Atherosclerosis 269 (2018) 21–28

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Metabolic phenotyping of human atherosclerotic plaques: Metabolic alterations and their biological relevance in plaque-containing aorta

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ARTICLE INFO

Article history:
Received 12 June 2017
Received in revised form
10 November 2017
Accepted 30 November 2017
Available online 6 December 2017

Keywords:
Atherosclerosis
Metabolomics
Lipidomics
Quinic acid

ABSTRACT

Background and aims: Atherosclerosis is a chronic inflammatory disease characterized by thickening of the arterial wall. However, a limited number of studies have been conducted on metabolic profiling of human aortic tissue.
Methods: We applied liquid chromatography/mass spectrometry to perform global and targeted profiling of plaque-containing aortic tissue. The aorta samples included plaque-containing (n = 18) and control plaque-free (n = 24) aortic tissue from patients undergoing aortic surgery.
Results: The metabolic patterns of atherosclerotic and control vessels were significantly different. Metabolites in the purine and glutathione pathways showed dysregulation of oxidative stress in plaques, and levels of glucosylceramide, tryptophan, and kynurenine, which are related to inflammation, were also altered. Interestingly, an increased level of quinic acid was observed in plaques ($p < 0.000$), and we demonstrated an inhibitory effect of quinic acid on inflammatory activation and oxidative stress in macrophages.
Conclusions: Our study provides insight into the disease mechanism and potential markers of atherosclerosis through comprehensive metabolic profiling of human aortic tissue samples containing plaque.

1. Introduction

Atherosclerosis, a major cause of human morbidity and mortality, is caused by several factors. Pathologically, it is a chronic inflammatory disease characterized by intimal thickening and plaque formation in arteries. The pathogenesis of atherosclerosis is multifactorial [1–3], and understanding the biochemical interactions and molecular processes of this pathology may be helpful in reducing the risk.

Metabolomics and lipidomics can be applied to comprehensively analyze the composition of metabolites and lipid species in a biological system and its multifarious responses to changes in cellular metabolism [4,5]. Furthermore, metabolomics can also be applied to quantitatively determine the absolute concentrations of metabolites in biological fluids and tissues by using modern analytical platforms. Metabolic profiling in intact tissue provides insights into the distribution of metabolites associated with specific diseases. It also contributes to the understanding of the metabolic

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https://doi.org/10.1016/j.atherosclerosis.2017.11.034
0021-9150/

changes that lead to specific pathological conditions, providing more in-depth knowledge of their etiology [6,7]. Profiling of metabolites in atherosclerosis has been applied to clinical studies primarily using body fluids such as blood [8–10]. A limited number of studies with human tissues have identified metabolites and pathways possibly associated with atherosclerosis [11,12].

In the current study, we explored metabolites that are differentially expressed in plaque-containing and control plaque-free human aortic tissues. Furthermore, *in vitro* analysis was conducted to demonstrate the biological relevance of the metabolites identified in the analysis. Characterization of metabolic alterations in plaque-containing aorta with considerable diversity in physicochemical properties may help understand the mechanisms of atherosclerosis.

2. Materials and methods

2.1. Study subjects

The study protocol was approved by the institutional review board of Severance Hospital, Seoul, Korea, and methods were performed in accordance with the relevant institutional guidelines. All participants provided written informed consent (no. 4-2013-0688). From January 2014 to December 2015, consecutive patients, who underwent aortic graft replacement surgery for thoracic aortic aneurysm, were consecutively included. When patients or their guardians refused enrollment, they were excluded. Samples were then classified by an experienced pathologist, according to the presence of atherosclerotic plaques. First, we examined the lesion by gross examination. After the identification of the lesion, we took four to five sections at 5-mm segments. Then, hematoxylin-eosin stain, trichrome, and Elastic van Gieson stain was performed. A pathologist classified the lesions according to the modified classification of the American Heart Association proposed by Virmani et al. without knowledge of the specimens (Supplementary Fig. 1) [13]. Initially, 60 samples were obtained (from 44 patients); 42 samples (from 33 patients) were appropriate for and used in quantitative analysis. Of those 42 samples, 25 (from 22 patients) were analyzed by means of global profiling.

