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The regional ratio of cholesteryl palmitate to cholesteryl oleate measured by ToF-SIMS as a key parameter of atherosclerosis

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ABSTRACT

Objective: Changes in cholesterol ester (CE) content regulate the progression of atherosclerosis. However, the spatial dynamics of CE subsets and their quantitative changes during lesion progression are not well understood due to a lack of appropriate imaging techniques. In this study, we developed an imaging–based analysis method to map the distribution of CE subsets using time-of-flight secondary ion mass spectrometry (ToF-SIMS).
Methods: Serial sections of atherosclerotic aortic sinuses from apolipoprotein E knock-out mice ($n = 15$) fed a 0.15% high-fat diet for 12–20 weeks were examined by ToF-SIMS.
Results and conclusion: We found that the ratio of cholesteryl palmitate (Ch-PA) to cholesteryl oleate (Ch-OA) increased by approximately 99% ($p = 0.02$) as atherosclerosis progressed, whereas the ratios of cholesteryl linoleate ($p = 0.09$) and cholesteryl stearate ($p = 0.22$) to Ch-OA did not change significantly. In advanced atherosclerotic plaques, *in situ* and *in-vitro* cell death assays showed that local Ch-PA densities were highly correlated with an increase in the number of apoptotic cells. These results suggest that increased Ch-PA may contribute to the formation of a necrotic core by increasing cell death. Our results indicate that the regional ratio of CEs as measured by ToF-SIMS might be a valuable new marker of atherosclerotic progression.

1. Introduction

The abnormal accumulation of lipids is considered a major risk factor for atherosclerosis [1]. As atherosclerosis progresses, both the composition and distribution of lipids in the plaque change, affecting the functions of adjacent vascular cells [2,3]. Lipids in the initial stages of atherosclerosis (type I–II, according to the histological classification endorsed by the American Heart Association) lead to the differentiation of macrophages into foam cells to form fatty streak lesions [4,5]. The aortic plaques at the intermediate stages of atherosclerosis (type III–IV) are characterized by a necrotic core, which is a mixture of apoptotic and necrotic cells with extracellular lipids composed mainly of free cholesterol, cholesterol esters, triglycerides, phospholipids, and FAs [6,7]. At advanced stages (type V–VI), lipid cores that are more complicated, often including cholesterol crystals, calcification, and intrahemorrhages, can form in the deep intima, eventually destabilizing the plaque [8]. Therefore, systemic studies of the changes in the lipid contents and functions during the progression of atherosclerosis are important to better elucidate the mechanism of plaque development.

Quantitative analyses of lipids based on tissue extraction methods are subject to several important limitations. First, any spatial information about the lipids in the tissue is lost. Second, other histological analyses, including immunohistochemistry, cannot be performed simultaneously, precluding the collection of additional data on the relationship between atherosclerotic lipids and the adjacent vascular cells. More recently, Zaima et al. demonstrated the feasibility of the use of matrix-assisted laser desorption/ionization (MALDI) mass spectrometry for the histological examination of atherosclerotic lesions in the aortic roots of apolipoprotein E–deficient ($apoE^{-/-}$) mice [9]. Although MALDI

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can recognize a wide variety of biomolecules, especially in high mass ranges (>*m/z* 800), its spatial resolution is still limited (>10 μm). Therefore, subtle changes in the regional distribution of individual lipids are required to finely assess the progression of atherosclerosis only beyond the identification of the lesion area.

Using time-of-flight secondary ion mass spectrometry (ToF-SIMS), we have achieved a higher spatial resolution than currently possible with other imaging mass spectrometry techniques. Secondary ion images with a spatial resolution of ≈1 μm can be obtained by rastering the tissue samples with a Bi_3^+ primary ion beam [10]. Additionally, due to the high sensitivity of lipid signals, ToF-SIMS can be readily applied in studies of atherosclerosis, obesity and other lipid metabolic disorders [11–14]. For example, Malmberg et al. determined the lipid distribution in healthy rat aortas using ToF-SIMS [11], and Mas et al. investigated the lipid cartography of the human carotid artery by ToF-SIMS [12]. Although previous studies have identified and localized individual lipids in animal or human atherosclerotic lesions, the roles of individual lipids in the progression of atherosclerosis have not been studied systemically.

CEs are the most abundant lipids in the atherosclerotic plaque in the mouse, rabbit [15] and human [16] aorta. Ch-OA (cholesterol + C18:1) and Ch-PA (cholesterol + C16:0) are the major CEs in the aorta. The composition of CE pools with different fatty acyl chains determines the pathophysiology of atherosclerosis [17]. Changes in CE pools, such as the quantitative variation of their saturated long-chain fatty acids, can result in a phase transition between the liquid and liquid-crystalline phases, modulating disease progression [18]. However, little is known about the regional and quantitative change of CEs with atherosclerotic progression.

In this study, we aimed to identify the changes in the distribution and composition of atherosclerotic lipids during lesion progression by ToF-SIMS. ToF-SIMS imaging was used to characterize the molecular distribution of CEs in early, intermediate and advanced lesions from the aortic sinuses of $apoE^{-/-}$ mice fed a high-fat diet. ToF-SIMS spectra revealed differences in the ratios of major CEs during lesion development. Combined histochemical analysis showed that the changes to the major CE ratio affected cellular lipotoxicity. These results may provide insight into the role of each CE in the progression of atherosclerosis.

