Atherosclerosis 237 (2014) 769–776

Contents lists available at ScienceDirect

Atherosclerosis

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

Lipid crystals mechanically stimulate adjacent extracellular matrix in advanced atherosclerotic plaques

Eun-Soo Lee [a, 1], Joo Hyun Park [a, b, 1], Sang-Won Lee [a], Junhee Hahn [c], Haea Lee [d], Soo-Won Chae [d], Tae Geol Lee [a, b], Dae Won Moon [e], Se-Hwa Kim [a, b, *]

[a] Center for Nanosafety Metrology, Korea Research Institute of Standards and Science, Daejeon, Republic of Korea
[b] Department of Nanobio Surface Science, Korea University of Science and Technology, Daejeon, Republic of Korea
[c] Center for Energy Materials Metrology, Korea Research Institute of Standards and Science, Daejeon, Republic of Korea
[d] Department of Mechanical Engineering, Korea University, Seoul, Republic of Korea
[e] Department of New Biology, Daegu Gyeongbuk Institute of Science and Technology, Daegu, Republic of Korea

ARTICLE INFO

Article history:
Received 12 May 2014
Received in revised form
29 September 2014
Accepted 6 October 2014
Available online 31 October 2014

Keywords:
Lipid crystal
Foam cell
Multimodal nonlinear optical imaging
Elastin
Finite element analysis

ABSTRACT

Objective: Although lipid crystals (LCs) have received attention as a causative factor of plaque rupture, the mechanisms by which they increase plaque vulnerability are unknown. We examined whether solid-state LCs physically affect the adjacent extracellular matrix (ECM) using a combination of multimodal nonlinear optical (MNLO) imaging and finite element analysis (FEA). **Methods**: The changes of ECMs affected by lipids in atherosclerotic arteries in apolipoprotein E-deficient mice ($n = 32$) fed a high-fat diet for 20–30 weeks were micro-anatomically visualized by a 3D MNLO imaging platform including CARS for lipids, TPEF for elastin, and SHG for collagen. **Results and Conclusion**: The TPEF signal of elastin was increased at the peripheral regions of LCs (<10 μm) compared with foam cell regions. In order to confirm the increase of elastin, biochemical assay (western blot) was performed. The protein level of elastin was increased approximately 2.25-fold ($p = 0.024$) in LC-rich arteries. Under the hypothesis that the increase of elastin resulted from the mechanical stimulus from solid-state LCs, MNLO images were subjected to FEA to simulate the displacement according to the expanding magnitude of the vessel during cardiac cycles. We found that microscale focal stress was increased specifically around the LCs. These FEA results corresponded with the increase of elastin observed by TPEF. These data suggest that LCs mechanically stimulate the adjacent ECM to alter the composition of ECM and cause vessel remodeling. The combination of MNLO imaging and FEA has great potential to verify the mechanical predictions in cardiovascular diseases.

1. Introduction

Atherosclerotic plaque rupture, an underlying cause of most myocardial infarctions, is typically characterized by a thin fibrous cap and a large lipid-rich necrotic core [1,2]. Numerous studies have focused on the thin fibrous cap in terms of the role of matrix metalloproteinase [3,4]; however, the lipid core, a dominant factor in atherosclerotic lesions, has been relatively understudied due to the technical challenges in imaging intact tissues at a fine resolution, such as nano- and micro-resolution.

Several methods, such as magnetic resonance imaging [5] and near-infrared spectroscopy [6,7], have been used to evaluate lipid core plaques, although these methods lack the spatial and temporal resolution needed to define detailed plaque compositions. Nonlinear optical (NLO) techniques have recently emerged as an appropriate tool for investigating the micro-anatomic features of biosamples [8,9]. NLO methods include multiphoton excitation fluorescence (MPEF), second-harmonic generation (SHG), and coherent anti-Stokes Raman scattering (CARS). The ability to visualize the fluorescence materials of MPEF can be utilized to image the intrinsic autofluorescence of elastin fibers, and SHG is specialized for visualizing collagen fibrils based on unique structural features [10–12]. Among the diverse range of lipids that compose lipid

* Corresponding author. Korea Research Institute of Standards and Science, 267 Gajeong-Ro, Yuseong-Gu, Daejeon 305-340, Republic of Korea.
E-mail address: shkim@kriss.re.kr (S.-H. Kim).
[1] Both are equally contributed.

http://dx.doi.org/10.1016/j.atherosclerosis.2014.10.018
0021-9150/

cores, lipid crystals (LCs) have recently been perceived as a factor that induces both local and systemic inflammation leading to plaque rupture [13,14]. We previously reported some micro-anatomical features of atherosclerotic lesions and characterized four major types of atherosclerotic lipids, including two distinct types of LCs, based on multiplex CARS analysis [15]. Suhalim et al. recently reported the physiochemical properties of cholesterol crystals using stimulated Raman scattering (SRS) and SHG combined microscopy [12]. From this perspective, NLO imaging could be a suitable system for concomitantly investigating correlations between LCs and important structural proteins, such as elastin and collagen.