2.2. Aortic tissue extraction

Tissue samples were cut into 5 × 5 mm^2-sized pieces, and adventitial fat was removed. Human aortic tissue specimens with 2.8-mm zirconium oxide beads were homogenized twice with 1:1 v/v methanol/water mixture at a ratio of 3 ml solvent/g tissue using a Precellys 24 tissue homogenizer at 5000 rpm for 15 s each [14]. The homogenate was incubated at 4 °C for 10 min and an equal volume of chloroform was added. After homogenization, the tissue lysate was incubated at 4 °C for 10 min and centrifuged for 10 min at 13,000*g* and 4 °C. The supernatant (the aqueous extract) was transferred to a new 1.5-ml tube and dried in a vacuum concentrator for 2 h. The lipid extract, under the pellet, was transferred to a new 1.5-ml tube and evaporated under a stream of nitrogen. For liquid chromatography/mass spectrometry (LC/MS) analysis, the aqueous extracts were diluted with an acetonitrile/water mixture (2:8, v/v) and the lipid extracts were diluted with an isopropanol/acetonitrile/water mixture (2:1:1, v/v/v). The dissolved samples were centrifuged for 10 min at 13,000*g* and 4 °C, and transferred into vials.

2.3. Metabolomic and lipidomic analysis using LC-MS

For global metabolic profiling of human aortic tissues, ultra-performance LC (UPLC)/quadrupole time-of-flight (QTOF) MS analysis was performed on a triple TOF™ 5600 MS/MS System (AB Sciex, Canada) combined with a UPLC system (Waters, USA). For quantitative targeted analysis of metabolites, ultra-high performance LC (UHPLC)/triple quadrupole (TQ) MS analysis was performed on Agilent 1290 Infinity LC and an Agilent 6495 triple quadrupole MS system equipped with an Agilent Jet Stream electrospray ionization source (Agilent Technologies, USA). For further details, see Supplementary Data.

2.4. Cell culture

Dulbecco's modified Eagle medium (DMEM; Welgene, Korea), gentamicin (Sigma, USA), fetal bovine serum (FBS; Welgene) and phosphate-buffered saline (PBS) were purchased from Gibco (Grand Island, USA). The murine macrophage-like cell line J774A.1 (abbreviated J774) was provided by Prof. Yury I. Miller of the Department of Medicine, University of California, San Diego, CA, USA. J774 cells were maintained in DMEM supplemented with 10% FBS and 50 μg/ml gentamicin.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Cell culture medium was collected, and the secreted interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were quantified by using ELISA kits (R&D Systems, USA). Ninety-six-well plates were coated with 1 mg/well capture antibody. The coated plates were washed with PBS containing 0.05% Tween 20 and incubated with culture medium followed by incubation with biotin-conjugated secondary antibody. The plates were read at an absorbance of 450 nm. The target proteins were analyzed according to the manufacturer's specifications. Appropriate specificity controls were included, and all samples were run in duplicate.

2.6. Measurement of reactive oxygen species (ROS) generation

Generation of intracellular ROS was determined using CM-H2DCFDA. J774 cells in 12-well plates (3 × 10^5 cells/well) were treated with 2–2000 μM quinic acid for 24 h. After staining with 5 M CM-H2DCFDA in PBS for 45 min at 37 °C, cells were incubated in the presence or absence of 100 M H_2O_2 for 20 min. ROS generation was then determined with a flow cytometer. The mean fluorescence intensity was determined measuring 10,000 cells in the FITC channel.

2.7. Immunoblot analysis

Cells were solubilized in lysis buffer containing 1 M HEPES (pH 7.5), 5 M NaCl, 0.5 M EDTA, 1% Triton X-100, and protease inhibitor cocktail (Roche, USA). Protein concentration was determined using a bicinchoninic acid protein assay (Pierce Biotechnology, USA). Cell lysates were loaded onto a 12.5% SDS-PAGE gel and proteins were transferred to a polyvinylidene difluoride membrane (EMD Millipore, USA). The membrane was blocked to prevent nonspecific binding and incubated overnight at 4 °C with primary antibodies in 3% BSA/TBS with 0.05% Tween. The next day, the membrane was washed with TBS containing 0.05% Tween and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Bands were visualized by using an ECL kit (Thermo Fisher Scientific).

2.8. Statistical analysis

Multivariate statistical analysis was performed with unit variance scale by using SIMCA-P+ software, version 12.0 (Umetrics, Sweden). Metabolites with variable influence on projection (VIP)