2. Methods

2.1. Animal procedures

Six-week-old male $apoE^{-/-}$ mice (n = 15) were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained under specific pathogen–free conditions at the Korea Research Institute of Bioscience and Biotechnology. $ApoE^{-/-}$ mice were fed a Western high-fat diet containing 0.15% cholesterol (21% fat, 50% carbohydrate, and 20% protein) from Research Diets (Diet#D12079B, New Brunswick, NJ) for 12–20 weeks.

2.2. Sample preparation

Mice were sacrificed by CO_2 inhalation. The vasculature was then flushed with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} for 10 min. The heart, including the aortic sinus, was extracted, promptly frozen in liquid nitrogen and then stored at -80 °C. To minimize contaminants for ToF-SIMS measurement, OCT compound (Sakura Finetek Europe, Netherlands) was only used to attach the specimen to a cutting block. The frozen tissues were cut into 10 μm sections with a Leica cryostat (Leica Microsystems SA, Rueil-Malmaison, France) at -20 °C. Serial sections were alternately deposited on silicon wafers for ToF-SIMS analysis and on silane-coated glass slides for histological assays. The serially sectioned samples were used for a correlative study using ToF-SIMS and histological assays, such as Oil-red O and hematoxylin staining. After staining, we analyzed the samples according to the American Heart Association guidelines and determined the size of the lesion area in which the ToF-SIMS analysis was performed. After sectioning, tissue slices for ToF-SIMS analysis were transferred directly into a vacuum chamber, dried for 1 h and then immediately analyzed by ToF-SIMS. Slices for histological assays were stored at −80 °C until use. For each examination, 3 ∼ 7 samples from independent mice were used for the ToF-SIMS analysis.

2.3. ToF-SIMS analysis

Arterial cryosections were analyzed using a ToF-SIMS V instrument (ION-TOF GmbH, Münster, Germany) equipped with a bismuth liquid metal ion gun (LMIG). A Bi_3^+ primary ion beam at 25 keV in low-current bunched mode (pulse width ∼ 1.13 ns, beam diameter ∼ 1.1 μm) with a target current of 0.13 pA and a cycle time of 150 μs was used to acquire chemical images of atherosclerotic lesions with high mass resolution. The primary ion dose density (PIDD) was 5×10^{11} ions·cm^{-2}. The analysis area was 400 × 400 μm^2 (128 × 128 pixels) and was charge-compensated by an electron flooding gun. Positive and negative ion spectra were internally calibrated using CH_3^+, $C_2H_3^+$, $C_3H_5^+$, $C_{27}H_{45}^+$ peaks and CH^-, C_2H^-, C_4H^-, C_6H^-, $C_{27}H_{45}O^-$ peaks, respectively.

For the extraction of ToF-SIMS spectra, regions of interest (ROIs) were manually selected from the atherosclerotic lesion area to eliminate information from adjacent non-lesion tissue. When multiple ROIs were selected in the same image, all ROIs were the same size and their mass spectra were normalized to the total ion count. Each image was normalized to the intensity of the brightest pixel.

The lipid reference compounds PA, OA, Ch-PA, and Ch-OA were purchased from Sigma–Aldrich and used to generate ToF-SIMS reference spectra. Each compound was dissolved in ethanol and adjusted to a concentration of 10 μM. The lipid solutions were loaded by droplet deposition onto silicon wafers that were cleaned in piranha solution, and then solvent (ethanol) was evaporated before the measurement of ToF-SIMS spectra. The PIDD was 5×10^{11} ions·cm^{-2}, and the analysis area was 200 × 200 μm^2.

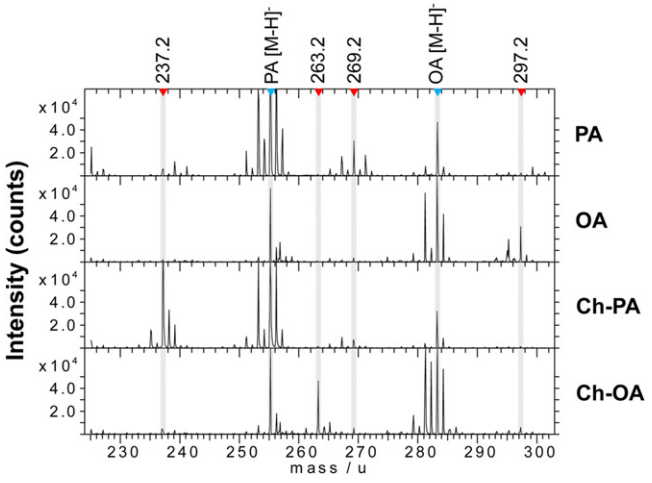

Fig. 1. Negative ion mass spectra of lipid reference compounds in the *m/z* range of 230–300 were obtained by ToF-SIMS. The blue arrows indicate conventional ion peaks of PA $[M-H]^-$ (*m/z* 255.2) and OA $[M-H]^-$ (*m/z* 281.2) respectively. The red arrows indicate fragmented ions that discriminate among four lipids. PA, palmitate (*m/z* 269.2); OA, oleate (*m/z* 297.2); Ch-PA, cholesteryl palmitate (*m/z* 237.2); Ch-OA, cholesteryl oleate (*m/z* 263.2).