Elastin is a principal component of the internal elastic lamina (IEL), which serves as a barrier between the intima and media in the vascular wall [16–18]. It can transmit environmental stimuli (i.e., tensile, compressive, and shear stress) by working as a vigorous autocrine regulator of smooth muscle cells (SMCs), ultimately leading to physiological responses [19,20]. Therefore, structural and quantitative changes to elastin are believed to be promising markers for plaque development [21,22]. Using NLO imaging, Chen et al. reported the characteristics of the layered structure of coronary adventitia under various mechanical loads [8]. The ability of CARS to achieve label-free detection of molecular specificity could also be useful for visualizing atherosclerotic lipids [23,24]. Wang et al. reported that CARS-based multimodal NLO microscopy was capable of analyzing various pathological components of atherosclerotic lesions without any probes [23].

In this study, we used a combinatorial method, MNLO imaging-based finite element analysis (FEA), to understand how LCs influence the circumferential ECM in advanced atherosclerotic plaques. We hypothesized that LCs mechanically stimulate the ECM to alter its structure and composition in response to cardiac cycles. The MNLO imaging system developed herein was used to concomitantly image LCs and ECM (elastin and collagen). Foam cells (FCs) filled with intracellular lipid droplets were compared as a control. The resulting MNLO images were subjected to FEA to demonstrate the mechanical influence of LCs on the adjacent ECM.

2. Methods

2.1. Development of multimodal nonlinear optical microscopy

A schematic of our MNLO imaging system is shown in Fig. 1. Detailed methods are available in the Online Supplement.

2.2. Preparation of samples for MNLO imaging

Male apolipoprotein E deficient (ApoE$^{-/-}$) mice ($n = 32$) and C57BL/6 mice ($n = 10$) were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained under pathogen-free conditions at the Korea Research Institute of Bioscience and Biotechnology. The ApoE$^{-/-}$ mice ($n = 27$) were fed a high-fat diet (#D12079B, Research Diets, New Brunswick, NJ) for 20–30 weeks. C57BL/6 mice were also fed high-fat diet (HFD, $n = 5$) or normal diet (ND, $n = 5$) and used as negative control groups for atherosclerotic mice. All animal studies conformed to the tenets of the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology. The aorta was isolated as described in our previous study [15]. The atherosclerotic lesions in descending aorta (DA) were promptly measured within 30 min in a label-free manner after the arteries were isolated. For *en face* 3D image construction, a longitudinally opened DA was mounted lumen side down on a coverglass-bottom chamber, covered with a coverslip, and measured until 40–120 μm in z-depth.

2.3. Immunofluorescence assay

Cross-sectioned descending aorta samples (10 μm of thickness) from ApoE$^{-/-}$ mice fed an HFD for 30 weeks were used for immunofluorescence assays. After fixation in 4% paraformaldehyde for 15 min, the tissue slices were permeabilized and blocked with a

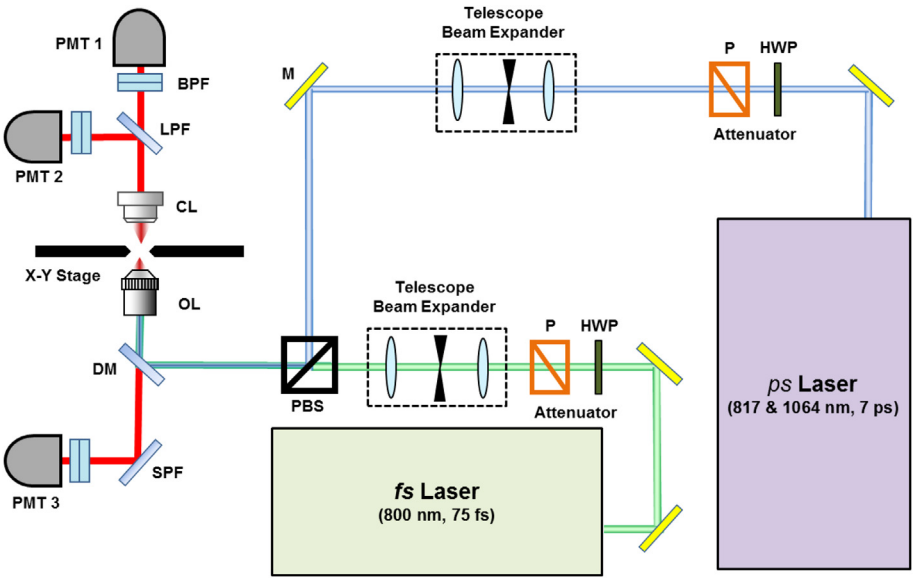

Fig. 1. A schematic diagram of the MNLO microscopy system. HWP: half-waveplate; P: Glan-Thompson polarizer; PBS: polarizing beam splitter; DM: dichroic mirror; OL: objective lens; CL: collection lens; SPF: short-pass filter; LPF: long-pass filter; BPF: bandpass filter; PMT: photon multiplier tube. Our multimodal NLO microscope acquired two-dimensional (2D) *en face* images with a maximum field of view of 250 × 250 μm^2 and a spatial resolution of 0.4 μm in the lateral (x–y) plane and 1.3 μm along the axial direction (z). Depth-sectioned images consisting of 512 × 512 pixels were obtained at a frame rate of 1.2 fps.