrometer. Lipid molecular identification was performed by using exact mass measurements, peak peaking and spatial filtering combined with Lipidsmap database using a tolerance of ≤0.005 Da, as previously published [26,27].

2.3. MALDI-MSI differential analysis of arterial layers and control/atherosclerotic tissue

Two approaches were performed by MALDI-MSI: 1) in-depth comparison of histologically-distinct, regions of interest (media, intima, plaque), and 2) comparison of control and atherosclerotic tissue. For comparison between control and atherosclerotic tissue, only those masses specifically defining an arterial region of interest (intima, media, or plaque) were selected. Additionally, we selected only those present in a minimum of two thirds of the total number of assayed samples. A random selection of the whole spectra sets from these regions were then imported into ClinProTools 3.0 (Bruker Daltonik) where they underwent smoothing, baseline subtraction, mass spectral alignment and normalization. In both cases, proteins and lipids, recalibration was performed using 2000 ppm of maximal peak shift and 10% match to calibrant peaks. The resulting peak intensity data was then subject to statistical analysis using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). We performed D'Agostino–Pearson normality test but the number of values was too small. As Gaussian distribution could not be assumed, we perform more stringent Mann–Whitney non-parametric test to calculate p-values.

2.4. Histochemical analysis and correlation with MALDI-MSI images

After the MALDI experiments the remaining matrix was removed by immersion in a 70% ethanol solution. The tissue sections were then stained with hematoxylin and eosin (H&E) and scanned with a digital iScan Coreo slide scanner (Roche, Ventana Medical Systems Inc., Arizona,USA). In FlexImaging, the H&E stained sections were co-registered to the MALDI-MSI results to align mass spectrometric data with the histological features. Consecutive sections were subjected to H&E staining, red alizarin staining to visualize calcium deposits, and oil red staining to localize lipids. α-Actin (Dako) immunohistochemistry (IHC) was performed to localize vascular smooth muscle cells (VSMCs).

2.5. Human aortic tissue collection

Human healthy and atherosclerotic aortic tissues were from necropsy origin collected in a timeframe of 4–10 h at Hospital Virgen de la Salud (Toledo, Spain). Tissue samples were obtained from 10 subjects (male:7, average age: 76) with atherosclerotic lesions previously characterized [28] which show a well formed lipid core, presence of an inflammatory infiltrate, and evidence of sporadic calcification. Control arteries were obtained from 4 individuals who did not die from cardiovascular events and with no evidences of atherosclerotic lesions in any of the most frequently affected arteries (male: 2, average age: 48). Samples were immediately washed in saline, embedded in OCT, frozen with liquid nitrogen and stored at −80 °C until use. Procedures of sample collection were approved by the local ethics committee.

2.6. Immunohistochemistry analysis of TMSB4X in rabbit and human aortic tissue

Sections of 4 μm of formalin-fixed, paraffin-embedded FFPE rabbit aortic tissue from control (n = 4) and pathological (n = 4) were analyzed using a dilution 1/100 of primary antibody against TMSB4X (Abnova Corporation). Sections of 6 μm of OCT embedded human aortas from control (n = 4) and atherosclerotic tissue (n = 10) were analyzed using a dilution of 1/10,000 of primary antibody against TMSB4X (Abcam, Cambridge, UK). Detection was performed by using EnVision + DAB system (Dako). Image analysis of the staining was conducted with Image-Pro Plus software (Media Cybernetics, Inc.). For this purpose the stained slides were scanned at ×20 (human tissue) or ×40 (rabbit tissue) objective magnification in a digital slide scanner (iScan Coreo slide scanner, Roche, Ventana Medical Systems Inc., Arizona, USA).